Investigation of Mechanisms of Breast Cancer Invasion – the Role of Matrix Metalloproteinases and Cell Adhesion Molecules

Thesis submitted for the degree of

Doctor of Philosophy (Pathology)

at the University of Leicester

by

Janet Louise Jones

BSc. MB ChB. MRCPath.

Department of Pathology

University of Leicester

September 1999

UMI Number: U536319

UMI

Dissertation Publishing

UMI U536319

ProQuest®

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Investigation of Mechanisms of Breast Cancer Invasion – the Role of Matrix Metalloproteinase and Cell Adhesion Molecules

A defining feature of cancer is the ability of malignant cells to invade and metastasise. Orchestrated changes in cell adhesion molecules (CAM) and proteolytic enzymes are associated with the onset of cell invasion in both physiological and pathological situations. The hypothesis is that (i) co-ordinated changes in Matrix Metalloproteinases (MMP) enzymes and E-Cadherin and integrin cell adhesion molecules (CAM) are important in the control of breast cancer invasion, (ii) a factor contributing to these changes is the tumour-fibroblast relationship, and (iii) re-establishing a more normal CAM profile or altering the tumour-fibroblast relationship would therefore inhibit tumour invasion.

Breast carcinomas (114) were examined for expression of the MMP-2/TIMP-2/MT1-MMP and MMP-9/TIMP-1 systems using immunohistochemistry, in-situ hybridisation and zymography. All components are expressed by fibroblasts, but the level of tumour cell membrane MMP-2 and MT1-MMP correlates both with MMP-2 activity and the presence of lymph node metastases. MMP-9 expression is associated with the infiltrating lobular phenotype and with reduced E-Cadherin. Reduced $\alpha 2, \alpha 3$ and $\beta 1$ integrin is related to loss of tumour differentiation, and expression of $\alpha 6$ and $\beta 4$ integrin is associated with high tumour grade.

Evidence from in-vitro studies confirms a relationship between cell adhesion, MMP expression and invasion. Lack of E-Cadherin in MDA-MB 231 and MDA-MB 468 cells is associated with MMP-9 expression and disrupting E-Cadherin in MCF-7 and T47D cells induces MMP-9. The high invasive capacity of MDA-MB 231 is $\alpha 6 \beta 4$ dependent, however, the redistribution of $\alpha 6 \beta 4$ to hemidesmosome-like structures is associated with a significant reduction in invasion and increase in both MMPs and TIMPs.

Tumour cell-fibroblast co-culture enhances tumour cell invasion and is associated with up-regulation of MMP expression in both cell populations with increased net proteolytic activity. Introducing normal myoepithelial cells into this culture system inhibits tumour cell invasion and MMP expression in tumour cells and fibroblasts.

These results indicate the importance of co-ordinate changes in MMPs and CAM in the process of invasion in-vivo and in-vitro, and identify a potential invasion-suppressor role for the myoepithelial cell

Acknowledgements

I am indebted to my supervisor, colleague and friend, Dr. Rosemary Walker, for her constant guidance and support. Her enthusiasm and commitment are inspirational and what first attracted me to breast pathology – a decision I have never regretted. I am particularly grateful to her for 'taking the load' whilst I have tackled this task. I owe you one!.

I want to thank my co-workers Steve Chappell, Howayda Hassan and Tom Walsh. Steve taught me molecular biology with typical thoroughness, and we shared many joys and disappointments with our experiments. I will always cherish those days. I am grateful to Sheila Dearing who keeps us in order, and is full of useful information. Also to the other members of the BCRU –Jacqui, Linda, Matt, Suzanne, Afsana, Karen, Bharti and Giles – who make this such a great place to work. I have spent many hours at the confocal microscope and enjoyed tea, biscuits and the stories. Most especially, I am grateful to Chris d'Lacey, who has helped enormously with the photographic work . Thanks.

Much needed help and support come in the form of Malcolm Rae – thank-you. And to Howard Pringle for being so interested in everything, and full of ideas.

I very much appreciate the continuing support of Profesor Ian Lauder, and other members of the Department, and also to my NHS colleagues – especially Salli Muller, who has freed up vital time for me recently.

I am extremely grateful to the Royal College of Pathologists for honouring me with the first BUPA Research Fellowship

Finally, to Alan, who knows almost as much about this project as I do. Thank you for everything. Without you, I think this truly may not have been completed.

List of Contents

List of Figures

List of Tables

Abbreviations

ABC	Avidin-Biotin-Complex
AJ	Alec Jeffries
AP-1	Activator Protein - 1
APS	Ammonium Persulphate
BM	Basement Membrane
Bp	Base Pairs
BSA	Bovine serum Albumin
CALLA	Common Acute Lymphoblastic Leukaemia Antigen
CDNA	Complementary DNA
CK	Cytokeratin
CM	Conditioned Medium
CNS	Central Nervous System
CTB	Cytotrophoblast
DAB	Diaminobenzidine
dATP	2'-Deoxyadenosine 5'-Triphosphate
dd	Deionised
DMEM	Dulbeccos Modified Eagles Medium
DNA	Deoxyribonucleic Acid
dNTP	2' Deoxynucleotide 5'-Triphosphate
dUTP	2' Dooxyuridine 5'Triphophate
E-CD	E-Cadherin
ECM	Extracellular Matrix
EDTA	Disodium Ethylene Diamine Tetraacetate
EGFR	Epidermal Growth Factor Receptor
EHS	Engelbroth –Holm Swarm
RT	Reverse Transcriptase
ELOSA	Enzyme Linked Oligonucleotide Assay
EMA	Epithelial Membrane Antigen
EMMPRIN	Extracellular MMP Inducer
ER	Oestrogen Receptor
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FITC	Fluoro-isothiocyanate
GAPDH	Glyceraldehyde –3- Phosphate Dehydrogenase
GLB	Gel Loading Buffer
HBAR	Heat Based Antigen Retrieval
HD1	Hemidesmosome 1/Plectin
HRP	Horseradish Peroxidase
HSB	High Salt Buffer
ID	Integrated Density
IDC	Infiltrating Ductal Carcinoma
IF	Intermediate Filaments
IHC	Immunohistochemistry
ILC	Infiltrating Lobular Carcinoma
IMS	Industrial Methylated Spirit
Kb	Kilo base
Kda	Kilo dalton
LCIS	Lobular Carcinoma in-situ
LMP	Last Menstrual Period
MAPK	Mitogen Activated Protein Kinase
MES	2-[N-Morpholino] ethanesolfonic acid
Mg	Magnesium
MMP	Matrix Metalloproteinase
MPC	Magnetic Particle Concentrator

MT-MMP	Membrane Type- Metalloproteinase
Nm	Nanometer
NRS	Normal Rabbit Serum
NSS	Normal Swine Serum
OD	Optical Density
PAGE	Poly Acrylimide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PgR	Progesterone Receptor
PK	Proteinase K
RNA	Ribonucleic acid
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SMA	Smooth Muscle Actin
Taq	ThermusAquaticus
TGFβ	Transforming Growth Factor Beta
TIMP	Tissue Inhibitor of Metalloproteinase
TRITC	Rhodamine
μl	Microlitre
μm	Micrometer

Chapter 1

Introduction

1.1 Breast Cancer - the problem

Worldwide, breast cancer remains the commonest malignancy in women, and comprises 18% of all female cancers. In the U.K. the age standardised incidence is the highest in the world, with 33,000 new cases diagnosed each year and 15,000 deaths (Boring et al, 1993), and the incidence is increasing, particularly in elderly women, by 1-2% per year (Miller, 1992). It is estimated that a woman has a 1 in 12 chance of developing the disease in her life. The age specific incidence begins to rise over the age of 35 years, and the disease is a major cause of morbidity and mortality in women over the age of 50 years. Whilst the mortality rate from breast cancer has been increasing world-wide, there have been recent reports of declining mortality in the UK, USA, Norway and Sweden (Beral et al, 1995; Herman & Beral, 1996), probably due to earlier diagnosis and better treatment. However, in spite of this, breast cancer accounts for 20% of female cancer deaths in the UK. Mortality from breast and other cancers is due to spread of the malignant cells, therefore an understanding of the mechanisms that control tumour cell invasion and metastasis is important in attempts to identify key regulatory events that may provide efficient targets for therapeutic intervention. The focus of this thesis is on the factors that control breast tumour cell invasion, however, before considering these factors it is necessary to have an understanding of the normal breast and its response to physiological events.

1.2 Development, Structure and Function of the Normal Breast

1.2.1 Embryogenesis and Early Breast Development

The human mammary gland develops from the mammary ridges, or milk lines, which are thickenings of the epidermis that arise on the ventral surface of the 5 week-old foetus. At around 15 weeks, under the influence of testosterone, mesenchymal condensation occurs around an epithelial stalk and cords of epithelium grow into the mesenchyme, producing a group of solid epithelial columns, each of which will give rise to a lobe in the mammary gland. The papillary layer of the dermis continues to encase these growing epithelial cords and ultimately forms the vascularised intralobular and periductal stroma, whilst the less cellular more collagenised interlobular stroma is formed from the reticular dermis. In the last 2 months of gestation, canaliculisation of the epithelial cords occurs followed by early development of branching lobuloalveolar glandular structures (Ham & Cormack, 1979). Cells at the tips of the terminal end buds show features which suggest they generate both basal and luminal epithelial populations (Osin et al, 1998).

1.2.2 Adolescent Breast Development

With the onset of puberty, oestrogen-dependent elongation and branching of breast ducts occurs (Topper & Freeman, 1980). Differentiation of the oestrogen dependent intralobular and periductal stroma also occurs at this time. Growth hormone, insulin, progesterone and glucocorticoids all contribute to the lobuloalveolar differentiation that starts at this stage and continues into the early 20s (Topper & Freeman, 1980).

1.2.3 The Adult Breast

The mature adult breast is composed of 15-25 lobes corresponding to the parenchyma

associated with each of the major lactiferous ducts which terminate in the nipple. The

lactiferous ducts extend through a series of branches to the terminal ductal-lobular units

(TDLU), which are embedded in the hormonally responsive specialised stroma. The ducts and

lobular acini are lined by a layer of cuboidal/columnar epithelial cells supported by an

underlying layer of myoepithelial cells (fig.1.1). The latter form an attenuated layer in intimate

contact with the basal lamina, and are frequently indistinct on routine haematoxylin and eosin

sections (Nagato et al, 1980). The intralobular stroma contains more capillaries and is less

densely collagenised than the interlobular stroma, and it has been suggested that the

intralobular fibroblasts have a paracrine effect on the epithelium (Eyden et al, 1986).
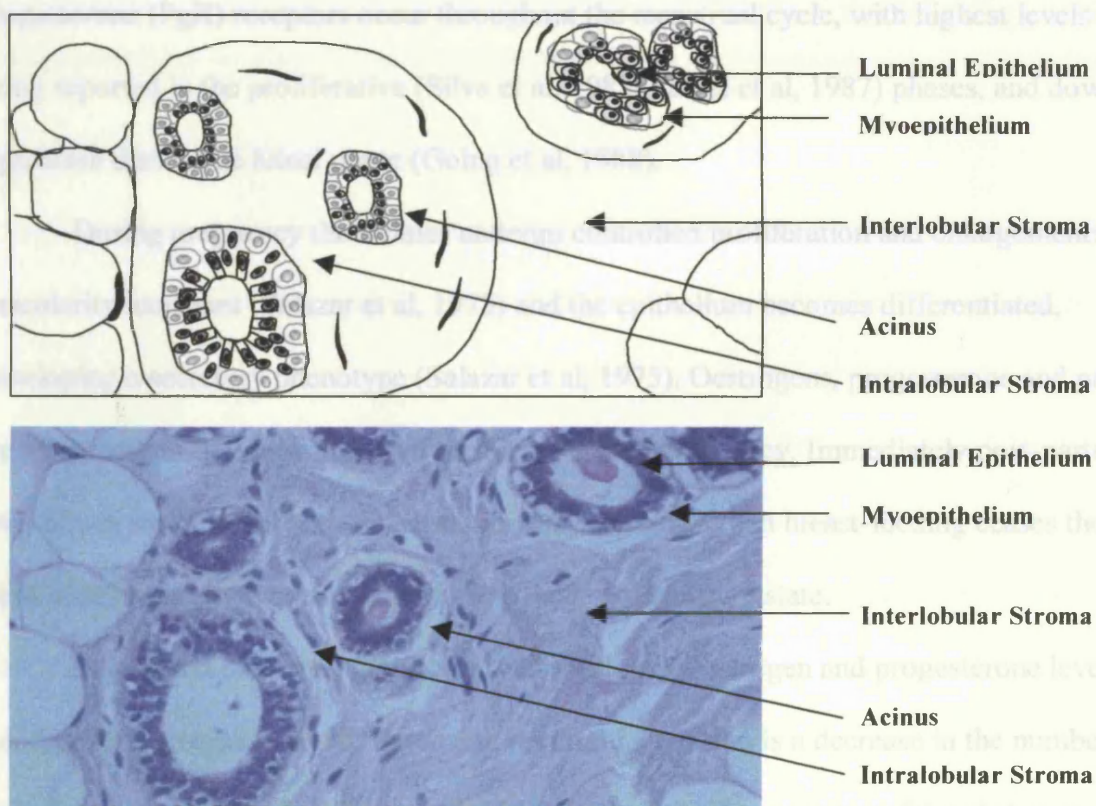
Fig.1.1: Structure of Normal Adult Breast

1.2.4 Cyclical Variations in the Breast

The normal histology of the breast is not constant because it is subject to changes associated with the menstrual cycle, pregnancy, lactation and the menopause.

The structural effects of the cyclical menstrual changes are manifested clinically by fluctuations in breast size and texture, and changes occur in both the epithelial and stromal compartments. In the proliferative phase (day 3-7) there is a high level of mitosis and apoptosis in the lobular epithelium, whilst the stroma is dense and hypovascular (Ferguson & Anderson, 1981; Longacre & Bartow, 1986). Following ovulation, at day 15-20, there is a small peak in mitotic activity followed by an increase in apoptosis (at the onset of the menstrual phase). The intralobular stroma becomes oedematous and highly vascular prior to the menstrual phase, when it once again becomes compact. Differences in the levels of oestrogen (ER) and progesterone (PgR) receptors occur throughout the menstrual cycle, with highest levels of ER being reported in the proliferative (Silva et al, 1983; Fabris et al, 1987) phases, and down-regulation during the luteal phase (Going et al, 1988).

During pregnancy the lobules undergo controlled proliferation and enlargement: stromal vascularity increases (Salazar et al, 1975) and the epithelium becomes differentiated, developing a secretory phenotype (Salazar et al, 1975). Oestrogens, progesterone and prolactin are all important in development of the breast during pregnancy. Immediately post-partum the level of sex steroids fall and prolactin initiates lactation. When breast-feeding ceases there is rapid involution of the breast and return to the pre-pregnancy state.

During and after the menopause, with declining oestrogen and progesterone levels, but maintained testosterone levels, the major structural alteration is a decrease in the number and size of the lobules (Huseby & Thomas, 1954), and a relative increase in fat and stroma.

1.3 Breast Cancer

Breast cancer is a heterogeneous group of diseases: some tumours grow slowly, do not metastasise and do not greatly influence life expectancy, whilst other tumours are highly aggressive, spread widely and cause significant morbidity and mortality. Predicting the behaviour of a breast tumour has been the focus of surgeons, pathologists and scientists for many decades. This drive continues and is a major focus of research in the field of breast cancer, and to date, a number of features have emerged as useful indicators of disease behaviour, including histopathological characteristics of tumours, extent of spread and certain markers of biological behaviour. For the purpose of this study, it is necessary to outline the features commonly used to indicate the nature and behaviour of a given tumour, namely tumour type, grade and extent of spread, which will be discussed briefly in the following sections.

1.3.1 Classification of Malignant Breast Disease

Over 90% of carcinomas of the breast are derived from the epithelium, and traditionally lesions have been divided into those arising from ducts and those from lobules. However, it is now accepted that the majority of cancers, whatever their morphological pattern, arise in common from the terminal ductal-lobular unit (TDLU) (Wellings et al, 1975). In spite of this, current classifications still separate ductal and lobular lesions, partly because of their distinct morphology but more importantly because of their different clinical implications. Table 1.1 summarises the classification of malignant breast lesions, with an indication of their relative frequencies and different prognosis.

The classification most widely used by pathologists is based on that of the World Health Organisation (WHO, 1981). Non-invasive lesions are those in which the malignant cells are

6

confined within the duct or lobule without evidence of invasion through the basement

membrane. They are characterised as either lobular or ductal on cytological features and growth

patterns.

Table 1.1: Histological Classification of Malignant Breast Disease, Relative Frequency and Survival Rates.

TYPE OF CARCINOMA	RELATIVE FREQUENCY(%)	10 YEAR SURVIVAL (%)
Ductal carcinoma in-situ	4.5	92
Lobular carcinoma in-situ	0.1	NA
Infiltrating ductal carcinoma NST	47	47
Infiltrating lobular carcinoma	15	54
Tubular carcinoma	2.3	90
Medullary carcinoma	2.7	51
Mucinous carcinoma	0.9	80
Mixed ductal & lobular ca.	4.7	40
Mixed ductal & special type	2.5	64

NST : No Special Type   NA: Not Appropriate
Data taken from: Ellis et al, 1992.

Ductal carcinoma in-situ (DCIS) is itself a heterogeneous disease (Holland et al, 1994;

Lennington et al, 1994; Poller et al, 1994) but virtually always involves a single duct system.

Because in the past most women with a diagnosis of DCIS underwent mastectomy, there is

limited data on the extent of progression of DCIS to invasive disease. *Lobular carcinoma in-*

situ (LCIS) is a distinctive entity in which lobules become distended by a proliferation of

neoplastic cells that are regular and uniform and lack distinct cell boundaries (Foote & Stewart,

1941). It is typically a multicentric disease, affecting both breasts (Lambird & Shelley, 1969)

and generally an incidental finding in biopsy material performed for other reasons, and as such

the true incidence of the lesion is unknown. A number of studies have indicated that the

presence of LCIS confers a tenfold risk of developing carcinoma in comparison to the general

population (Andersen et al, 1974; Wheeler et al, 1974; Page et al, 1991). The risk of developing

an invasive carcinoma applies to both breasts (Page et al, 1991).

Infiltrating ductal carcinoma of no special type is the most common type of breast

cancer, forming between 50% and 70% of most series. This is essentially a category of

exclusion, if the tumour does not fulfil the criteria for the "special type' carcinomas. *Special*

Type carcinomas are a group of tumours with distinctive histological patterns. The importance

of this category is the improved prognosis of most of these tumours over those of no special

type.

Infiltrating lobular carcinoma (ILC) is classically composed of small round cells, many

possessing intracytoplasmic lumina, invading the stroma in a diffuse manner in the form of

single strands or Indian files. They were originally thought to derive from lobular epithelium,

but this remains unproven. ILC is more frequently associated with bilateral disease than ductal

carcinomas.

1.3.2 Histological Grading of Tumours

The power of histological grading of breast cancer differentiation was evident as early

as 1925, when Greenhough demonstrated a clear association between the morphological

features of a tumour and clinical outcome (Greenhough, 1925). Since then many grading

systems have been developed and currently the system commonly used is a modification of the

grading system described by Bloom and Richardson in 1957 (Bloom & Richardson, 1957;

Elston & Ellis, 1991). This is based on assessing the extent of tubule formation in a tumour, the

degree of nuclear pleomorphism and the frequency of mitotic figures. Each of these parameters is given a score of 1-3, the values combined and converted into three groups: grade I/well differentiated (score 3-5); grade II/moderately differentiated (score 6-7) and grade III/poorly differentiated (score 8-9). There is a strong correlation between histological grade and patient survival (Elston & Ellis, 1991).

1.3.3 Staging of Breast Cancers

The process of staging a cancer involves measuring the extent of spread of disease according to a defined set of criteria. The two main systems used are the International Classification of Staging and the TNM (Tumour, Node, Metastasis) system. Histological involvement of axillary lymph nodes, and the number of nodes involved shows significant correlation with prognosis (Valagussal et al, 1978; Ferguson et al, 1982). A large study by the National Cancer Institute concluded that tumour size and lymph node status were both independent but additive prognostic indicators (Carter et al, 1989; Table 1.2).

Table 1.2: Five Year Survival Rates (%) in Relation to Tumour Size and Lymph Node Status

LN STATUS	SIZE (mm)				
	<5	5-10	10-19	20-50	>50
Negative	99.2	94.9	90.6	89.4	82.2
1-3 nodes positive	95.3	94.0	86.6	79.9	73.0
4+ nodes positive	59.0	54.2	67.2	58.7	45.5
Total	96.2	94.9	90.6	79.8	62.7

Taken from: Carter et al, 1989.

9

1.4 Tumour Invasion and Metastasis

Irrespective of tumour type or grade, the absolute hallmark of malignancy in all carcinomas is the ability of the tumour cell to invade, and the potential to metastasise. In normal tissue, epithelial cells form intact sheets held together by homotypic adhesions, and separated from the surrounding stroma by a basement membrane (BM), to which they adhere. With development of an invasive tumour, changes occur in the adhesive interactions between tumour cells themselves, between tumour cells and other cell types, and between tumour cells and the extracellular matrix (ECM). Initial invasion requires penetration of BM and active migration of the malignant cells through the surrounding stroma, which involves overcoming the restrictions imposed by normal homotypic cell adhesion, and the ability to make and break specific bonds with the extracellular matrix proteins. Induction of new vessel formation (angiogenesis) is a vital skill for tumour cells, both to provide nutrition and also to promote access of the tumour cells to the vascular compartment for dissemination, and this process also involves stromal proteolysis and cell migration. Acquisition of metastatic competence requires tumour cells to penetrate further basement membrane barriers both during the process of vascular invasion (either blood vessel or lymphatic) and extravasation at a distant site. A further requirement for successful tumour spread is evasion of the hosts' immune response (Hart & Saini, 1992).

Although a malignant tumour displays an apparently anarchic relationship with the host tissue, it is the co-ordinated expression of molecules involved in the processes of cell adhesion, cell motility and proteolysis that enables a tumour to successfully achieve invasion and metastasis. Elucidation of the molecules involved in these steps, their inter-relationship and the factors controlling them is of fundamental importance to our understanding of the factors controlling tumour progression.

10

(I) Normal gland

(ii) Gland containing in-situ carcinoma

(iii) Early invasion
through basement membrane

(iv) Invasion through
extracellular matrix

(v) Angiogenesis

(vi) Access to vascular
compartment

(vii) Overcoming host
immune system

(viii) Exit at distant site

(ix) New growth at
distant site (metastasis)
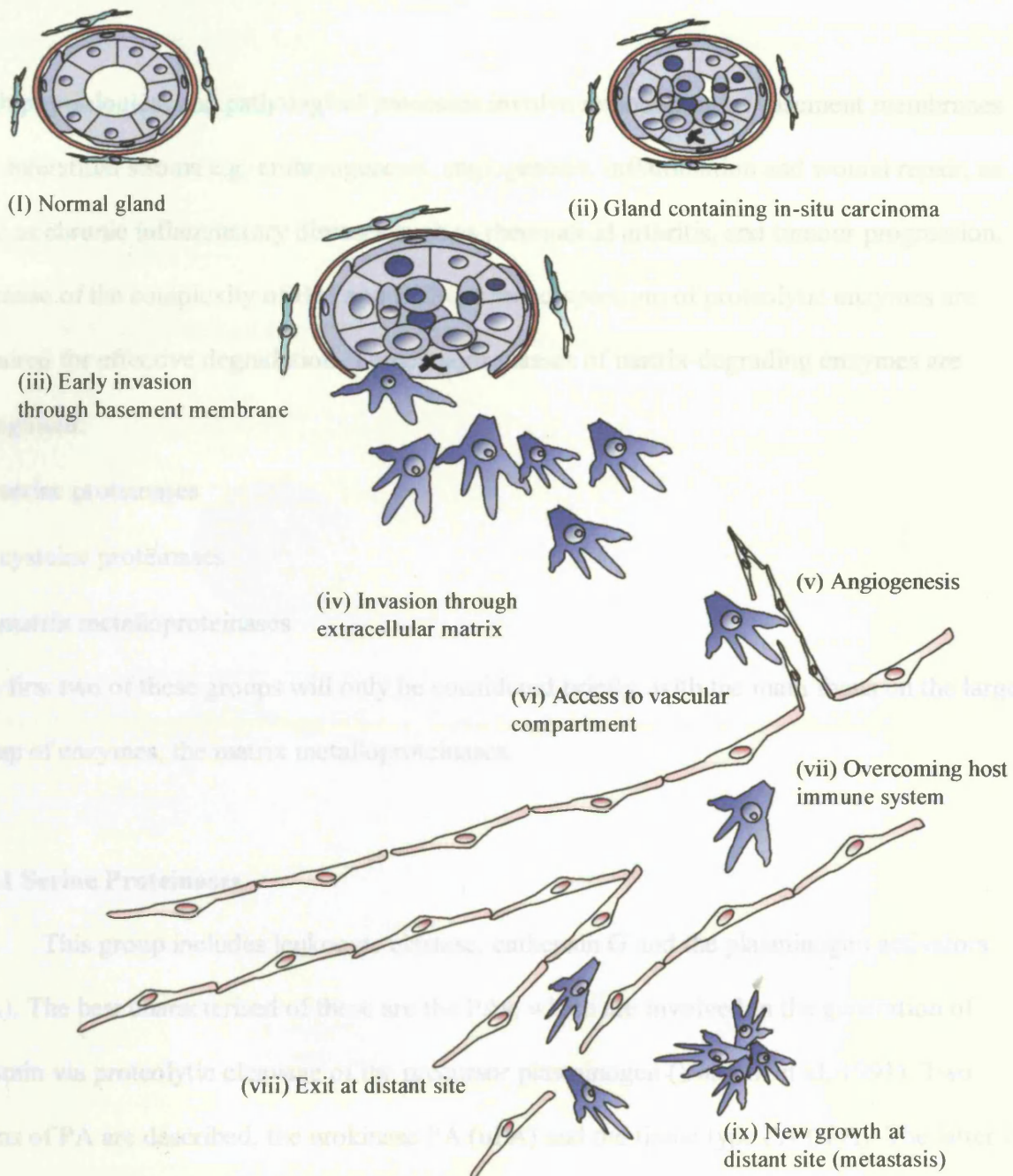
Fig. 1.2: Schematic Model Outlining Putative Steps of Tumour Invasion and Metastasis

1.5 Proteolytic Remodelling of the Extracellular Matrix

Both physiological and pathological processes involve remodelling of basement membranes and interstitial stroma e.g. embryogenesis, angiogenesis, inflammation and wound repair, as well as chronic inflammatory diseases such as rheumatoid arthritis, and tumour progression. Because of the complexity of BM and ECM, a broad spectrum of proteolytic enzymes are required for effective degradation. Three major classes of matrix-degrading enzymes are recognised:

- serine proteinases

- cysteine proteinases

- matrix metalloproteinases

The first two of these groups will only be considered briefly, with the main focus on the largest group of enzymes, the matrix metalloproteinases.

1.5.1 Serine Proteinases

This group includes leukocyte elastase, cathepsin G and the plasminogen activators (PA). The best characterised of these are the PAs, which are involved in the generation of plasmin via proteolytic cleavage of the precursor plasminogen (Vassalli et al, 1991). Two forms of PA are described, the urokinase PA (uPA) and the tissue type PA (tPA). The latter is primarily involved in intravascular thrombolysis, whereas uPA is bound and activated by a specific plasma membrane receptor (uPA-R), and mediates pericellular proteolysis (Cubellis et al, 1986; Appella et al. 1987). Like other enzyme systems, uPA is secreted as an inactive precursor (pro-uPA) which is activated at its receptor by limited proteolysis (Cubellis et al, 1986). Once activated, uPA, also known as urokinase, cleaves plasminogen to yield plasmin.

12

This is a highly efficient reaction, partly because urokinase is held in close proximity to its

substrate (Plow et al, 1986; fig. 1.3), but also because cleavage of plasminogen initiates an

amplification cascade, with very low levels of plasmin being capable of further activating pro-

uPA (Peterson et al, 1988). Plasmin itself is a serine protease and whilst its classical substrate is

fibrin, it is now recognised to have a broad, trypsin-like substrate profile (Werb et al, 1980).
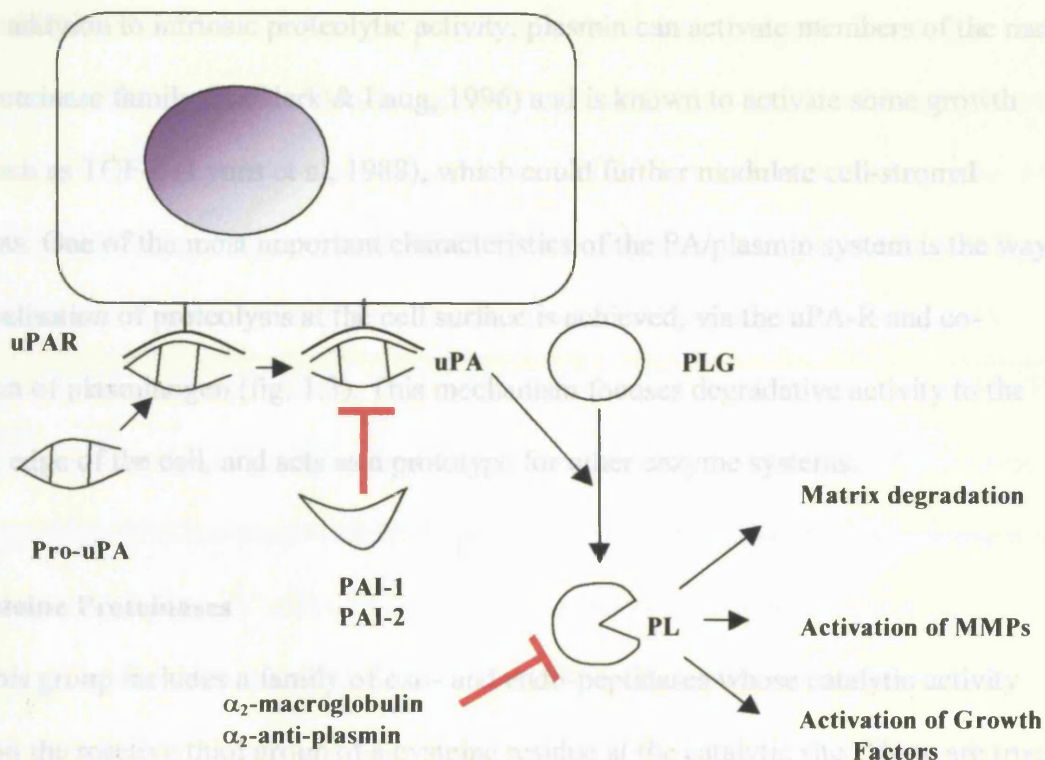
Fig. 1.3: The Urokinase Plasminogen Activator System.
UPAR: plasminogen activator receptor; uPA: urokinase plasminogen activator; PAI: plasminogen activator
inhibitor; PLG: plasminogen; PL: plasmin

The action of PAs and plasmin is subject to control at multiple levels. Expression of uPA is regulated at the transcriptional level by several agents including growth factors, retinoids, prostaglandins and cAMP (Dano et al, 1985), by activation of the latent pro-uPA, and also via the action of specific inhibitors, PAI-1 and PAI-2 (Sprengers & Kluft, 1987). A further level of control is mediated by inhibition of plasmin activity which is achieved via α2-anti-plasmin and α2-macroglobulin, both of which are abundant in plasma and interstitial fluid (De Bruin et al, 1988).

In addition to intrinsic proteolytic activity, plasmin can activate members of the matrix metalloproteinase family (DeClerk & Laug, 1996) and is known to activate some growth factors, such as TGF-β (Lyons et al, 1988), which could further modulate cell-stromal interactions. One of the most important characteristics of the PA/plasmin system is the way in which localisation of proteolysis at the cell surface is achieved, via the uPA-R and co-localisation of plasminogen (fig. 1.3). This mechanism focuses degradative activity to the migrating edge of the cell, and acts as a prototype for other enzyme systems.

1.5.2 Cysteine Proteinases

This group includes a family of exo- and endo-peptidases whose catalytic activity depends on the reactive thiol group of a cysteine residue at the catalytic site. These are true intracellular enzymes and the major members are the lysosomal cathepsins, which are small glycoproteins (20-42 kDa) active against a wide range of small peptides and larger proteins (Bond & Butler, 1987). As with the serine proteases, so cysteine proteases are controlled by specific inhibitors, referred to as cystatins (Barrett, 1987). The different cystatins show different tissue distributions and may be intracellular or extracellular. When intracellular they are found in the cytoplasm and thus occupy a different space to the enzymes that they inhibit.

14

Inhibition only occurs when compartmentalisation fails, i.e. if lysosomal enzymes leak into the cytoplasm (Barrett, 1987).

1.5.3 Matrix Metalloproteinases

There is a growing body of evidence to indicate a role for the Matrix Metalloproteinase (MMP) family of proteolytic enzymes in the remodelling of the ECM. To date 21 MMP enzymes have been described which between them can degrade most if not all components of the ECM (Mignatti & Rifkin, 1993). The enzymes can be loosely grouped according to their substrate specificity (see Table 1.3), although there is considerable functional overlap between individual enzymes. The *collagenases*, which include MMP-1 or interstitial collagenase, MMP-8 or polymorphonuclear collagenase and MMP-13 or collagenase 3, degrade collagens Type I and III as well as collagens Types VII and X, but they cannot digest gelatins or Type IV collagen (Miller et al, 1976; Goldberg et al, 1986; Schmid et al, 1986; Frieje et al, 1994). The *gelatinases*, also known as Type IV collagenases, comprise two members, the 72kDa form (MMP-2) and the 92kDa enzyme (MMP-9). As the name implies these have a prominent role in the degradation of Type IV collagen: MMP-2 and MMP-9 have identical substrate specificity profiles in-vitro -they cleave native Type IV collagen at a single site into one fourth and three-fourth size fragments, and both enzymes degrade denatured collagen (gelatin) and Type IV collagen as well as fibronectin, but have no effect on interstitial collagens (Salo et al, 1983; Fessler et al, 1984; Collier et al, 1988; Wilhelm et al, 1989; Murphy et al, 1991). There are three *stromelysins* (STR), STR-1 (MMP-3), STR-2 (MMP-10), and STR-3 (MMP-11), (Chin et al, 1985; Bassett et al, 1990). With the exception of STR-3, the stromelysins have the broadest substrate specificity, being capable of degrading fibronectins, laminin, elastin, proteoglycans and collagens IV, V, VIII and IX, though only in the non-helical regions (Okada

15

Table 1.3: Classification of Matrix Metalloproteinases and their Substrate Specificity

ENZYME	*MW(kDa)	MMP	SUBSTRATE	REFERENCE
Interstitial collagenase	52	MMP-1	Collagens I, II, III, VII, X	Goldberg et al, 1986
Polymorphonuclear collagenase	85	MMP-8	Collagens I, II, III	Hasty et al, 1990
Collagenase-3	60	MMP-13	Collagen II (I & II),denaturec collagens & aggrecan	Freije et al, 1994 Knauper et al, 1997
72kDa Gelatinase (Type IV collagenase)	72	MMP-2	Denatured collagens Native collagens IV, V, VII, X, fibronectin, elastin	Collier et al, 1988 Seltzer et al, 1989
92kDa gelatinase (Type IV collagenase)	92	MMP-9	Denatured collagens Native collagens IV, V	Wilhelm et al, 1989
Stromelysin-1	57	MMP-3	Proteoglycan core protein fibronectin, laminin, denatured collagens. Collagen IV, V, IX, X	Chin et al, 1985
Stromelysin-2	53	MMP-10	Denatured collagens Collagens III, IV, V (weak)	Breathnach et al, 1987 Muller et al, 1988
Stromelysin-3	61	MMP-11	Fibronectin & Laminin	Basset et al, 1990
Matrilysin (PUMP-1)	28	MMP-7	Proteoglycan core protein fibronectin, laminin, denatured collagens.	Quantin et al, 1989 Miyazaki et al, 1990
Metalloelastase	54	MMP-12	Elastin	Senior et al, 1982 Senior et al, 1989
Collagenase	58	MMP-18	Collagens I, II, III, VII Denatured collagens	Cossins et al, 1996
MMP-19	57	MMP-19	Stromelysin-like profile	Pendas et al, 1997
Enamelysin	54	MMP-20	Amelogenin	Llano et al, 1997

16

Table 1.3: Classification of Matrix Metalloproteinases and their Substrate Specificity (contd)

ENZYME	*MW(kDa)	MMP	SUBSTRATE	REFERENCE
Membrane Type 1-MMP	63	MMP-14	Activates proMMP-2 & pro-MMP-13 Fibronectin, aggrecan, nidogen, collagens I & III	Sato et al, 1994 Knauper et al, 1996 dOrtho et al, 1997
Membrane Type 2-MMP	64	MMP-15	Activates pro-MMP-2 Laminin	Will & Hinzmannn, 1995 dOrtho et al, 1997
Membrane Type 3-MMP	64	MMP-16	Activates pro-MMP-2	Takino et al, 1995
Membrane Type 4-MMP	70	MMP-17	?activates pro-MMP-2	Puente et al, 1996
Membrane Type 5-MMP	65	MMP-21	activates pro-MMP-2	Llano et al, 1999 Pei, 1999

et al, 1986; Wilhelm et al, 1987). Although structurally distinct from the stromelysins,

matrilysin (MMP-7, or PUMP-1 - putative metalloproteinase -1) can be placed in this group

due to considerable overlap in its substrates targets (Collier et al, 1988; Quantin et al, 1989).

Metalloelastase (MMP-12) targets elastin as its only substrate, though both MMP-7 and the

gelatinases are also capable of degrading elastin (Shapiro et al, 1992; Shapiro et al, 1993). An

expanding subgroup in the MMP family is the *Membrane Type-MMP* (MT-MMP) subset of

enzymes, which are characterised by the presence of an integral transmembrane domain (Sato

et al, 1994). Five members of this group are described, termed MT1-MMP to MT5-MMP,

respectively (Sato et al, 1994; Will & Hinzmann, 1995; Takino et al, 1995; Puente et al, 1996;

Llano et al, 1999; Pei, 1999), and as well as structural homology, MT1-, MT2-, MT3- and

MT5-MMP also share the same substrate target, being activators of MMP-2 (Sato et al, 1994;

Takino et al, 1995; Butler et al, 1997; Pei, 1999). In addition to their function as activators,

MT1-MMP and MT2-MMP have recently been shown to possess a broad proteolytic action including gelatinolytic activity (dOrtho et al, 1997; see Table 1.3). The MMP family is continuing to expand, with the characterisation of a new collagenase-like enzyme, MMP-18 (Cossins et al, 1996), and MMP-19 which also shows similarities to collagenases and stromelysins, but appears structurally distinct and has been suggested to represent a further MMP subfamily (Pendas et al, 1997). Enamelysin has been designated MMP-20, and was cloned from human odontoblastic cells (Llano et al, 1997). Its expression is highly restricted to dental tissue and it degrades amelogenin, the major protein of enamel matrix.

It must be emphasised that there is considerable overlap in the enzymatic activity of the different MMPs. Thus whilst interstitial collagenase was so named because of its ability to cleave the three interstitial collagens (types I, II and III), it also digests casein, and many species of the enzymes will digest gelatins, some at a similar efficiency to its digestion of interstitial collagens (Fields et al, 1990).

Despite their diverse targets, the MMPs have a number of common characteristics:

(i)   similar functional domains can be identified in each family member;

(ii)   the enzymes are synthesised as latent precursors (zymogens) and, with the exception of STR-3 and the MT-MMPs, they are secreted in an inactive form;

(iii)   enzyme activity is zinc dependent;

(iv)   expression of enzyme activity is tightly controlled at multiple levels.

1.5.3.1 Domain Structure of MMP Family

An understanding of the functional significance of the structural domains of the MMPs has shed light onto their common mechanisms of action as well as helped to elucidate the basis of their differing substrate specificities.

Each enzyme has: (i) a transient *signal peptide*, which directs the translation product to the endoplasmic reticulum; (ii) a *propeptide* domain, which contains the conserved amino acid sequence PRCGXPDV located at the site of an unpaired cysteine residue. Cleavage of the propeptide domain plays a critical role in the activation of the latent MMP, as described in section 1.8.3.2 (Springman et al, 1990; Stetler-Stevenson et al, 1991); (iii) the *catalytic* domain which contains the zinc-ion binding site, and, with the exception of matrilysin/PUMP-1, (iv) a *hemopexin/vitronectin-like COOH-terminal domain*, separated from the catalytic domain by a hinge region (fig. 1.4).
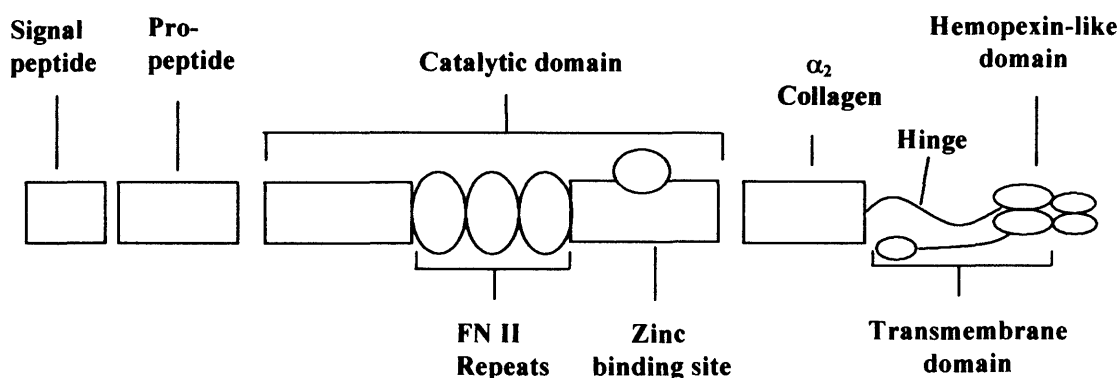
Fig. 1.4: Domain Structure of Matrix Metalloproteinases.
Only MMP-2 and MMP-9 possess the fibronectin type II-like repeats, and only MMP-9 has an $\alpha2$-collagen-like domain. The transmembrane domain is restricted to the MT-MMP subgroup.

The hemopexin domain is thought to be involved in defining substrate specificity, as demonstrated by studies employing COOH-terminally truncated enzymes which lose the ability to degrade their normal substrate (Marcy et al, 1991; Murphy et al, 1992). It also appears to play a role in the binding of MMPs with their specific inhibitors - loss of the COOH-terminal domain of MMP-2 prevents it binding to its naturally occurring inhibitor (Fridman et al, 1992). Matrilysin is unique amongst the MMPs in that it lacks the hemopexin-like domain, whilst the

19

gelatinases possess additional domains to other MMPs. MMP-9 has a fibronectin domain composed of fibronectin type II repeats, plus a collagen domain with sequence homology to the α2 chain of type IV collagen. MMP-2 also possess a fibronectin domain though lacks a collagen domain. Again these domains are thought to be involved in conferring substrate specificity on the enzyme (Clark & Cawston, 1989; Sanchez-Lopez et al, 1993). The MT-MMPs are unique among the MMPs in possessing a membrane spanning sequence in the fourth and last pexin-like repeat of the carboxy-terminal domain (Cao et al, 1995; Sato et al, 1994).

1.5.3.2 Structural Basis of MMP Activation - the cysteine-switch mechanism

The MMPs are synthesised as latent precursors and with the exception of STR-3 and the MT-MMPs, they are secreted as inactive zymogens, which are subsequently activated in the extracellular/pericellular environment. The cysteine-switch model was proposed to explain the complex activation process of MMP zymogens (Van Wart & Birkedal-Hansen, 1990; Springman et al, 1990; fig. 1.5).

In the high molecular weight latent form, the highly conserved propeptide is covalently bonded to the active site zinc atom through the thiol group of an unpaired cysteine residue near the end of the 80 amino acid propeptide. Disruption of the cysteine-zinc bond is a critical step in the activation process and may be achieved either by conformational perturbation, e.g. by agents such as SDS, or by limited proteolysis, e.g. by plasmin or trypsin.

Proteolysis destabilises the cysteine-zinc bond enough to free the zinc-binding site. Autocatalytic cleavage then occurs, converting the MMP to an active form. The extent of the activity of the enzyme is dependent on the position of cleavage. Thus, plasmin will cleave MMP-1 propeptide, but does not convert the enzyme to a fully active form and further cleavage by MMP-3 is required to yield full enzyme activity (Suzuki et al, 1990; Grant et al, 1987).

Other agents can directly cleave the propeptide domain without generation of an intermediate

form, or the requirement of another enzyme (Weiss, 1989).
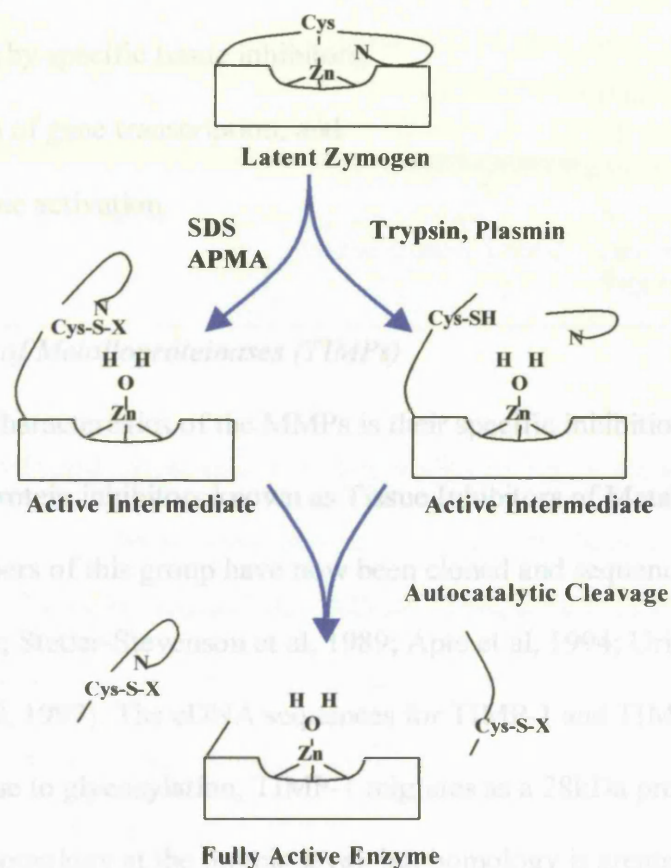
Fig. 1.5: Cysteine-Switch Mechanism for Activation of MMPs.
Cysteine in the pro-enzyme domain contacts Zinc (Zn) to maintain latency. Agents such as sodium dodecyl sulphate (SDS) and Aminophenylmercuric acid (APMA) unfold the structure to expose Zn. Alternatively proteolytic enzymes can cleave the propeptide, followed by autocatalytic cleavage to yield the fully active enzyme. (Adapted from Woessner, 1991).

Whilst the cysteine switch model is widely accepted as the mechanism of pro-enzyme

activation, other studies have suggested that the situation may be more complex, and that bonds

other than the cysteine-zinc bond may play a role in stabilising the latent enzyme (Chen et al,

1993; Freimark et al, 1994).

1.5.3.3 Control of MMP Activity

Uncontrolled action of proteolytic enzymes would lead to catastrophic effects in tissues. It is essential, therefore, that the action of these enzymes is tightly controlled. Control of MMP activity is achieved by several mechanisms:

- inhibition by specific tissue inhibitors,

- regulation of gene transcription, and

- pro-enzyme activation.

- *Tissue Inhibitors of Metalloproteinases (TIMPs)*

One of the defining characteristics of the MMPs is their specific inhibition by tight binding naturally occurring protein inhibitors known as Tissue Inhibitors of Metalloproteinases (TIMPs). Four members of this group have now been cloned and sequenced from human cells (Docherty et al, 1985; Stetler-Stevenson et al, 1989; Apte et al, 1994; Uria et al, 1994; Greene et al, 1996; Leco et al, 1997). The cDNA sequences for TIMP-1 and TIMP-2 code for 21kDa proteins, however, due to glycosylation, TIMP-1 migrates as a 28kDa protein. TIMP-1 and TIMP-2 show 38% homology at the protein level, but homology is greater between the NH-terminal domains than the COOH-terminal domains (Carmichael et al, 1986; Stetler-Stevenson et al, 1990; Apte et al, 1995). TIMP-3 shares 45% identity with TIMP-1 and TIMP-2 (Uria et al, 1994), whereas TIMP-4 shares 37% homology with TIMP-1 but 51% with TIMP-2 and TIMP-3 (Greene et al, 1996; Table 1.4). All of the TIMPs have 12 conserved cysteine residues paired into 6 disulphide bonds which divides the protein into two domains, thought to have distinct functions (Williamson et al, 1990).

Table 1.4: Tissue Inhibitors of Metalloproteinases (TIMPs)

INHIBITOR	MW	INHIBITORY ACTION	REFERENCE
TIMP-1	28kDa (glycosylated)	All active MMPs Active & latent MMP-2	Docherty et al, 1985
TIMP-2	21kDa	All active MMPs Active & latent MMP-9	Stetler-Stevenson et al, 1989
TIMP-3	21.7kDa	All active MMPs ?Active & latent MMP-2 ?Active & latent MMP-9	Uria et al, 1994 Apte et al, 1995
TIMP-4	26kDa	All active MMPs Active & latent MMP-2	Greene et al, 1996 Leco et al, 1997 Bigg et al, 1997

Whilst the TIMPs are characterised by their ability to inhibit active MMPs by forming essentially irreversible 1:1 molar stoichiometric complexes with the active enzymes (Cawston et al, 1981; DeClerk et al, 1991), there is also evidence of their interaction with latent gelatinases (Stetler-Stevenson et al, 1989; Wilhelm et al, 1989; Goldberg et al, 1989; Bigg et al, 1997). It is predicted that the COOH-terminal TIMP domain is gelatinase specific, thus TIMP-1 interacts specifically with latent MMP-9 (Goldberg et al, 1992), and TIMP-2 and TIMP-4 interact specifically with latent MMP-2 (Goldberg et al, 1989; Greene et al, 1996).

With the exception of MT1-MMP, which is efficiently inhibited by TIMP-2 and TIMP-3 but not by TIMP-1 (Will et al, 1996), all members of the TIMP family have a similar inhibitory action towards all active MMPs. The proposed model for the mechanism of interaction between TIMPs and the gelatinases is illustrated in figure 1.6, and is derived from a series of experiments including use of truncated enzymes and cross-linking techniques (Stetler-Stevenson et al, 1993).
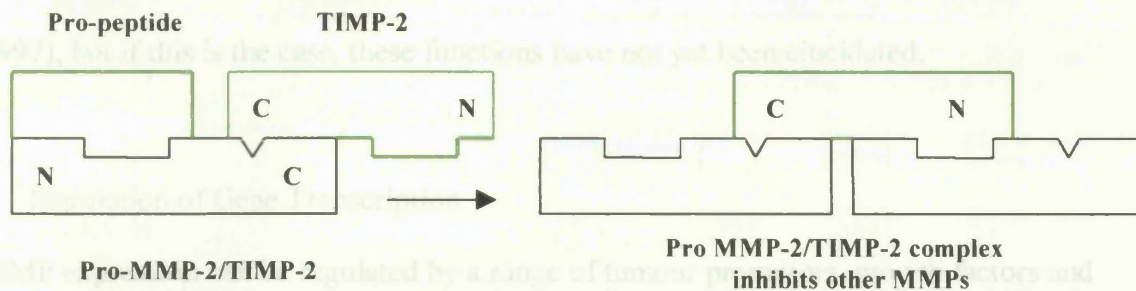
Fig 1.6: Interaction of TIMP-2 with Latent MMP-2 and other Active MMPs
The carboxy-terminal domain (C) of TIMP-2 binds specifically to latent MMP-2 and still maintains inhibitory action against other MMPs via the amino-terminal domain (N).
(Adapted from Stetler-Stevenson et al, 1993).

Taken together these studies suggest that TIMP-2 binds to the COOH-terminal domain of pro-MMP-2 and that this binding site is distinct from the active site of the enzyme. When the active site of the enzyme is revealed after activation of pro-MMP-2, TIMP-2 now forms a complex with MMP-2 including the active site, resulting in complete enzyme inhibition (Kleiner et al, 1992; Kolkenbrock et al, 1991; Curry et al, 1992). Similar experiments have indicated that the interaction of pro-MMP-9 with TIMP-1 is likely to be similar to this model (Goldberg et al, 1992).

The identification of TIMP-3 and TIMP-4 raises an interesting issue, which is the apparent existence of functional redundancy amongst the inhibitors. It is recognised that both TIMP-1 and TIMP-2 possess growth-promoting properties in addition to their MMP-inhibitory action (Gasson et al, 1985; Docherty et al, 1985; Hayakawa et al, 1992; Hayakawa et al, 1994, Saika et al, 1998), although in other situations the TIMPs have been shown to inhibit tumour cell growth (Imren et al, 1996; Watanabe et al, 1996; Wang et al, 1997). Recently, a further role for TIMP-2 has been described as it has been shown to provide a receptor mechanism for the activation of pro-MMP-2 (Strongin et al, 1995). These observations have led to the suggestion

that TIMP-3 and TIMP-4 may also have functions other than their inhibitory action (Bigg et al,

1997), but if this is the case, these functions have not yet been elucidated.

- Regulation of Gene Transcription

MMP expression can be regulated by a range of tumour promotors, growth factors and

cytokines (Brenner et al, 1989; Kerr et al, 1990; Masure et al, 1990; Guerin et al, 1997; Pilcher

et al, 1997; Uria et al, 1998), and functional studies of the promotor regions of some of the

genes have helped to elucidate the mechanisms of control. Many of the MMPs possess a TPA-

responsive element (TRE), or AP-1 binding site which appears to play an important role in

mediating the effects of cytokines and growth factors (McDonnell et al, 1990; Lafyatis et al,

1990; Huhtala et al, 1991; Birkedal-Hansen et al, 1993; Schiavolino et al, 1994; fig. 1.7). AP-1

transcription factors include members of the Fos and Jun family which through the formation of

homo- and heterodimers bind DNA with different affinities. This provides a mechanism for a

wide range of responses in gene activity according to the precise nature of the signal (Angel &

Karin, 1992; Karin et al, 1997).

    With the exception of MMP-2, most of the other MMP promotors contain conserved

PEA-3 elements, which binds members of the c-ets family of proteins which in turn are

inducible by ras and src activation (Gutman & Wasylyk, 1990). This responsiveness to proto-

oncogene products links MMP expression to cellular growth control, and this is further

supported by the induction of some MMPs by phorbol esters (Frisch et al, 1987; Moll et al,

1990), and growth factors such as EGF (Kondapaka et al, 1997; Festuccia et al, 1996). A

putative TGF-β1 inhibitory element has also been described in the MMP-9 promotor region

(Kerr et al, 1990), further linking control of MMP expression to growth factor signalling.
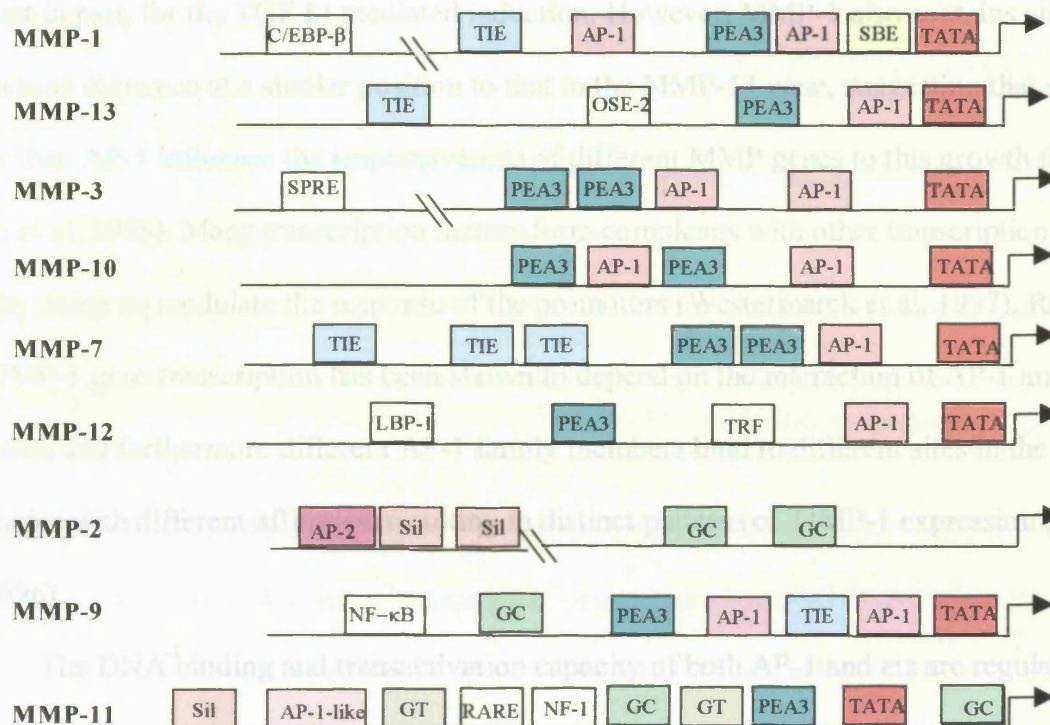
Fig. 1.7: Regulatory Elements of Promotor Regions of Human MMP Genes.
Boxes indicate the following elements: **AP-1**: activator protein-1; **PEA3**: Polyoma enhancer A binding protein-3; **TIE**: TGF-β inhibitory element; **GC**: Sp-1 binding site; **SBE**: STAT binding element; **C/EBP-b**: CCAAT/enhancer binding protein-β; **OSE-2**: osteoblast-specific element; **SPRE**: stromelysin-1 PDGF-responsive element; **TRF**: octamer binding protein; **Sil**: silencer sequence; **NF-κB**: nuclear factor κB; **NF-1**: nuclear factor-1; **RARE**: retinoic acid responsive element. (adapted from Westermarck & Kahari, 1999).

In some situations MMPs are co-ordinately regulated e.g. in the case of phorbol ester induction of MMP-3 and MMP-1 in endothelial cells (Frisch et al, 1987), or the induction of MMP-1 and MMP-13 by IL-1β (Uria et al, 1998). In other cases, however, there is divergent regulation of MMP genes, e.g. in fibroblasts, TGF-β1 markedly down regulates MMP-1 transcription but enhances expression of the closely related MMP-13 (Uria et al, 1998). Functional studies have revealed that an AP-1 binding site in the MMP-13 gene is responsible,

26

at least in part, for the TGF-β1 mediated induction. However, MMP-1 also contains an AP-1

consensus sequence at a similar position to that in the MMP-13 gene, suggesting that sequences

other than AP-1 influence the responsiveness of different MMP genes to this growth factor

(Uria et al, 1998). Many transcription factors form complexes with other transcription factors

and by doing so modulate the response of the promotors (Westermarck et al, 1997). Regulation

of TIMP-1 gene transcription has been shown to depend on the interaction of AP-1 and ets

proteins, and furthermore different AP-1 family members bind to different sites in the TIMP-1

promotor with different affinities, resulting in distinct patterns of TIMP-1 expression (Logan et

al, 1996).

The DNA binding and transactivation capacity of both AP-1 and ets are regulated by

phosphorylation under the control of mitogen activated protein kinases (MAPKs). MAPKs

mediate signals triggered by growth factors, cell-cell and cell-matrix interactions (Robinson &

Cobb, 1997). This link between cell adhesion and regulation of MMP gene expression has been

demonstrated by the induction of MMP-13 in fibroblasts grown in collagen gels via a MAPK-

dependent pathway (Ravanti et al, 1999).

Perhaps one of the most striking features is the tissue-specific response to different

factors. Thus TGF-β1 inhibits MMP-1 expression in fibroblasts but induces transcription in

keratinocytes (Mauviel et al, 1996). Many studies have shown βFGF to induce MMP

expression in a range of cell types including fibroblasts, smooth muscle cells, osteoblasts and

endothelial cells (Sasaki, 1992; Cornelius et al, 1995; Hurley et al, 1995), however, MMP-1 is

inhibited by βFGF in basal keratinocytes (Pilcher et al, 1997). In summary, the literature

indicates a highly complex control system for MMP and TIMP expression which for any given

situation cannot easily be predicted.

- Pro-enzyme Activation

It is evident from the previous discussion that activation is a key step for MMPs to function in-vivo. In-vitro studies have identified a range of factors capable of activating pro-MMPs including organomercurials, HOCl, acid exposure and growth factors (Birkedal-Hansen et al, 1993). Serine proteases such as plasmin, trypsin, kallikrein, leukocyte elastase or cathepsin G may be particularly important activators in-vivo, since they have the ability to activate many of the pro-MMPs (Suzuki et al, 1990; Nagase et al, 1991; Okada et al, 1992). In addition to this activation by other protease classes, MMPs can activate each other, thus pro-MMP-9 can be activated by matrilysin, interstitial collagenase, stromelysin-1 and MMP-2 (Ogata et al, 1995; Sang et al, 1995; Fridman et al, 1995). In some situations the site of cleavage in the propeptide domain can result in so-called "superactivation" of an enzyme, e.g. stromelysin-1 cleaves ~15 residues from the COOH-terminal region of pro-MMP-1 to yield a five- to eight fold enhanced collagenase activity, compared to the plasmin activated enzyme (He et al, 1989). A further example of superactivation was recently demonstrated by the highly efficient activation of pro-MMP-9 by human trypsin (Sorsa et al, 1997). A complex activating cascade has recently been identified for pro-MMP-9 in which plasmin generates active MMP-3, which is itself a highly potent activator of latent MMP-9 (Ramos-DeSimone et al, 1999).

However, pro-MMP-2 is unique in that it cannot be directly activated by the serine proteases (Mignati & Rifkin, 1993), and whilst the precise mechanisms of its activation are still not fully clarified, the existence of membrane receptors (Emonard et al, 1992) and dependence on a membrane-associated system has been recognised for some time (Azzam et al, 1993; Brown et al, 1993; Strongin et al, 1995), and the discovery of the Membrane-Type-MMPs (MT-MMP) has allowed the details of this mechanism to start to be dissected.

1.5.4 MT-MMPs - a new family of MMPs

In 1994, using degenerate PCR primers with homology to conserved amino acid regions in the cysteine switch and catalytic domains of known MMP genes, Sato et al (1994) isolated a novel MMP from a human placenta cDNA library. In common with other MMPs it encodes a signal domain, a propeptide sequence, a core-enzyme domain with a potential zinc binding site and a hemopexin-like domain. However, uniquely amongst the MMPs, at the C-terminus is a potential transmembrane domain composed of 24 hydrophobic amino acids, and on this basis the gene was termed Membrane-Type Matrix Metalloproteinase (MT-MMP). Evidence for the function of this transmembrane domain was provided by transfection of the MT-MMP plasmid into COS-1 cells, with the protein product being localised to the cell surface (Sato, 1994). Its gelatinase activating potential was demonstrated by transfection of human fibrosarcoma HT1080 cells and mouse fibroblast NIH3T3 cell lines: these cell lines secrete pro-MMP-2 and pro-MMP-9, but are not able to activate these species. Transfection with MT-MMP generated active MMP-2 but not active MMP-9. Further confirmation of the role of MT-MMP as a membrane activator of pro-MMP-2 was provided by co-transfecting COS-1 cells with pro-MMP-2 and MT-MMP plasmids and revealing active MMP-2 which was co-localised with MT-MMP at the cell membrane (Sato et al, 1994).

Using a similar approach of degenerate PCR primers, four further MMPs, each with the characteristic transmembrane domain, have been identified, and termed MT2-, MT3-, MT4- and MT5-MMP respectively (Will & Hinzmann, 1995; Takino et al, 1995; Puente et al, 1996; Llano et al, 1999; Pei, 1999). All but MT4-MMP have been shown to activate pro-MMP-2 (Takino et al, 1995; Butler et al, 1997; Llano et al, 1999; Pei, 1999). It is possible that in cases where cell-mediated activation of pro-MMP-2 is evident in the absence of MT1-MMP, other members of this subfamily may be involved.

1.5.4.1 Regulation of MT-MMP Expression

- Regulation of Gene Expression

It is evident that factors controlling MT1-MMP gene expression are cell type specific. MT1-MMP is up-regulated by TNF-α in synovial cells (Migita et al, 1996), and by TNF-α, IL-1β, EGF and βFGF in human embryonic lung fibroblasts (Lohi et al, 1996). The lectin Concanavalin A induces MT1-MMP activity in some cells, including fibroblasts. Only a modest increase in mRNA levels is detected and part of the effect of this lectin may be to enhance the cross-linking of MT1-MMP receptors (Yu et al, 1995). Recent studies have demonstrated that MT1-MMP expression can be regulated by changes in cytoskeletal organisation (Tomasek et al, 1997) and also by certain ECM components, such as fibronectin (Stanton et al, 1998).

- Post-Translational Regulation

A distinct feature of all members of the MT-MMP family is a conserved Arg-Arg-Lys/Arg-Arg sequence (so called RRKR sequence) sandwiched between the pro-and catalytic domains (Sato 1994). This sequence, which is also present in stromelysin-3 (STR-3) at the same position (Bassett et al, 1990) is characteristic of a recognition sequence for a family of precursor processing endoproteases (Pei & Weiss 1995). Furin, one of the most ubiquitously expressed of these endoproteases (Steiner et al, 1992; Seidah et al 1993), has been implicated in the activation of the MT1-MMP pro-enzyme in the transgolgi network (Pendas et al, 1997). The ability of plasmin to cleave soluble pro-MT-MMP within the RRKR recognition motif has recently been demonstrated (Okumuru et al, 1997), and this plasmin-activated MT-MMP exhibits pro-MMP-2 activating properties. Plasmin does not itself directly activate pro-MMP-2. Thus there are at least two mechanisms for the activation of MT-MMP - intracellular activation

30

via furin and extracellular activation via plasmin, which is likely to be of importance in the fine regulation of a cell's proteolytic activity.

1.5.4.2 Mechanism of pro-MMP-2 activation via MT-MMP

Studies using mutant proteins and GST-fusion proteins have started to unravel the complex mechanisms involved in the MT-MMP-mediated activation of MMP-2. A number of studies have demonstrated that the C-terminal domain of pro-MMP-2 is required for binding of the zymogen to TIMP-2, and is vital for activation of pro-MMP-2 by cell membranes (Murphy et al, 1992; Fridman et al, 1992; Willenbrock et al, 1993; Strongin et al, 1993; Ward et al, 1994). Strongin et al (1993) found low levels of TIMP-2 enhanced ability of MT-MMP to activate pro-MMP-2, whilst inhibiting this same process at higher concentrations. Using the haemopexin-like domain fragment of MMP-2, Strongin et al (1995) isolated TIMP-2 complexed with MT1-MMP from plasma membrane preparations, and proposed the formation of a trimolecular complex of pro-MMP-2/TIMP-2/MT1-MMP for activation. Butler et al (1998) demonstrated that interactions occur between the C-terminal domain of TIMP-2 and proMMP-2, and between the N-terminal domains of TIMP-2 and MT1-MMP and that whilst MT1-MMP-mediated activation was dependent on TIMP-2, it was also inhibited at higher TIMP-2 concentrations. On the basis of these findings they proposed the current model in which TIMP-2 binds to MT1-MMP via its N-terminal domain and through C-terminal domain interactions, co-localises and concentrates pro-MMP-2 at the cell membrane which is then activated by adjacent 'free' i.e. non-TIMP-2 bound, MT1-MMP molecules. Higher concentrations of TIMP-2 are thought to lead to binding and inactivation of all MT1-MMP receptors, which are then unable to activate pro-MMP-2 (fig. 1.8). TIMP-3 can also bind MT1-

MMP and pro-MMP-2, and it has been suggested that it may therefore provide an alternative

receptor mechanism for pro-MMP-2 activation (Will et al, 1996).

MT1-MMP   **MT-MMP**

TIMP-2   PRO-MMP-2

PRO-MMP-2   **PRO MMP-2**
RECEPTOR   **ACTIVATOR**
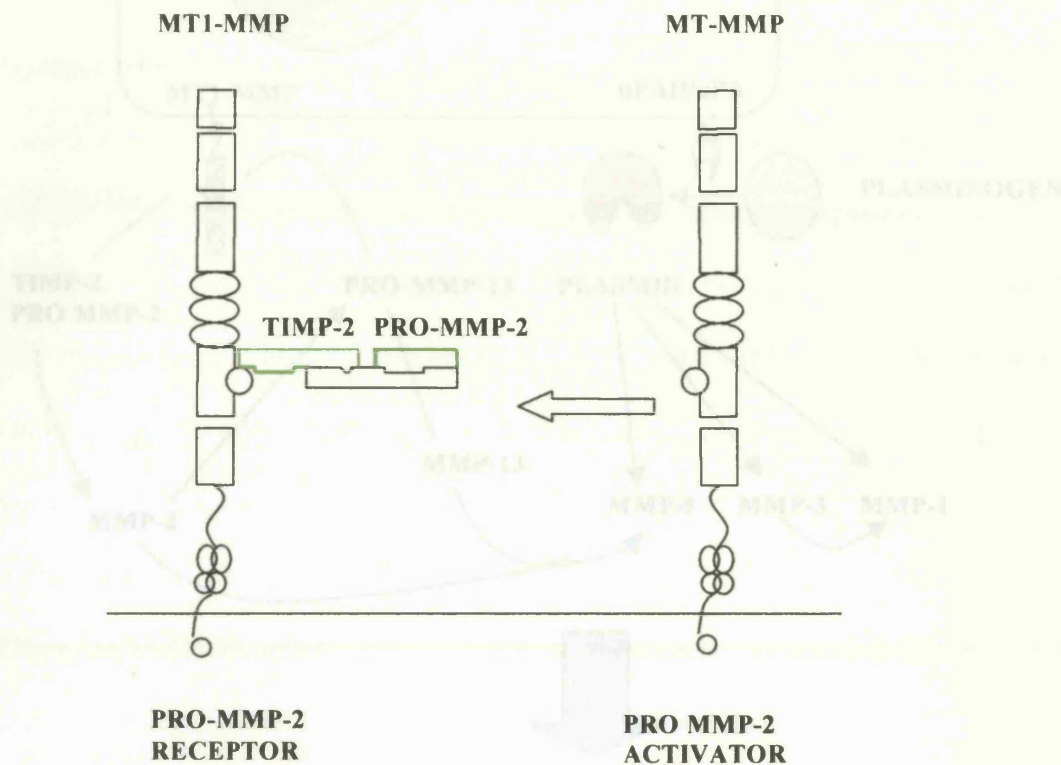
Fig. 1.8: Mechanism of MT1-MMP-mediated activation of pro-MMP-2
One MT1-MMP molecule acts as a receptor for the TIMP-2/MMP-2 complex, whilst an adjacent non-TIMP-2-
bound molecule then activates the latent MMP-2.

1.5.4.3 Interaction of MT1-MMP with other MMPs

Until recently, the membrane associated activation of pro-MMP-2 by MT-MMP was thought to

be totally specific, however, MT1-MMP has also been shown to directly activate MMP-13

(collagenase 3) in a process that is inhibited by TIMP-2 and TIMP-3 (Knauper et al, 1996;

Cowell et al, 1998). However, MMP-13 does not interact with the C-terminal domain of TIMP-

2, thus the mechanism by which MT1-MMP may activate MMP-13 may be profoundly

different to that described for pro-MMP-2 activation (Knauper et al, 1997).

32

MT1-MMP   uPAR/uPA

PLASMINOGEN

TIMP-2
PRO MMP-2   PRO-MMP-13   PLASMIN

MMP-13

MMP-2   MMP-9   MMP-3   MMP-1

MATRIX
DEGRADATION
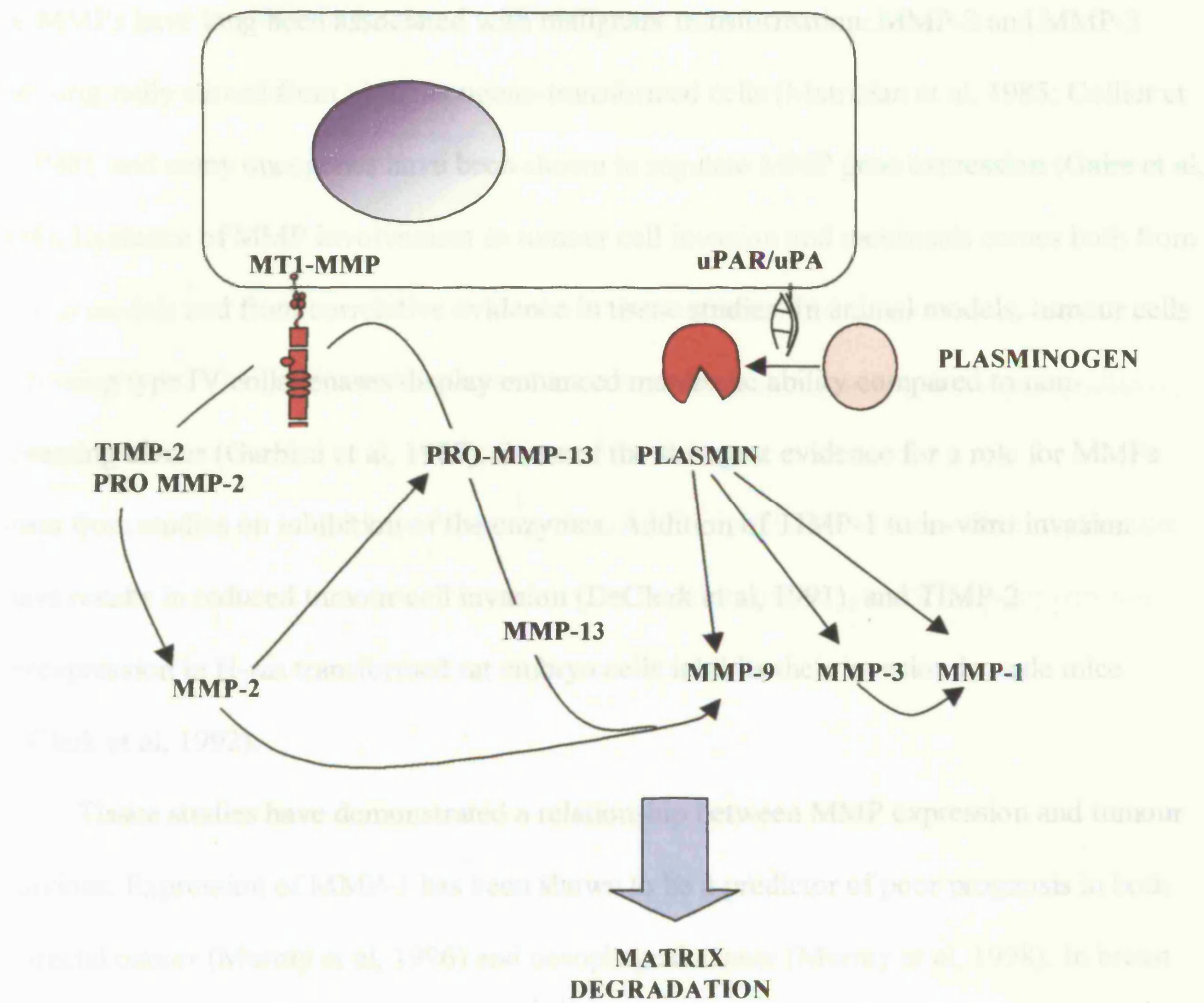
Fig. 1.9: Matrix Metalloproteinase Activation Cascades

The potential for MT1-MMP interaction with other activation cascades, such as the indirect activation of pro-MMP-9 by activated MMP-13 (Knauper et al, 1997b), as well as possessing intrinsic proteolytic activity (Ohuchi et al, 1997) suggests that these molecules may be pivotal factors in the orchestration of proteolysis (fig. 1.9).

1.5.5 The Role of MMPs in Cancer

The MMPs have long been associated with malignant transformation. MMP-2 and MMP-3 were originally cloned from viral oncogene-transformed cells (Matrisian et al, 1985; Collier et al, 1988), and many oncogenes have been shown to regulate MMP gene expression (Gaire et al, 1994). Evidence of MMP involvement in tumour cell invasion and metastasis comes both from in-vitro models and from correlative evidence in tissue studies. In animal models, tumour cells expressing type IV collagenases display enhanced metastatic ability compared to non-expressing clones (Garbisa et al, 1987). Some of the strongest evidence for a role for MMPs comes from studies on inhibition of the enzymes. Addition of TIMP-1 to in-vitro invasion assays results in reduced tumour cell invasion (DeClerk et al, 1991), and TIMP-2 overexpression in H-ras transformed rat embryo cells inhibits their invasion in nude mice (DeClerk et al, 1992).

Tissue studies have demonstrated a relationship between MMP expression and tumour behaviour. Expression of MMP-1 has been shown to be a predictor of poor prognosis in both colorectal cancer (Murray et al, 1996) and oesophageal cancer (Murray et al, 1998). In breast and bladder cancer, both high levels and enhanced activity of MMP-2 and MMP-9 correlate with high tumour grade (Davies et al, 1993a; Davies et al, 1993b) and a number of studies have correlated elevated gelatinase activity with the presence of lymph node metastases (Brown et al, 1993; Tokuraku et al, 1995; Iwata et al, 1996). MMP-11 is overexpressed in many human tumours including breast (Bassett et al, 1990) and head and neck carcinomas (Muller et al, 1993).

As well as having a role in modulating tumour invasion, it is evident that MMPs may contribute to tumour progression by a number of other mechanisms. MMP activity can promote the bio-availability of growth factors. The insulin-like growth factor-1 binding protein-1 (IGFBP-1) has recently been identified as the physiological substrate of MMP-3. Cleavage of

34

this binding protein releases IGF-1 and –II thus promoting cell survival and proliferation

(Manes et al, 1997). Via disruption of cell-BM interactions, MMP activity also influences

apoptosis (Boudreau et al, 1995; Witty et al, 1995). MMPs may promote tumour progression by

regulating tumour angiogenesis or neovascularisation, in particular MMP-2 (Schnaper et al,

1993; Itoh et al, 1998). Direct evidence that MMP-2 plays a significant role in tumour

angiogenesis was recently provided by the development of MMP-2 deficient mice. The rate of

angiogenesis and the growth and metastatic behaviour of melanoma cells and lung carcinoma

cells are markedly reduced in these mice (Itoh et al, 1998).

Thus it is evident that MMPs are involved at multiple levels in the evolution of cancer

and elucidation of the mechanisms that regulate expression and activity of these enzymes is

essential for understanding the process of tumour progression.

1.6 Cell Adhesion Molecules

Interactions between cells and between cells and matrix components are known to be crucial

for maintaining tissue structure and for the regulation of tissue differentiation and function in a

range of organisms. It is now clear that there are a large number of cell adhesion molecules

which, on the basis of conserved functional domains or sequence homology, can be placed into

protein families. The major families are indicated in Table 1.5, and the function of those groups

that are the focus of this study is discussed below.

35

Table 1.5: Classes of Cell Adhesion Molecules. Their Ligands and Tissue Distribution

FAMILY	EXAMPLES	LIGANDS	DISTRIBUTION
Cadherins	E-Cadherin P-Cadherin N-Cadherin Desmosomal Cadherins	Homophilic	Epithelia Placenta, skin, epithelia Neural & mesenchymal tissue Skin, epithelia
Immunoglobulins	ICAM-1 V-CAM N-CAM	$\beta2$ integrins VLA-4 integrin homophilic	Endothelium, fibroblasts Endothelium Neural & mesenchymal tissue
Integrins	$\beta1$ integrins (VLA-1-8) $\beta2$ integrins (LFA-1)	Extracellular matrix ICAM-1	Widespread - epithelia, mesenchymal Leukocytes
Selectins	L-Selectin E-Selectin P-Selectin	MadCAM, CD34 Sialyl Lewis a PSGL Sialyl Lewis x (all)	Leukocytes Activated endothelium Platelets, endothelium
CD44	Multiple isoforms due to alternative splicing	Hyaluronic acid, col, ln, homotypic.	Leukocytes (90kDa variant) Epithelial & mesenchymal cells (160kDa variant)

ICAM: Intercellular Cell Adhesion Molecule; V-CAM: Vascular Cell Adhesion Molecule; N-CAM: Neural cell Adhesion Molecule; MadCAM: Mucosal Addressin Cell Adhesion Molecule; PSGL: P-Selectin Glycoprotein Ligand

1.6.1 Cell-Cell Adhesion

Family members of the Cadherin, Immunoglobulin and Selectin groups are all involved in mediating cell-cell interactions. The Cadherins are of particular importance in the context of epithelial cell function and the following discussion will focus on this group of molecules.

1.6.1.1 Cadherins

The Cadherins are a family of transmembrane glycoproteins which are involved predominantly in mediating homophilic adhesive interactions in a Ca^{++} dependent manner (Takeichi, 1995).

36

On the basis of structural homology many new family members have been described, but for many a role in cell adhesion has not been demonstrated (Berndorff et al, 1994; Dantzig et al, 1994; Sugimoto et al, 1996). The most well characterised members are the classical Cadherins and of these the most extensively studied are E-Cadherin (also known as uvomorulin and L-CAM), P-Cadherin and N-Cadherin. These molecules are expressed in a tissue-specific manner and were named according to the cell types and tissues in which they were first discovered (Table 1.5). The classical Cadherins are glycoproteins of 115-140 kDa with a large N-terminal extracellular domain, a single membrane-spanning domain and a C-terminal cytoplasmic tail (Takeichi, 1995; fig 1.10). The extracellular domain can be divided into five repeated subdomains, termed EC1-5, which contain highly conserved Ca^{++}-binding motifs, and at the N-terminal end, an HAV motif (histidine, alanine, valine tripeptide), which appears to be involved in binding specificity (Blaschuk et al, 1990; Nose et al, 1990; Noe et al, 1999). The three-
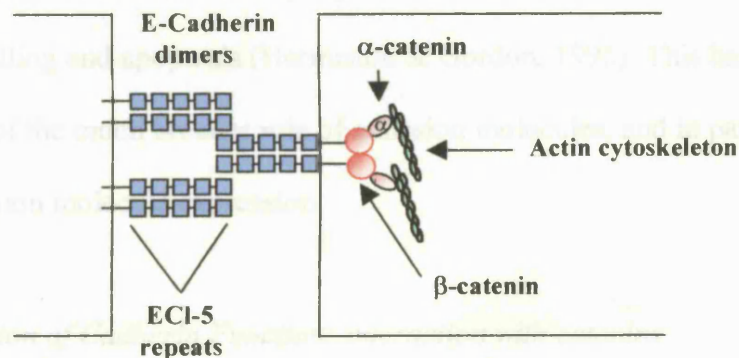
Fig. 1.10: Structure of E-Cadherin and Interaction with Catenins and Actin Cytoskeleton

dimensional structures of E-Cadherin and N-Cadherin suggest that the extracellular domains form dimers, with two monomers arranged in parallel at the plasma membrane, and each dimer interacts with another dimer on the opposite membrane (Overduin et al, 1995; Shapiro et al,

1995; fig 1.10). The classical Cadherins are defined by a highly conserved cytoplasmic domain and the capability to interact with the catenin family of cytoplasmic proteins, which links the cell adhesion molecule to the actin cytoskeleton (Ranscht, 1994).

Classical Cadherins have important roles in the formation and maintenance of tissues during gastrulation, neurulation and organogenesis, and are also implicated in the formation of segmental boundaries in the developing CNS (Takeichi, 1995). Knockout experiments have demonstrated that E-Cadherin is essential for the formation and maintenance of epithelia (Larue et al, 1994).

As well as adhesive function, a number of studies have supported a role for Cadherins in cell signalling. Homophilic adhesion of N-Cadherin activates a Fibroblast Growth Factor Receptor-dependent signalling pathway that triggers neurite outgrowth (Saffell et al, 1997). Furthermore, when mutant N-Cadherin lacking the extracellular domain is expressed in mouse intestinal epithelial cells, cell-cell contacts are disrupted and the polarised phenotype is lost, but in addition the cells enter a cell death programme, indicating a link between Cadherin mediated adhesion, signalling and apoptosis (Hermiston & Gordon, 1995). This has relevance for our understanding of the much broader role of adhesion molecules, and in particular the effects of abnormal adhesion molecule expression.

1.6.1.2 Regulation of Cadherin Function: interaction with catenins

The classical Cadherins interact with the actin cytoskeleton via a group of cytoplasmic proteins termed catenins, and this linkage is vital for adhesive function (Aberle et al, 1996). The catenins are members of the armadillo family of proteins and include the armadillo protein in Drosophila, β-catenin, plakoglobin, which is identical to γ-catenin (Knudsen & Wheelock, 1992), and $p120^{CAS}$. α-catenin is related both structurally and functionally to vinculin, and acts as a linker protein, binding directly to the actin cytoskeleton (Herrenknecht et al, 1991;

38

Nagafuchi et al, 1991). α-catenin does not bind directly to the Cadherins but is linked via β-catenin or plakoglobin, which form mutually exclusive complexes with Cadherins (Aberle et al, 1996; fig.1.3). The linkage of the Cadherins to the catenins is a dynamic process: following synthesis in the endoplasmic reticulum, E-Cadherin binds directly to β-catenin or plakoglobin and is then directed to the plasma membrane, where α-catenin is incorporated into the complex (Hinck et al, 1994). $p120^{CAS}$ binds directly to E-Cadherin in both the β-catenin-E-Cadherin and plakoglobin-E-Cadherin complexes (Daniel & Reynolds, 1995). Its role is not clear, but it is known to be a major src substrate and is phosphorylated in response to ligand stimulation of receptor tyrosine kinases (Aberle et al, 1996).

Under normal conditions, there is tight control of the level of free β-catenin in the cell (fig. 1.11). Excess β-catenin is phosphorylated by Glycogen Synthase Kinase-3β (GSK-3β) favouring binding by the Adenomatous Polyposis Coli (APC) gene product, which targets the β-catenin to the ubiquitin-proteosome pathway for degradation (Polakis, 1997; fig. 1.11). Truncating mutations in APC (for example in colon cancer) or mutations in β-catenin can prevent phosphorylation, resulting in its accumulation in the cytoplasm (Rubinfeld et al, 1997; Morin et al, 1997). Free β-catenin then binds to transcription factors of the T Cell Factor (Tcf) and Lymphocyte Enhancing Factor-1 (LEF-1) family, which translocate to the nucleus and activate gene expression (Jankowski et al, 1997; Peifer, 1997; fig. 1.11). The target genes affected by this event have not been fully identified, though E-Cadherin itself (Jankowski et al, 1997), c-myc (Pennisi, 1998) and MMP-7/matrilysin (Crawford et al, 1999) are amongst the genes so far reported to show a change in expression.

Evidence is rapidly accumulating that Cadherin function is regulated by signalling pathways. Activation of the Epidermal Growth Factor (Hoschuetzky et al, 1994) and Hepatocyte Growth Factor (Shibamoto et al, 1994) receptor tyrosine kinases induces tyrosine
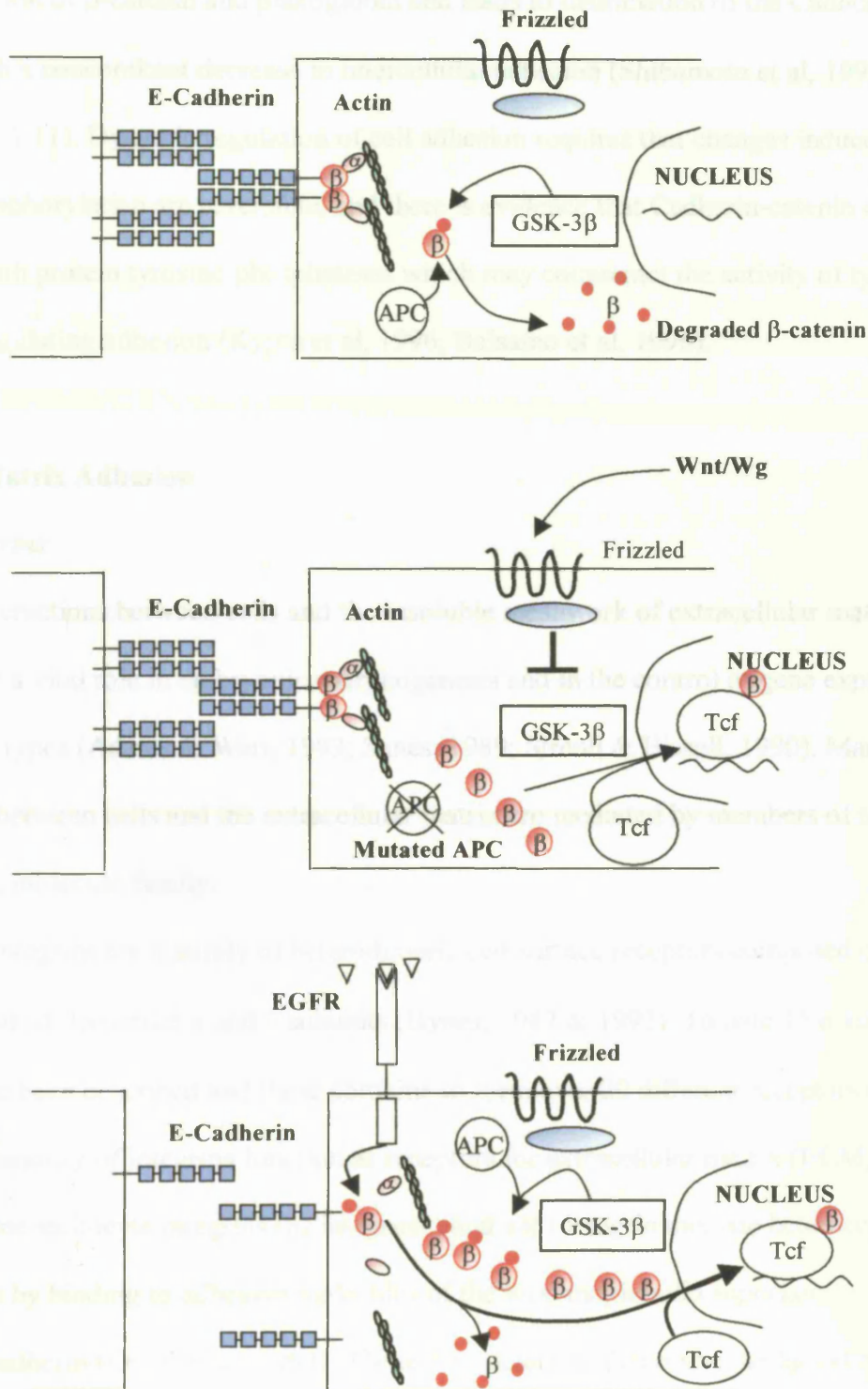
Fig. 1.11: Regulation of Cadherin – Catenin Complex.
Phosphorylation of free β-catenin by glycogen synthetase kinase –3β (GSK-3β) targets it for degradation. Activation of Wnt pathway or mutations of APC prevent β-catenin degradation which may activate gene transcription. Activation of Epidermal Growth Factor Receptor (EGFR) can dissociate the E-Cadherin-catenin complex and result in increased free β-catenin and subsequent gene transcription.

phosphorylation of β-catenin and plakoglobin and leads to dissociation of the Cadherin-catenin

complex with a concomitant decrease in intercellular adhesion (Shibamoto et al, 1995; Kinch et

al, 1995; fig. 1.11). Dynamic regulation of cell adhesion requires that changes induced by

tyrosine phosphorylation are reversible, and there is evidence that Cadherin-catenin complexes

are linked with protein tyrosine phosphatases which may counteract the activity of tyrosine

kinases in regulating adhesion (Kypta et al, 1996; Balsamo et al, 1996).

1.6.2 Cell-Matrix Adhesion

1.6.2.1 Integrins

Adhesive interactions between cells and the insoluble meshwork of extracellular matrix

proteins play a vital role in embryonic morphogenesis and in the control of gene expression in a

range of cell types (Adams & Watt, 1993; Sanes, 1989; Streuli & Bissell, 1990). Many of the

interactions between cells and the extracellular matrix are mediated by members of the integrin

cell adhesion molecule family.

The integrins are a family of heterodimeric cell surface receptors composed of non-

covalently linked dissimilar α and β subunits (Hynes, 1987 & 1992). To date 15 α and 8 β

subunits have been described and these combine to form over 20 different receptors (Hynes

1992). The majority of integrins function as receptors for extracellular matrix (ECM) proteins,

however, some leukocyte integrins (β2 integrins) and α4β1 integrin mediate heterotypic cell-

cell adhesion by binding to adhesion molecules of the immunoglobulin superfamily, and αEβ7

binds to E-Cadherin (Cepek et al, 1994). The α/β associations determine the ligand binding

specificities of the receptor for various extracellular matrix proteins (Table 1.6).

Whilst some integrins for example α5β1, the classical fibronectin receptor, recognises

only fibronectin (Brown & Juliano, 1985), more commonly an integrin is capable of binding

several ligands (Takada et al, 1989; Hynes, 1992). There is further functional overlap in that

several integrins may bind to the same matrix protein, for example, there are at least 7 different

receptors for fibronectin. In some cases the integrins bind to different regions of the ligand, as

do the α4β1 and α5β1 receptors (Guan & Hynes, 1990), whereas in other cases the integrins bind

to the same region of the protein, e.g. the binding of α5β1 and α3β1 to fibronectin (Elices et al,

1991). This apparent redundancy in the system suggests that integrins function as more than

mere sticky molecules, and considerable interest has focused on their role as potential

signalling molecules.

Integrins are well adapted for a signalling function, providing a transmembrane link

between the extracellular matrix and the cellular actin cytoskeleton (Horwitz et al, 1986; fig.

1.12). Both α and β subunits possess large extracellular domains - 675-700 amino acids in β

chains, 950-1100 amino acids in α chains. Each β subunit contains 56 cysteine residues, which

suggests a highly folded conformation, and in the N-terminal domain there is extensive internal

disulfide bonding. In α subunits there are seven repeats in the N-terminal part of the molecule

of which three or four are highly homologous to EF-hand-like motifs with calcium binding

properties. These cation-binding sites have a major impact on integrin-ligand binding. Some α

subunits have a further I domain to denote an insertion or interactive domain, which is also

thought to influence ligand binding. The adhesive motif of integrin receptors was elucidated by

Pierschbacher and Ruoslahti (Pierschbacher et al, 1981; Pierschbacher and Ruoslahti, 1984),

who found that the adhesive capacity of fibronectin was mimicked by a small synthetic peptide

containing the Arg-Gly-Asp (RGD) sequence. Since then other peptide ligands for integrins

have been identified that lack the RGD motif, however, most contain an aspartic acid residue

which is thought to be essential for function (Smith, 1994).

The cytoplasmic domains of both subunits of the integrin receptors interact with the

cytoskeleton. With the exception of the β4 subunit which is 1000 amino acids long, the β chain

42

Table 1.6: The Integrin Family of Receptors and their Ligands

RECEPTOR			LIGAND	DISTRIBUTION
$\beta 1$	VLA-1	$\alpha 1 \beta 1$	Ln, Col	Broad
	VLA-2	$\alpha 2 \beta 1$	Ln, Col	Broad
	VLA-3	$\alpha 3 A \beta 1$	Ln, Col, Fn, Epiligrin	Broad
		$\alpha 3 B \beta 1$	Ln, Col, Fn, Epiligrin	Broad
	VLA-4	$\alpha 4 \beta 1$	Fn (V25), VCAM-1	B & T cells, mac. neural crest cells.
	VLA-5	$\alpha 5 \beta 1$	Fn (RGD)	Broad
	VLA-6	$\alpha 6 A \beta 1$	Ln	Broad
		$\alpha 6 B \beta 1$	Ln	Broad
	VLA-7	$\alpha 7 A \beta 1$	?	?
		$\alpha 7 B \beta 1$	Ln	?
	VLA-8	$\alpha 8 \beta 1$	?	?
		$\alpha V \beta 1$	Fn, Vn	Epithelial cells
$\beta 2$	LFA-1	$\alpha L \beta 2$	ICAM-1, ICAM-2, ICAM-3	
	CR3, Mac-1	$\alpha M \beta 2$	C3bi, factor X, Fbn	Leukocytes
	p150,95	$\alpha X \beta 2$	C3bi, Fbn	
$\beta 3$	gpIIb/IIIa	$\alpha IIb \beta 3$	Fbn, Fn, vW factor, Vn, Thrsp	Platelets
	VNR	$\alpha V \beta 3$	Fbn, Fn, vW factor, Vn, Thrsp, osteopontin	Endothelial cells, osteoclasts.
$\beta 4$		$\alpha 6 A \beta 4$	Laminin 5	Epithelial cells
		$\alpha 6 B \beta 4$	Laminin 5	Epithelial cells
$\beta 5$		$\alpha V \beta 5$	Vn	Carcinoma cells
$\beta 6$		$\alpha V \beta 6$	Fn	?
$\beta 7$		$\alpha 4 \beta 7$,	Fn, VCAM-1, MadCAM-1	Active B & T cells,
		$\alpha E \beta 7$	E-Cadherin	intraepithelial lymphocytes.
$\beta 8$		$\alpha B \beta 8$	?	?
		$\alpha 9 \beta 8$	?	?

Ln: laminin; Col: collagen; Fn: fibronectin; VCAM: vascular cell adhesion molecule; ICAM: intercellular adhesion molecule; C3bi: activated complement component C3; Fbn: fibrinogen; vW factor: von Willebrand factor; Vn: vitronectin; Thrsp: thrombospondin; MadCAM: mucosal addressin cell adhesion molecule.

cytoplasmic domains are relatively short at 40-60 amino acids, and are highly conserved. The

β4 subunit is entirely novel having a larger cytoplasmic domain compared to other β chains, and

linking to intermediate filaments rather than to the actin cytoskeleton. In contrast to the

sequence similarities seen among the β chains, the α cytoplasmic domains, whilst containing

some similar motifs, show significant differences in sequence. From this diversity of receptor

structure it is possible to elicit a range of cellular responses through binding of a single ligand.
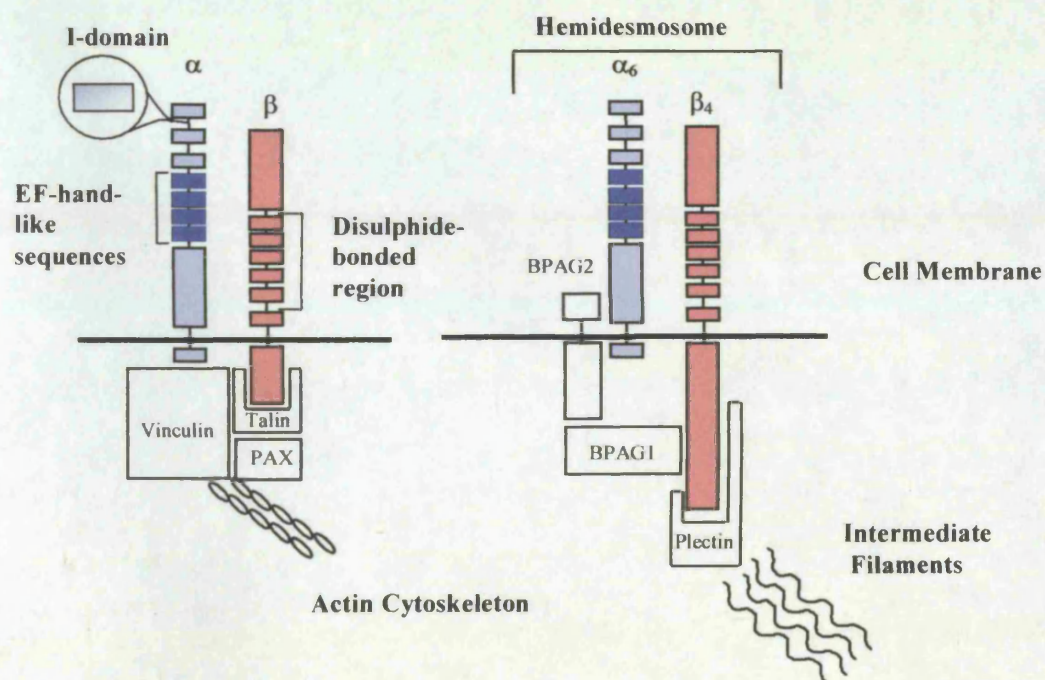
Fig. 1.12: Structure and Cytoskeletal Interactions of Integrin Heterodimers.
Most integrin heterodimers link to the actin cytoskeleton. α6β4 integrin is a component of hemidesmosomes and binds to the intermediate filament system.
PAX: Paxillin; BPAG1: Bullous Pemphigoid Antigen 1; BPAG2: Bullous Pemphigoid Antigen 2

1.6.2.2 Signalling by Integrin Receptors

Signals of two kinds can be elicited by integrin receptors, so called "inside-out" signalling and "outside-in" signalling (Ginsberg et al, 1992; Juliano & Haskill, 1993; fig. 1.13). Inside-out signalling regulates the affinity state of the integrin receptor (Schwartz et al, 1995) and is proposed to involve the propagation of conformational changes from the cytoplasmic domains of integrins to the extracellular binding site in response to intracellular signalling events (O'Toole et al, 1994). Several regions within the cytoplasmic domains of both α and β subunits have been shown to be involved in regulating the affinity state of the integrin receptor. There is evidence that conformational changes within these regions, brought about through phosphorylation or dephosphorylation events, allow the association of other proteins which can regulate integrin activity state (Coppolino et al, 1996; Dedhar & Hannigan, 1996). Thus certain interactions between integrins and cytoskeletal components may restrict movement of integrin receptors in the cell membrane; release of these interactions allow integrin receptors to cluster and increase adhesive function (Yauch et al, 1997).

Outside-in signalling mediates signals from the extracellular matrix following integrin ligation and involves regulation of many fundamental cellular processes including cell survival and proliferation, cellular differentiation, morphogenesis and cell migration (Damsky & Werb, 1992; Juliano & Haskill, 1993; Clark & Brugge, 1995; Roskelley et al, 1995). It involves integrin-ligand binding and receptor clustering with subsequent assembly of the focal adhesion plaque - a complex of cytoskeletal proteins and signalling molecules including paxillin, talin, vinculin, α-actinin, tensin and Focal Adhesion Kinase (FAK) (Dedhar & Hannigan, 1996; fig. 1.13). This process is dependent on the GTPase Rho A (Burbelo et al, 1995; Nobes & Hall, 1995). Phosphorylated FAK can also lead to activation of the MAP Kinase pathway, probably via Ras activation, which can then influence gene expression (Chen et al, 1994; Zhu & Assoian, 1995).

45

Different integrins induce specific events, for example, whilst some β1 integrins induce expression of cell cycle proteins with consequent induction of proliferation, the β4 integrin appears to provide signals which induce cell cycle arrest and apoptosis (Clarke et al, 1995).
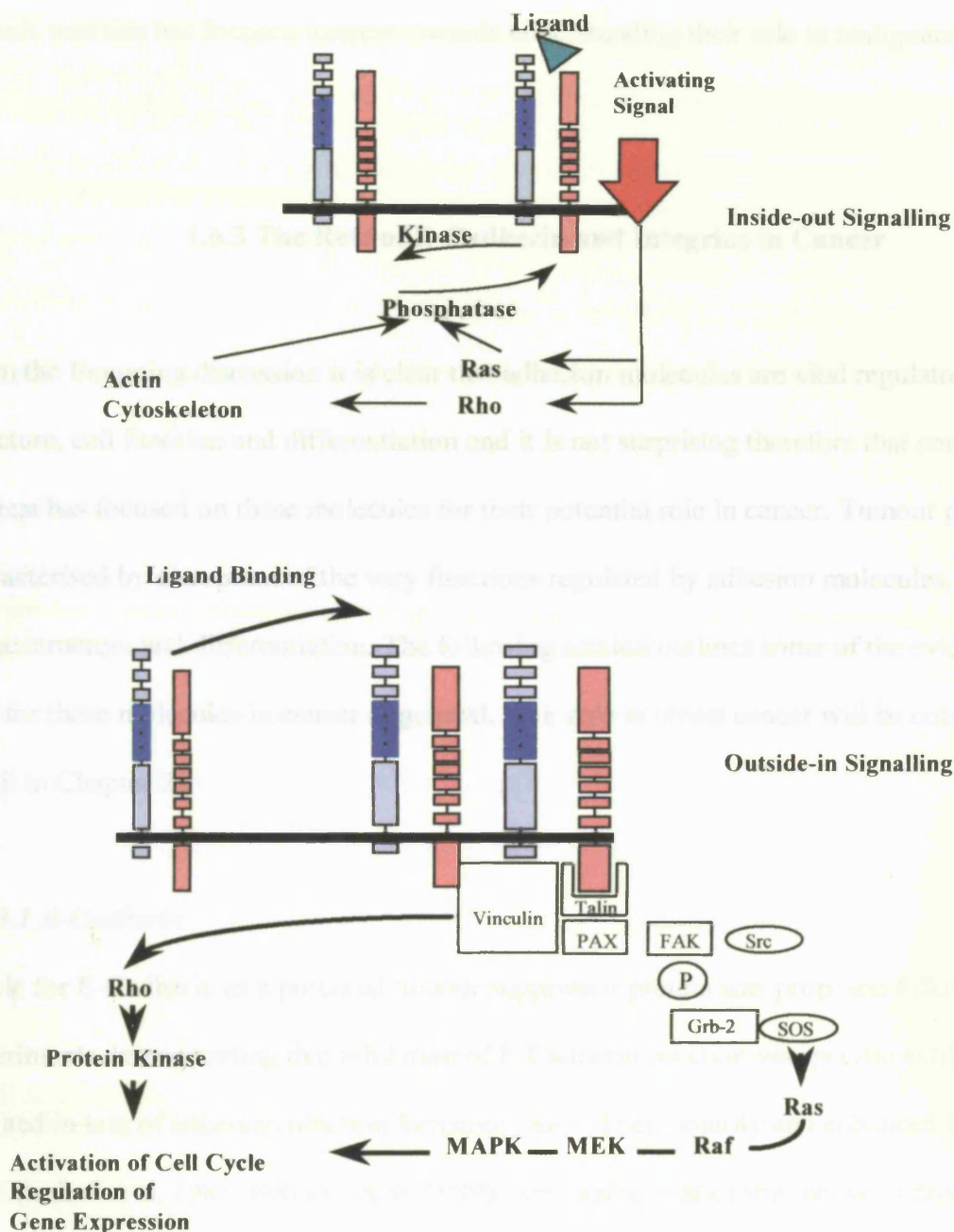
Fig. 1.13: Integrin Signalling Mechanisms.
Inside-out signalling is mediated by intracellular events initiated either by Rho-dependent reorganisation of the cytoskeleton or phosphorylation of integrins. Outside-in signalling is generated by integrin-ligand binding with subsequent activation of MAP Kinase pathway, or Rho-dependent cytoskeletal reorganisation.
FAK: Focal Adhesion Kinase; MAP: Mitogen Active Protein; MEK: MAP kinase kinase; P: phosphorylation.

Integrin-mediated cell -matrix interactions are also involved in maintaining cell differentiation as demonstrated by the laminin-dependent induction of milk protein gene expression in mouse mammary epithelial cells (Roskelley et al, 1994; Streuli et al, 1995). It is evident that integrins are key players in the co-ordinated response of cells to extracellular stimuli, and this has focused interest towards understanding their role in malignancy.

1.6.3 The Role of E-Cadherin and Integrins in Cancer

From the foregoing discussion it is clear that adhesion molecules are vital regulators of tissue structure, cell function and differentiation and it is not surprising therefore that considerable interest has focused on these molecules for their potential role in cancer. Tumour progression is characterised by disruption of the very functions regulated by adhesion molecules, that is, tissue structure and differentiation. The following section outlines some of the evidence for a role for these molecules in cancer in general, their role in breast cancer will be considered in detail in Chapter 2.

1.6.3.1 E-Cadherin

A role for E-Cadherin as a potential tumour suppressor protein was proposed following experiments demonstrating that inhibition of E-Cadherin function via specific antibodies resulted in loss of adherens junction formation, loss of cell polarity and enhanced invasion in-vitro (Imhof et al, 1983; Behrens et al, 1989). An inverse relationship between expression of E-Cadherin and invasive behaviour in tumour cell lines was reported and this relationship further supported by inhibition of tumour cell invasion following transfection of cells with E-Cadherin cDNA (Vleminckx et al, 1991). In a number of studies invasion could be switched on and off

47

by down- and up-regulation of E-Cadherin within the same population of cells (Chen &

Obrink, 1991; Frixen & Nagamine, 1993).

Loss of E-Cadherin expression or function may result from a number of different

events. Certain tumours are associated with allelic deletions or loss of heterozygosity on

chromosome 16 where E-Cadherin is located (Chr 16q22). Single mutations have been

identified in gastric carcinomas (Becker et al, 1994) and in endometrial carcinomas (Risinger et

al, 1994), which affect the extracellular domain of E-Cadherin and thus its adhesive function.

However, mutations in this gene appear to be infrequent events.

Whilst a clear role for E-Cadherin in preventing tumour invasion has emerged from in-

vitro studies, the relationship between E-Cadherin expression and tumour behaviour in-vivo is

inconsistent. In head and neck squamous carcinomas (Schipper et al, 1991), bladder tumours

(Bringuier et al, 1993) and in prostate carcinomas (Umbas et al, 1992), loss of E-Cadherin

expression has been associated with loss of tumour differentiation and with the presence of

lymph node metastasis. However, in other epithelial tumours, there is no clear association

between down-regulation of E-Cadherin and tumour behaviour. An immunohistochemical

study in lung carcinomas found no alteration of E-Cadherin expression either in the primary

tumours or in metastases (Shimoyama et al, 1989), and in some tumours high levels of

Cadherin have been associated with enhanced metastatic potential (Oka et al, 1992).

1.6.3.2 Integrins

Integrins are involved in a number of cellular processes that impact on the development of

tumours, such as proliferation, apoptosis, differentiation and migration. The dependence of

normal cells in-vitro on contact with the substratum for growth and survival is well recognised,

and acquisition of anchorage-independent growth is characteristic of tumour cells (Dike &

Farmer, 1988; Meredith et al, 1993; Brooks et al, 1994; Boudreau et al, 1995). The integrin

α5β1 has been shown to regulate both tumour cell invasion and proliferation. Clones of Chinese hamster ovary (CHO) cells overexpressing α5β1 grow less rapidly in nude mice compared to clones lacking the fibronectin receptor (Schreiner et al, 1989). Furthermore, transfection of CHO cells with α5β1 inhibits anchorage-independent growth, reduces proliferation and leads to a nontumourigenic phenotype when injected into nude mice (Giancotti & Ruoslahti, 1990). Varner et al (1995) expressed α5β1 in HT29 colon cancer cells that normally lack this integrin. Following transfection, the cells were either nontumourigenic or significantly less tumourigenic than control transfectants, and proliferated at half the rate of control cells. In contrast, some integrins appear to positively regulate proliferation. When melanoma cells were selected for lack of expression of αvβ3 integrin, the cells exhibited significantly reduced proliferation and tumourigenicity, which could be restored by re-expression of the integrin (Felding-Habermann et al, 1992).

Resistance to apoptosis is a feature of many malignant cells and such resistance may be due to aberrant integrin signalling. In rhabdomyosarcoma, down-regulation of the integrin-associated FAK induces apoptosis in these cells (Xu et al, 1996) whilst FAK-overexpressing MDCK cells are rescued from apoptosis and become anchorage-independent and tumourigenic (Frisch et al, 1996).

A major role for integrins has been demonstrated in the processes of invasion and metastasis. Many malignancies show altered integrin expression (Pignatelli et al, 1990; Hall et al, 1991; Pignatelli et al, 1991; Jones et al, 1992; Koukoulis et al, 1993), but the most clear-cut association between altered integrin profile and tumour progression has been demonstrated for malignant melanoma. The collagen receptor α2β1 is more strongly expressed in invasive melanoma compared to in-situ melanoma (van Duinen et al, 1994) and there is consistent, strong correlation between acquisition of αvβ3 integrin and both the vertical growth phase of melanoma and metastasis (Nesbit & Herlyn, 1994). Further evidence for the role of integrins in

49

malignancy is demonstrated by the specific inhibition of migration and invasion of glioma cells with blocking antibodies to α3β1 integrin (Fukushima et al, 1998).

In some situations, expression of integrin receptors can have a direct effect on differentiation. Mammary carcinoma cell lines grown in collagen gels can be made to undergo glandular differentiation following transfection with α2 integrin, and this effect is abrogated by antisense α2 mRNA (Zutter et al, 1995).

At its simplest level, the change in adhesion molecule expression on tumour cells favours cell migration by reducing adhesion to other cells and to basement membrane, and enhancing interaction with the surrounding stroma. However, there is increasing evidence that integrins in particular may be involved in controlling another aspect of cell migration - that of extracellular matrix remodelling, and in doing so, link the processes of cell motility and matrix degradation.

1.7 Relationship between Matrix Proteolysis and Cell Adhesion

If efficient cell migration is to occur, it is vital that the processes of matrix degradation and subsequent matrix attachment are co-ordinated. It is recognised that cellular interaction with certain matrix proteins can regulate MMP expression, and evidence is accumulating that this regulation is an integrin-dependent phenomenon. In rabbit synovial fibroblasts, two integrin receptors - α4β1 and α5β1 - co-operate in the control of three MMPs, MMP-1, MMP-3 and MMP-9: cross-linking of α5β1, or interaction of the receptor with its target sequence in fibronectin, strongly induces expression of these MMPs (Werb et al, 1989). When the cells are grown on intact fibronectin, integrin α4β1 binds to another distinct domain in fibronectin and suppresses these MMPs (Huhtala et al, 1995). MT1-MMP gene expression is also induced in some cells by growth on fibronectin (Stanton et al, 1998). In osteogenic sarcoma cell lines

50

induction of MMP-1 in response to growth in a collagen matrix has been shown to be

dependent on $\alpha2\beta1$ integrin, and MMP-1 synthesis is inhibited when the cells are transfected

with antisense $\alpha2$ integrin cDNA (Riikonen et al, 1995). Similarly keratinocytes only express

MMP-1 when cultured on a collagen matrix (Sudbeck et al, 1994), and in human mucosal

keratinocytes MMP-9 induction is an $\alpha3\beta1$ integrin-dependent process (Larjava et al, 1993).

Thus integrin-mediated interactions appear to co-ordinate proteolytic behaviour. As already

described, tumour cells frequently display an altered integrin repertoire compared to their

normal counterparts, and this may be a mechanism whereby cells can control expression and

secretion of proteolytic enzymes. In human melanoma cell lines, the differential expression of

$\alpha5\beta1$ integrin and $\alpha v\beta3$ integrin modulates release of MMP-2 and subsequent invasive

behaviour (Seftor et al, 1993).

Signalling through integrin-linked pathways has also been reported to promote

urokinase expression (Lengyel et al 1996; Irigoyen et al, 1997) and factors which up-regulate

integrins have been shown to simultaneously up-regulate uPAR (Lund et al, 1991). These

reports suggest an inter-relationship between the two groups of proteins, and this has been

further supported by the recent evidence of a physical association between uPAR and $\beta1$

integrin (Wei et al, 1996). These two proteins, together with the membrane protein caveolin

form a tripartite complex which functions as an adhesive and signalling unit at the cell surface

(Wei et al, 1996) and it has been suggested that in this way uPAR can modulate the function of

integrin receptors (Wei et al, 1996; Yebra et al, 1996; Xue at al, 1997).

Although the role of cell-cell adhesion molecules in the control of proteolytic enzymes

is certainly less well established than that of integrins, in some experimental systems, E-

Cadherin has been shown to regulate enzyme expression. The HMSV- induced skin cancer cell

line HaCa4 lacks E-Cadherin, shows constitutive expression of MMP-9 and has a highly

metastatic phenotype, whereas the subclone E24, generated by transfection with E-Cadherin

51

cDNA, does not express MMP-9 and is non-metastatic. Transfection of the E24 clone with

antisense E-Cadherin results in re-establishment of MMP-9 synthesis (Llorens et al, 1998).

Recent evidence has indicated that MMP-7 gene expression is induced by β-catenin/Tcf

transcriptional activation, and this induction can be reversed by restoration of E-Cadherin-

mediated adhesion (Crawford et al, 1999). Taken together these studies indicate an intimate

relationship between the expression of cell adhesion receptors and the elaboration of proteolytic

enzymes, and further support the concept that invasive behaviour is a highly co-ordinated

process.

1.8 Models of Cell Invasion

From the previous discussion it is evident that changes in cell adhesion molecules influence the

migratory capacity of a cell, and there is growing evidence to indicate that certain CAM

profiles can modulate elaboration of proteolytic enzymes. What is not so clear is how these

events are orchestrated in any one system to achieve cell invasion, and the control points that

limit invasion. The following sections present the evidence, in both a physiological and

pathological situation, for the co-ordinated changes that occur during the process of cell

invasion.

1.8.1 Physiological Invasion - placental implantation

The process of placental implantation shows many striking similarities to tumour cell invasion.

During the early stages of placentation, trophoblast cells adhere to, detach from, penetrate and

finally adhere again to basement membranes in a variety of locations, and in this regard share

many of the properties of metastatic tumour cells (Liotta et al, 1986). The striking difference

between trophoblast invasion and tumour invasion is the temporal and spatial control of the

former, with invasion being confined spatially to the uterine wall and temporally to the first and

early second trimester of pregnancy. Study of this process may, therefore, provide valuable

clues to the process of invasion by malignant cells and the apparent loss of control in this

situation.

From the earliest stages of implantation, two forms of trophoblast are evident at the

periphery of the embryo (Hertig et al, 1956), the mononuclear cytotrophoblast (CTB) and the

overlying syncitiotrophoblast. Branching morphogenesis produces trees of chorionic villi

generating two types of chorionic villi - the floating villus, which is bathed in maternal blood

and not directly connected to the uterus, and the anchoring villus which is embedded in the

uterine wall and attaches the fetus to the mother. At the tips of the anchoring villi, a population

of proliferating CTB undergo a complex - and not fully understood - differentiation process

resulting in the acquisition of a highly invasive phenotype (Aplin, 1991; Damsky et al, 1993;

Pijnenborg et al, 1980). This CTB population forms the CTB columns, which are aggregates of

non-polarised mononuclear cells, and from here penetrate deeply through the maternal

endometrium, into spiral arterioles and into the myometrium. Not surprisingly, the onset of

CTB invasion is heralded by a panoply of changes in cell-cell and cell-matrix interactions and

also considerable remodelling of the uterine stroma (fig. 1.14). Studies on placental bed

biopsies and on explant cultures of human placenta have shown that whereas villus-associated

CTB expresses E-Cadherin and $\alpha 6 \beta 4$ integrin, CTB columns exhibit down-regulation of these

cell adhesion molecules and switch on expression of $\alpha 1 \beta 1$ and $\alpha 5 \beta 1$ integrins (Damsky et al,

1992), and blocking antibody studies have indicated that these integrins have a profound

influence on CTB invasion (Librach et al 1991b).

Co-ordinate with this change in adhesion molecule expression, proteolytic

enzymes, particularly MMPs, are elaborated. CTB cultures derived from first trimester placenta

degrade basement membrane-like substrates in vitro, however, CTB from second trimester

Floating villus

Anchoring villus

Basement membrane

Cytotrophoblast

Syncitiotrophoblast

Cytotrophoblast columns (CTB)

Decidual cell

BM

α6β4

E-Cadherin

Non-invading trophoblast
Cyto-and syncitio-trophoblast associated with floating villus and non-tip area of anchoring villus.
Cells are polarised and express E-Cadherin and $\alpha_6\beta_4$ integrin.

MT-MMP/MMP-2

MMP-2

$\alpha 5\beta 1$
$\alpha 1\beta 1$

MMP-9

Invading trophoblast
Invasive cytotrophoblast of columns are non-polarised, lack E-Cadherin and $\alpha_6\beta_4$ integrin but express $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrins. They produce MMP-9 and MT1-MMP which binds and activates decidua-derived MMP-2.

Fig. 1.14: Physiological Invasion – Placental Implantation
This illustrates the cell adhesion molecule profile of non-invading and invading trophoblast, and the putative relationship between cell adhesion molecule expression and MMP expression with invasion.

placenta invade more slowly and less extensively, whereas those from term placentas are non-invasive (Fisher et al, 1989). This correlates with CTB invasive behaviour in-vivo. Mirroring the change in invasive potential, changes in a complex array of proteolytic enzymes has also been reported, with the most consistent changes being observed in the levels of MMP-9 (Hurskainen et al, 1996). Furthermore inhibitors of MMP-9 were found to abolish trophoblast invasion in in-vitro assays (Fisher et al, 1989). Invading trophoblast has also been shown to utilise the MMP-2/MT1-MMP system with high levels of MT1-MMP expression in the invasive CTB population, and the protein localised both to cell cytoplasm and cell membranes, in some areas co-localising with MMP-2 protein (Hurskainen et al, 1998). In contrast to MT1-MMP, MMP-2 is not synthesised by CTB, so the surface staining of this protein on CTB cells is consistent with its binding to a cell surface receptor.

Trophoblast invasion is a tightly controlled process and whilst the mechanisms of this control are not fully understood certain elements likely to contribute to the modulation of invasion have been described. The decidual microenvironment of invading trophoblast has a marked effect on CTB behaviour - it has been known for many years that trophoblast invasion is more aggressive at extrauterine sites without the modulatory influence of the decidua (Kirby, 1965). More recently Lala & Graham (1990) demonstrated that first trimester human decidual cells produce factors that block trophoblast invasion, and this suppression is TGF-β dependent. Neutralising antibodies to TIMPs promoted trophoblast invasion, and it was suggested that TGF-β induced TIMP expression which then abrogated invasive activity by blocking MMP action (Lala & Graham, 1990). In keeping with this, TIMP-1, -2 and -3 have all been localised to decidual cells in human placenta and it seems likely that controlled cellular invasion probably requires production of inhibitors as well as degradative enzymes (Hurskainen et al, 1996).

1.8.2 Pathological invasion - progression of malignant melanoma

Sequential changes in cell adhesion molecule expression and elaboration of proteolytic

enzymes have probably been best characterised in human malignant melanoma. The evidence

comes from both in-vivo observations and in-vitro models. Whilst it is not generally agreed that

melanocytic naevi are the precursors of melanomas, many tissue studies have placed these

lesions at the 'benign' end of the melanoma spectrum.

Normal melanocytes adhere to adjacent keratinocytes by E-Cadherin-mediated adhesion

(Tang et al, 1994). The most consistent finding in melanoma is loss of E-Cadherin (which is

also seen in naevus cells) and acquisition of Melanoma Cell Adhesion Molecule (Mel-

CAM/MUC18), which is seen in up to 80% of advanced primary and metastatic melanomas

(Lehmann et al, 1988; Shih et al, 1994). The collagen receptor $\alpha2\beta1$ is frequently up-regulated

in malignant melanoma, which reflects in-vitro studies, where $\alpha2\beta1$ expression on melanoma

cell lines is associated with enhanced invasion (Klein et al, 1991a; Klein et al, 1991b).

Furthermore, $\alpha2\beta1$ has been shown to modulate the level of MMP-1 gene expression in some

cell systems (Riikonen et al 1995) and the invasive behaviour of melanoma cells in-vitro has

been shown to be at least in part dependent on the expression of MMP-1 (Durko et al, 1997).

The $\alpha3\beta1$ receptor is also increased in advanced and metastatic melanomas, and the degree of

expression correlates with the extent of dermal invasion (Natali et al, 1993).

There is a consistent, strong correlation between acquisition of $\alpha v\beta3$ integrin and both

the vertical growth phase of melanoma and metastasis (Albeda et al, 1990; Nip et al, 1992;

Nesbit & Herlyn, 1994), and the presence of $\alpha v\beta3$ on melanoma cells correlates with their

tumourigenic potential in athymic mice (Cheresh, 1991). Cell culture experiments have shown

that interaction of $\alpha v\beta3$ with ligand or antibody stimulates production of MMP-2 and enhances

melanoma invasion (Seftor et al, 1993). It has also recently been demonstrated that melanoma

cells express MT1-MMP and utilise this receptor to activate MMP-2 (Kurschat et al, 1999). A
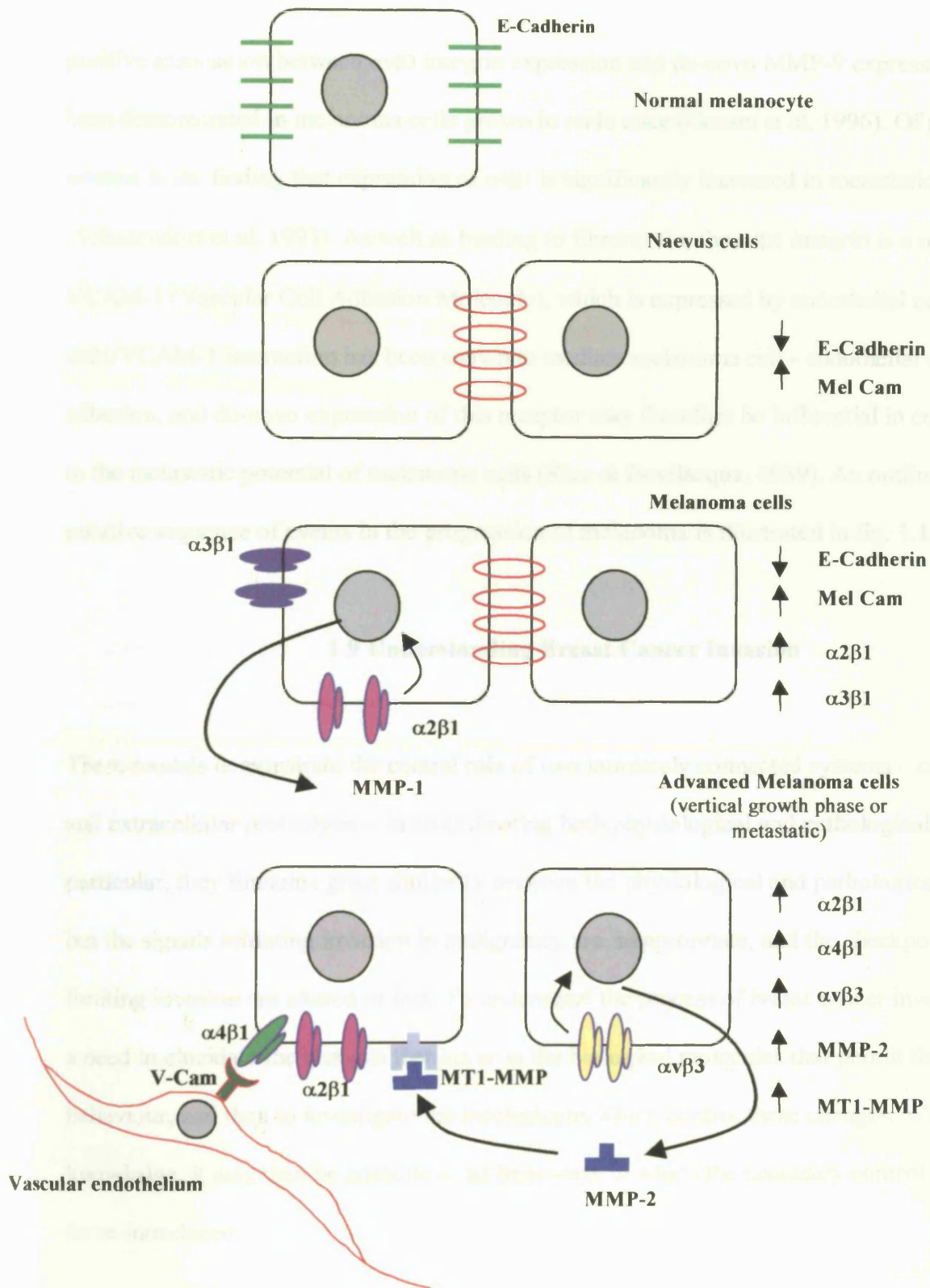
Fig. 1.15: Pathological Invasion – Progression of Malignant Melanoma.
Schematic outline of changes in cell adhesion molecule profile and MMP expression in relation to stages in the development and progression of malignant melanoma.

positive association between $\alpha v \beta 3$ integrin expression and de-novo MMP-9 expression has also

been demonstrated in melanoma cells grown in nude mice (Gouon et al, 1996). Of particular

interest is the finding that expression of $\alpha 4 \beta 1$ is significantly increased in metastatic melanomas

(Schadendorf et al, 1993). As well as binding to fibronectin, the $\alpha 4 \beta 1$ integrin is a receptor for

VCAM-1 (Vascular Cell Adhesion Molecule), which is expressed by endothelial cells. The

$\alpha 4 \beta 1$/VCAM-1 interaction has been shown to mediate melanoma cell - endothelial cell

adhesion, and de-novo expression of this receptor may therefore be influential in contributing

to the metastatic potential of melanoma cells (Rice & Bevilacqua, 1989). An outline of the

putative sequence of events in the progression of melanoma is illustrated in fig. 1.15.

1.9 Understanding Breast Cancer Invasion

These models demonstrate the central role of two intimately connected systems – cell adhesion

and extracellular proteolysis – in co-ordinating both physiological and pathological invasion. In

particular, they illustrate great similarity between the physiological and pathological situation,

but the signals initiating invasion in malignancy are inappropriate, and the checkpoints for

limiting invasion are altered or lost. To understand the process of breast cancer invasion there is

a need to elucidate the changes that occur in the biological molecules that permit this

behaviour, and then to investigate the mechanisms which control these changes. With this

knowledge, it may then be possible to address ways in which the necessary control points could

be re-introduced.

1.10 Hypothesis and Aims

The hypothesis is that co-ordinated changes in the extracellular proteolytic system and in cell adhesion molecule expression are important in the control of breast cancer invasion, and one of the factors contributing to these changes is the co-operative relationship between tumour cells and host stroma. Re-establishing a more normal cell adhesion molecule profile, or altering the tumour-stromal cell relationship would therefore be expected to inhibit invasion.

To investigate this hypothesis, the specific aims of the project are:

• To correlate breast tumour behaviour with changes in MMP and cell adhesion molecule expression. Specifically to examine the expression and distribution of the two gelatinase systems, MMP-2/MT1-MMP/TIMP-2 and MMP-9/TIMP-1, and to relate the immunohistochemical pattern of expression to enzyme activity. To examine changes in expression of integrin receptors and E-Cadherin, and to relate the cell adhesion molecule profile to the pattern of MMP expression.

• To develop an in-vitro model system to quantify breast cancer cell invasion and to relate the level of invasion to:

(i) the level of MMP and TIMP expression

(ii) the expression profile of integrins and E-Cadherin

• To manipulate E-Cadherin and Integrin function in these cell lines and analyse the effects on invasion and on MMP/TIMP expression.

• To introduce into the model system

(i) stromal fibroblasts, and

(ii) normal breast myoepithelial cells

and to analyse the effect of these cellular interactions on tumour cell invasion and on MMP/TIMP expression and activity.

59

Chapter 2

Expression of Matrix Metalloproteinases and Cell Adhesion

Molecules in Normal and Malignant Breast Tissue

AIM: To correlate breast tumour behaviour with changes in MMP and cell adhesion molecule expression. Specifically to examine the expression and distribution of the two gelatinase systems, MMP-2/MT1-MMP/TIMP-2 and MMP-9/TIMP-1 at protein and mRNA level and to relate the immunohistochemical pattern of expression with enzyme activity. To examine changes in expression of Integrin receptors and E-Cadherin, and to relate the cell adhesion molecule profile to the pattern of MMP expression.

2.1 Introduction

There is considerable evidence particularly from in-vitro studies to show that over-expression of MMPs can promote tumour cell invasion and that this effect is perturbed by the action of MMP inhibitors. Many studies have been performed to address the potential value of analysing MMP expression and activity in tissue specimens to provide relevant prognostic data. The association or not of any enzyme with tumour grade, stage or patient outcome is variable and many of the studies are not directly comparable owing to the use of different techniques which provide different information e.g. immunohistochemistry, in-situ hybridisation, zymography, Northern blotting or RT-PCR. Since the first barrier malignant epithelial cells must overcome is BM, type IV collagenase activity would appear to be a prerequisite for tumour invasion. BM degradation is also vital for angiogenesis, on which tumours depend for growth, and also for tumour cell access to blood vessels and lymphatics in the metastatic process. In keeping with this, one of the more consistent findings in breast - and other tumours - is an association of tumour behaviour with gelatinase expression. Davies et al (1993a) showed a positive correlation between high grade breast carcinoma and MMP-9 expression, and a clear relationship between disease severity and active MMP-2, as assessed on zymography. Iwata et

al (1996) demonstrated a relationship between expression of MMP-9 and the presence of lymph node metastasis, and found that whilst MMP-2 was widely expressed by carcinomas, only the level of activated MMP-2 correlated with metastatic disease. It is evident that the level of inhibitor in relation to enzyme has a profound influence on determining net proteolytic effect. Onisto et al (1995), demonstrated a significant change in balance of MMP-2: TIMP-2 in favour of net proteolysis in lymph node positive breast carcinomas. In individual cases this was due either to enhanced MMP-2 expression or a reduction in TIMP-2 expression. Thus for any meaningful assessment of the role of MMP in tumour progression, both enzyme and inhibitor levels must be analysed. However, activation of the latent precursor is also a key step in determining enzyme function in-vivo. Many factors are capable of activating MMP zymogens, but of particular interest is the membrane-associated mechanism of activation for MMP-2 via MT1-MMP (Sato et al, 1994), which not only activates enzyme but focuses activity to the cell surface. Five MT-MMPs are now described, but the clearest association with tumour behaviour has been demonstrated for MT1-MMP (Nomura et al, 1995; Yamamoto et al, 1996).

Changes in E-Cadherin and integrin expression have been shown to influence epithelial tumour cell invasion and tumourigenic potential (Behrens et al, 1989; Giancotti & Ruoslahti, 1990; Vleminckx et al, 1991; Natali et al, 1993; Nesbit et al, 1994). An altered cell adhesion molecule profile has been described in breast carcinomas compared to normal and benign tissue (Pignatelli et al, 1992; Lipponen et al, 1994; Gluckhova et al, 1995), and in some studies this altered expression has been related to tumour spread (Oka et al, 1993; Gui et al, 1997). A previous study in this laboratory showed altered expression of the β4 integrin receptor in apparently normal tissue taken from cancer-containing breast (Jones et al, 1992), suggesting that alterations in adhesion molecule expression may occur at the earliest stages in tumour development.

The process of tumour invasion and metastasis is likely to involve a dynamic interaction between cell adhesion receptors, the extracellular matrix and its remodelling by proteases, growth factors, cytoskeletal proteins and other signalling molecules. Regulatory links between cell adhesion receptor expression or activation and proteolysis have been demonstrated (Huhtala et al, 1995; Xie et al, 1998; Bafetti et al, 1998; Niu et al, 1998). The aim of this part of the study is to address whether changes in MMP/TIMP expression and cell adhesion molecule expression relate to breast tumour cell behaviour.

The study will use well-established immunohistochemical techniques to analyse the extent and distribution of the two gelatinase systems, MMP-2/MT1-MMP/TIMP-2 and MMP-9/TIMP-1, members of the integrin family and E-Cadherin. In-situ hybridisation using oligonucleotide probes will be employed to investigate the site of synthesis of the enzyme systems.

Since these techniques do not provide information on the functional state of enzymes, substrate gel zymography will be employed to relate the pattern of staining for the MMPs to the level of enzyme activity. A number of techniques are available for the identification and quantitation of proteases in biological samples, each having their own advantages and disadvantages (see Table 2.1). Substrate gel zymography was the technique of choice in this study because it is highly sensitive and allows relative quantitation of both active and latent enzyme forms, so providing information of the activating potential of the sample.

Whilst it is recognised that such a static approach to address a dynamic situation will provide correlative data only, in addition to assessing the potential predictive value of such information, any relationships identified would focus subsequent in-vitro experiments.

Table 2.1: Summary of Different Techniques for Detecting and Measuring Proteolytic Activity

TECHNIQUE	ADVANTAGES	DISADVANTAGES
Substrate Gel Zymography	• Active & latent enzymes detected • TIMP-complexed enzyme detected • No antibodies required • Sensitive (4 ng/ml)	• Reproducibility requires extreme care • Not specific due to overlapping enzyme activity • Absolute quantitation difficult • Labour intensive
Radiolabelled substrate assay	• Sensitive (4 ng/ml)	• Poor reproducibility • Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes
Chromophore assay	• Some methods are highly sensitive	• Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes
In-situ zymography	• Sensitive • Localisation of enzyme activity	• Difficult to ensure reproducibility • Non-specific due to overlapping enzyme substrate specificities
ELISA	• Sensitive • Rapid, multiple sample analysis • Absolute quantitation possible	• Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes • Dependent on antibody specificity

64

2.2 Materials

2.2.1 Tissue

All breast tissue was obtained within 30 mins of surgical excision at Glenfield Hospital

Trust during the period of 1990-1996. Representative areas of tissue were selected by a

pathologist (Dr. R.A.Walker or myself) and samples were snap frozen in liquid nitrogen onto

cork in OCT embedding compound and stored in the vapour phase of a liquid nitrogen freezer.

For all specimens, if more than 30 mins had lapsed since removal of the tissue, the case was not

included in the study in order to eliminate potential problems with loss of antigenicity and/or

degradation of RNA. Parallel slices of tissue were fixed in 10% formal saline for 24 hours at

room temperature and routinely processed through a series of graded alcohols and xylene to

paraffin wax.

Carcinomas were reported according to the Royal College of Pathologists

working party guidelines (1990), and graded using the modified Bloom and Richardson system

(Elston and Ellis, 1991). Clinicopathological features of the tumour cases studied are outlined

in Table 2.2

Table 2.2: Clinicopathological features of carcinomas studied

TYPE	GRADE	LN+	LN-	NK	ER+	ER-	NK	No.
Tubular	I	5	3	0	6	0	2	8
IDC	I	2	5	1	7	1	0	8
IDC	II	13	11	2	22	3	1	26
IDC	III	21	20	4	15	26	4	45
ILC	II	12	11	3	20	3	3	26
ILC	III	1	0	0	1	0	0	1
TOTAL		54	50	10	71	33	10	114

Normal tissue was obtained from reduction mammoplasty procedures, and dealt with in the same way, though under aseptic conditions, since tissue was also taken from these specimens for tissue culture (see Chapter 4).

2.2.2 Antibodies

Anti- MMP-2 Antibodies

Rabbit polyclonal antibody which recognises active, latent and TIMP-bound MMP-2, received as a gift from Dr. W.G.Stetler-Stevenson, N.I.H., Bethesda (Monteagudo *et al*, 1990).

Mouse monoclonal antibody which recognises active, latent and TIMP-bound MMP-2, clone 75-7F7, obtained from Oncogene Science Inc, Cambridge, MA.

Anti-MMP-9 Antibody

Rabbit polyclonal antibody which recognises active, latent and TIMP-bound MMP-9 received as a gift from Dr. W.G.Stetler-Stevenson, N.I.H., Bethesda (Monteagudo *et al*, 1990).

Anti-TIMP-1 Antibodies

Rabbit polyclonal antibody which recognises free and enzyme-complexed TIMP-1 received as a gift from Dr W.G. Stetler-Stevenson, N.I.H., Bethesda.

Mouse monoclonal antibody which recognises free and enzyme-complexed TIMP-1 from Oncogene Science Inc, Cambridge, MA.

Anti-TIMP-2 Antibodies

Rabbit polyclonal antibody which recognises active, latent and enzyme-complexed TIMP-2 received as a gift from Dr W.G. Stetler-Stevenson, N.I.H., Bethesda.

Mouse monoclonal antibody which recognises free and enzyme-complexed TIMP-2 from Oncogene Science Inc, Cambridge, MA.

Anti-MT1-MMP Antibody

Rabbit polyclonal antibody raised against a synthetic peptide corresponding to the amino acids at position 160-173 in the human MT1-MMP. The antibody was raised by Dr. P Glynn, MRC Toxicology Unit, University of Leicester, and characterised by Western Blotting of protein from Conconavalin A-stimulated HT1080 fibrosarcoma cells. The antibody recognises the propeptide, the active, membrane-associated form of MT1-MMP and a further N-terminally processed form of MT1-MMP.

Anti-α2 integrin antibody (CD49b)

Mouse monoclonal antibody, clone P1E6, obtained from Camfolio, Becton Dickinson. This is an anti-functional antibody and blocks VLA-2 mediated adhesion to collagen type I, II and IV (Carter *et al*, 1990).

Anti-α3 integrin antibody (CD49c)

Mouse monoclonal antibody, clone P1B5, obtained from Camfolio, Becton Dickinson. This is an anti-functional antibody and blocks VLA-3 mediated adhesion to epiligrin, collagen type I and IV (Carter *et al*, 1990; Carter *et al*, 1991).

Anti-α5 integrin antibody (CD49e)

Mouse monoclonal antibody, clone mAb 16, obtained from Camfolio, Becton Dickinson. This is an anti-functional antibody which inhibits fibroblast attachment to fibronectin but not laminin or vitronectin (Akiyama *et al*, 1989).

Anti-α6 integrin antibody

Rat monoclonal antibody, GoH3 clone, obtained from Serotec. This is an anti-functional antibody which blocks VLA-6 mediated adhesion to laminin (Sonnenberg *et al*, 1986).

67

Anti-β1 integrin antibody

Mouse monoclonal antibody, clone 3S3, obtained from Serotec. This is anti-functional and inhibits adhesion to collagen and fibronectin.

Anti-β4 integrin antibody

Mouse monoclonal antibody, clone 3E1. Produced from human foetal membranes that were used as immunogens in the production of antibodies to basement membrane components. This antibody is described as having function-altering behaviour (Hessle *et al*, 1984; Weaver *et al*, 1997).

Anti-E-Cadherin

Mouse monoclonal antibody, clone 6F91, obtained from EuroPath Ltd., U.K. This antibody has been shown to block cell-cell adhesion and promote epithelial cell invasion (Frixen *et al*, 1991).

QBEnd 10 (CD34)

Mouse monoclonal antibody reacting with haemopoietic stem cells and with endothelial cells. Obtained from Dako.

KP1 (CD68)

Mouse anti-human monoclonal antibody reacting with macrophages and monocytes. Obtained from Dako.

Secondary Antibodies

Biotinylated Rabbit Anti Mouse IgG obtained from Dako (Code no. E0354).

Biotinylated Swine Anti Rabbit IgG obtained from Dako (Code no. E0353).

Horseradish Peroxidase Swine Anti Rabbit IgG obtained from Dako

Tertiary Antibodies

Avidin Biotin Complex:

Streptavidin (Solution A) and Biotinylated Horseradish Peroxidase (Solution B) obtained as a kit from Dako (Code no. K0377).

2.2.3   Western Blotting

HT 1080 human fibrosarcoma cell line was received as a gift from Mr. M. Thompson, Department of Surgery, Leicester Royal Infirmary, Leicester. Dulbecco's Modified Eagles Medium without phenol red, L-Glutamine and Fetal Bovine Serum, γ-irradiated and mycoplasma screened, were all from Gibco Ltd. Bovine Serum Albumin (BSA, 20 mg/ml) was from Boehringer Mannheim. BioRad™ Protein Assay Dye Reagent was from BioRad Laboratories Ltd., and protein concentrators, 10kDa cut-off, were from Amicon. Protogel (30% acrylamide/ bis-acrylamide) was obtained from National Diagnostics. MultiMark Multi-Coloured Standard molecular weight markers were from Novex, San Diego, CA. Hybond-C Super Nitrocellulose Membrane from Amersham Life Science. Enhanced Chemiluminescence Western Blotting Detection Reagents Kit and Hyperfilm ECL high performance chemiluminescence film was obtained from Amersham Life Science.

2.2.4   Zymography

MMP Standards

Proenzyme MMP-2: human recombinant MMP-2 purified from mammalian cells, >95% purity on SDS-PAGE (Oncogene Research Products, Calbiochem-Novabiochem Ltd. UK)

Proenzyme MMP-9: human recombinant MMP-2 purified from mammalian cells, >95% purity on SDS-PAGE (Oncogene Research Products, Calbiochem-Novabiochem Ltd. UK)

Zymography

As for 2.2.3, and in addition:

Type III collagen from fetal calf skin was obtained from Sigma, and Coomassie Blue from BDH.

2.2.5 Northern Blotting and In-situ Hybridisation

Oligonucleotide Probes

Probes were designed, with the help of Dr. Howard Pringle and Dr. Jacqui Shaw, using the Molecular Biology Suite programme 'Primer', and each sequence was checked against the EMBL human sequence database for specificity. Oligonucleotides were synthesised by Gibco (Paisley, UK) or Genosys (Cambridgeshire, UK), and were purified to at least 98% purity by HPLC. The oligonucleotide sequences with their gene positions are shown in Table 2.3.

Labelling of Probes

Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (Dig-11-dUTP) and Tdt enzyme (20U/μl) were from Boehringer Mannheim.Terminal deoxynucleotidyl transferase buffer (Tdt) 5 x buffer was from Promega.

Test Strips, Northern Blotting and In-situ Hybridisation

Nitrocellulose membrane was obtained from Sartorius GmbH, Germany. Anti-digoxigenin-alkaline phosphatase (anti-dig-AP) was from Boehringer Mannheim. Salmon Sperm DNA (10 mg/ml) and Proteinase K was from Sigma. 3MM chromatography paper was from Whatmann and Apathy's mounting media from BDH.

2.2.6 Chemicals and Miscellaneous

All chemicals were 'Analar' grade and obtained from BDH, with the following exceptions: Diethyl pyrocarbonate, sodium dodecyl sulphate (SDS), ammonium persulphate, 3-aminopropyltriethoxysilane, Type XIV protease and monensin were all from Sigma. Acetone,

70

hydrogen peroxide and Triton X-100 were from Fisons. Normal Rabbit Serum (NRS) and

Normal Swine Serum (NSS) were from Dako.

Table 2. 3: Details of oligonucleotide Probes for In-situ Hybridisation

Gene Target	Oligonucleotide Sequence	Position
MMP-9	ACT GGC AGG GTT TCC CAT CAG CAT TGC CGT	868-897
	TCC GGC ACT GAG GAA TGA TCT AAG CCC AGC	1247-1276
	GTT GCA GGC ATC GTC CAC CGG ACT CAA AGG	1551-1580
	GCT CCC CCT GCC CTC AGA GAA TCG CCA GTA	1664-1693
	GCG GCT CCT CAA AGA CCG AGT CCA GCT TGC	1723-1752
MT1-MMP	CCC CTT GTA GAA GTA AGT GAA GAC TTC ATC	1590-1619
	CCT CAT CAA ACA CCC AAT GCT TGT CTC CTT	1327-1356
	CAT CCA GAA GAG AGC AGC ATC AAT CTT GTC	1414-1443
	AAT TTG CCA TCC TTC CTC TCG TAG GCA GTG	1286-1315

2.2.7 Buffers and Solutions

Details of buffers and solutions are given in Appendix 1.

2.3 Methods

2.3.1.1 Sample Preparation

Western blot analysis of non-commercial antibodies was carried out on the conditioned

medium (CM) of the HT 1080 human fibrosarcoma cell line, and for MT1-MMP, on the

cellular fraction of Concanavalin-A treated HT 1080 cells. Cells were maintained in 75cm^3

sterile tissue culture flasks in DMEM without phenol red with 10% FBS and 2mM L-

Glutamine at 37°C in a 5% CO_2 humidified atmosphere. At ~80% confluence, the culture

media was aspirated, centrifuged at 10 000r.p.m. at 4°C to remove any cell debris and stored at

-80°C in 1 ml aliquots until required. The cells were briefly washed in 5 ml pre-warmed (37°C)

sterile PBS then scraped from the bottom of the flask into 5 ml fresh cool (4°C) sterile PBS

using a rubber policeman. The resultant cell suspension was centrifuged and the pellet

resuspended into 250 μl cold (4°C) homogenising buffer. This cell suspension was then

subjected to 3 x 60 second cycles of homogenisation using a Pellet Pestle Motor (Kontes)

whilst keeping the specimen on ice. The resultant homogenate was briefly centrifuged at 13

000 r.p.m., the supernatant removed and stored at -80°C until required.

2.3.1.2 Protein Estimation

A standard curve of Absorbance : Protein Concentration was constructed using a dilution series

of protein concentrations 0.1μg/ml to 100.0 μg/ml from a 20 mg/ml stock solution of Bovine

Serum Albumin (BSA, Boehringer Mannheim) diluted in sterile PBS. Protein assays were

performed using the BioRad Protein Dye Assay Reagent based on the method described by

Bradford (1976). A 100 μl volume of each of the BSA solutions was added to 900 μl of 1:5 Dye

Reagent:PBS and the mix inverted then allowed to stand at room temperature for 10 mins prior

to recording the absorbance at 595 nanometres (nm) on a UV spectrophotometer (Ultrospec III, Pharmacia Biotech LKB). Measurement of sample protein concentrations was made in the same way and values obtained from the standard curve. All protein samples were normalised to 1 mg/ml protein concentration, either by diluting in sterile PBS, or by concentrating the sample using Chemicon microconcentrators with a 10kDa cut-off point.

2.3.1.3 SDS-PAGE and Protein Blotting

Electrophoresis of proteins was carried out using a Hoefer SE-250 Mighty Small II Mini-Gel Unit. Protein samples (1 mg/ml) were mixed 1:1 in 2 x Reducing Sample Buffer, denatured by heating at 100°C for 3 mins, placed on ice then centrifuged briefly at 13 000 r.p.m. to remove any precipitate. Samples (30 μl total volume) were loaded onto a 3.75% stacking gel and 10% resolving gel (appendix 2) and run in Reservoir buffer at 50V through the stacking gel and at 75 V for 3 1/2 hrs through the resolving gel. MultiMark Multi-Colored Standard molecular weight markers were run on each gel to allow size determination of the protein bands.

Following electrophoresis, the gel was transferred to Blotting buffer for 15 mins then overlaid with Hybond-C nitrocellulose membrane and blotted for 12 hrs at 300 mA in Blotting buffer.

2.3.1.4 Immunodetection

An Enhanced Chemiluminescence Detection system was used to demonstrate the separated proteins. The molecular weight marker lane was cut from the membrane which was then equilibrated in TBS for 5 mins after which the membrane was incubated in Blocking Solution for 1 hr at room temperature with agitation. The membrane was then rinsed twice in Washing buffer followed by washes of 1 x 15 mins and 2 x 5 mins (adopted as the standard washing

73

procedure), after which it was incubated in primary antibody diluted in blocking solution for 1 hr at room temperature with agitation. A range of antibody dilutions was tested in initial studies in order to achieve optimum staining with minimal background. Following the standard washing procedure, the membrane was incubated in secondary antibody (HRP-labelled swine anti rabbit immunoglobulin) for 1 hr at room temperature with agitation. Again, optimal antibody concentrations were determined in initial studies. The standard washing procedure was performed prior to ECL detection being carried out, according to the manufacturers recommendations. The membrane was then exposed to autoradiography film (Hyperfilm ECL) for variable exposure periods ranging from 30 secs to 10 mins.

2.3.2 Haematoxylin and Eosin Staining of Tissue Sections

Paraffin sections of 4μm were cut onto silane-coated slides, briefly fixed in 99% IMS, rinsed in tap water and stained with Harris' haematoxylin. Following a brief wash in Scots' Tap Water Substitute to enhance nuclear staining, cytoplasmic staining was achieved using 1% eosin. The sections were then dehydrated through graded alcohols, cleared in xylene and mounted under glass coverslips with XAM.

2.3.3 Immunohistochemistry

2.3.3.1 Silane Coating of Slides

Glass slides were coated with silane to increase adherence and reduce non-specific background staining. Detergent cleaned slides were washed in 96% Industrial Methylated Spirits (IMS), allowed to air dry and coated by immersion into a freshly prepared solution of 3-aminopropyltriethoxysilane for 5 seconds. Slides were then rinsed briefly in dry acetone, then in ultrapure water and allowed to air dry overnight at 42°C.

2.3.3.2 Immunohistochemistry on Paraffin sections

• Tissue sectioning

Immunostaining of tissues processed to paraffin wax was carried out on 4μm sections cut onto

silane-coated glass slides. The sections were allowed to dry for approximately 12 hours in a

37°C incubator. They were then dewaxed in xylene and hydrated through graded alcohols to

water. Immunohistochemistry was performed, either with no pre-treatment or following antigen

retrieval techniques.

• Antigen retrieval methods

(i) Protease Digestion

From water, sections were equilibrated in PBS previously warmed to 37°C for 5 minutes, after

which they were immersed in 0.05% Type XIV Protease solution at 37°C for 5, 10 or 15

minutes. The slides were then washed in tap water to halt proteolytic digestion, followed by

equilibration in PBS before continuing with immunohistochemistry.

(ii) Microwave Irradiation

From water, the slides were immersed in 0.01M citric acid buffer, pH 6.0, contained in

microwave-safe coplin jars. Using a Panasonic microwave (model NN-5450/5400) at

maximum power of 800 watts, a series of two or three five-minute cycles was performed.

During the microwave process, the level of citrate buffer was maintained, and replaced for each

cycle.

• Indirect Avidin Biotin Complex (ABC) Immunoperoxidase Technique

An Indirect ABC immunoperoxidase technique was employed for optimal sensitivity according

to methods described by Hsu *et al* (1981a and 1981b). Following the appropriate pre-

treatment, sections were equilibrated by washing twice in PBS and then blocked in a PBS

solution containing either 20% Normal Rabbit Serum (NRS) or 20% Normal Swine Serum

75

(NSS), according to the nature of the secondary antibody, for 30 mins at room temperature. The NRS/NSS was drained from the slides and 50 µl of the appropriate primary antibody diluted in blocking solution was applied to the corresponding section. A range of antibody dilutions was evaluated. The sections were incubated with the primary antibody for 12 hours at 4°C. Sections were then washed in PBS for 20 minutes at room temperature, after which the biotinylated secondary antibody -(swine IgG against rabbit primary antibodies, and rabbit IgG against mouse primary antibodies)- was applied at a dilution of 1:400 in PBS pH7.6, for 30 minutes. At this time the Streptavidin-Biotin-Complex (ABC) was made up in the following proportions and allowed to conjugate at room temperature for 30 minutes prior to use:

PBS	1000 µl
Streptavidin	1 µl
Biotinylated HRP	1 µl

After washing in PBS to remove the secondary antibody solution, the sections were incubated with the ABC for 30 minutes at room temperature. All incubations were carried out in humidity trays sealed with petroleum jelly. The sections were rewashed in PBS for 20 minutes prior to application of the filtered DAB solution, containing 0.5 mg/ml DAB plus 0.015% hydrogen peroxide in PBS. The reaction product was allowed to develop for 5 mins before a final wash in PBS, after which the sections were counterstained in Harris' haematoxylin for 30 seconds and after a final wash in tap water, dehydrated through graded IMS, cleared in Xylene and mounted under glass coverslips with XAM.

For each run of immunohistochemistry, positive and negative controls were included. Monensin-treated HT 1080 cells grown on glass coverslips and fixed in pre-cooled acetone for 10 mins were used as positive controls for MMP and TIMP antibodies in initial studies.

Cryostat sections of normal skin were used as positive controls for integrin and E-Cadherin antibodies. Omission of the primary antibody was used as a negative control in each case.

2.3.3.3 Immunohistochemistry on Frozen Tissue

- Tissue Sectioning

Cryostat sections were cut at 5 μm on a Bright cryostat onto silane coated slides, and allowed to air dry.

- Tissue Fixation

Two methods of fixation were assessed during initial experiments, with the method giving optimal tissue preservation associated with immunoreactivity being used for the remainder of the study.

(i)   Acetone fixation: sections were immersed for 10 mins at 4°C into pre-cooled acetone.

(ii)   Paraformaldehyde followed by methanol and acetone: sections were immersed into 3.7% paraformaldehyde in PBS for 10 mins followed by transfer to PBS. This was followed by immersion into cold (4°C) methanol for 4 mins followed by immersion into cold acetone for a further 2 mins.

- Indirect Avidin Biotin Complex (ABC) Immunoperoxidase Technique

Following fixation, the sections were transferred without drying to PBS. From this stage, frozen sections were treated in the same manner as paraffin sections, as described in section 2.3.3.1. In initial studies the same controls - HT 1080 cells and normal skin - were used, but when positive cases were identified, these were then used in future runs as positive controls.

2.3.3.4 Evaluation of Immunohistochemical Staining

The extent and distribution of reactivity for each antibody was recorded using a semi-quantitative scoring system. Staining was classed as 5 if all tumour cells were positive, 4 if <100% but >75% of cells stained, 3 if 50 - 75% of the tumour was positive, 2 for 20 - 50% reactivity, 1 for <20% staining and 0 if there was no reactivity in the tumour. This scoring system was applied to record the two patterns of reactivity observed in tumour cells - either membrane localisation or cytoplasmic. For MMPs and TIMPs, the extent of staining localised within the stromal compartment was recorded as sparse (1), moderate (2) or abundant (3). The presence or absence of staining in the wall of small calibre blood vessels was also recorded.

2.3.3.5 Statistical Analysis

Data entry and statistical analysis were done using Epi 5 (Epidemiological Package Version 5) and SPSS (Statistical Package for Social Sciences, SPSS Inc.) programs. The association between staining patterns and clinicopathological parameters of the tumours was assessed using both parametric and non-parametric tests, having evaluated the homogeneity of the tumour sample using Bartletts test for homogeneity of variance. Where the data showed a normal distribution, analysis of variances (ANOVA) test was used. If Bartletts test showed the variances in the samples to differ, the non-parametric Kruskal-Wallis test was applied. To assess the relationship between two sets of immunohistochemical staining, Kendalls tau correlation coefficient was used. A p value of < 0.05 was adopted as significant.

2.3.4 Zymographic Analysis of Breast Tissue

2.3.4.1 Zymography

Many of the MMPs, including the Type IV collagenases and the stromelysins, can degrade gelatin and this was therefore chosen as the protein substrate to be incorporated into the zymograms. To achieve a final substrate concentration of 1 mg/ml, 10 mg of type III collagen from calf skin in 4 mls double deionised water was denatured at 65°C for 1 hr. This was then added to a 10% SDS-polyacrylamide gel resolving gel (appendix 3) with a 3.75% stacking gel. The samples were mixed 1:1 with 2 x Non-reducing Sample Buffer and without prior denaturing were applied to the stacking gel. For each gel, MultiMark Molecular Weight Markers were applied, as was an internal standard which comprised either HT 1080 conditioned media, in early studies, and latterly recombinant pro-MMP-9 and pro-MMP-2. Electrophoresis was carried out at 75 V for 3 1/2 hrs in Running Buffer.

Once the proteins were separated, the SDS was removed from the gel by three washes of 15 mins in 2.5% Triton X-100 carried out at room temperature with agitation. After briefly rinsing in deionised water, the gel was then incubated in pre-warmed Incubation Buffer for 18 hrs at 37°C with gentle agitation. Following the incubation period, the gel was stained in 100 ml 0.05% Coomassie Blue staining solution for 4 hrs, and destained in three changes of Destaining Solution, washing for 15 mins, 30 mins and 60 mins respectively with each wash. The gel was then visualised on a light box, a record taken on a Gel Documentation system, and the gel was sealed in a polythene bag and stored for further densitometric analysis using a Pharmacia LKB Imagemaster scanning densitometer.

2.3.4.2 Quantitation of Zymograms

- Determination of Linear Range of Assay

To allow comparisons of gelatinolytic activity to be made between different samples, it was necessary to establish the linear range of the assay. To do this, a dilution series of purified, recombinant pro-MMP-2 and pro-MMP-9, ranging from 0.1 ng to 200 ng of protein, was prepared by diluting the stock protein of 1 mg/ml in sterile PBS. The samples were mixed 1:1 in 2 x Non-reducing Sample Buffer and applied to substrate gels, as described in section *2.3.4.1*. The gels were analysed densitometrically, and a graph of densitometric reading: MMP concentration was constructed, from which the linear range of the assay could be established.

- Assessment of Reproducibility

In order to examine the reproducibility of the technique samples of recombinant pro-MMP-2 and pro-MMP-9, at a concentration falling within the linear range of the assay, were subjected to replicate zymographic analysis on four separate gels on the same day. Densitometric analysis was performed on each gel and the values used to calculate standard deviation and coefficient of variation for inter-assay reproducibility.

2.3.4.3 Zymographic Analysis of Breast Tissue

- Sample Preparation

Selected cases which had been analysed immunohistochemically were subjected to zymographic analysis. In total, 20 IDC, which showed either low (0 - <20%) or high (>75%) level membrane staining for MMP-2 and MT1-MMP were examined. The same block of frozen tissue used for immunohistochemistry was selected for zymography. Four x 10 μm cryosections were transferred to pre-cooled sterile eppendorfs using the sharp end of a sterile glass micropipette. Protein was extracted and concentration measured as described in section 2.3.1.2,

and the samples normalised to 1 mg/ml protein. Samples were subjected to zymographic analysis immediately following protein extraction according to the method described in section 2.3.4.1 and the gels analysed densitometrically. The MMP-2 activation ratio (integrated density [ID] for 62kDa : ID for 72kDa + 62kDa) was calculated for each case, and the relationship between activity and membrane staining for MMP-2 and MT1-MMP was analysed using the Mann-Whitney U test.

2.3.5 In-Situ Hybridisation

2.3.5.1 Pre-treatment of Glassware and Solutions

All glassware, plasticware and solutions used for RNA work was treated to destroy RNases. Glassware and plasticware was soaked in 3% Hydrogen Peroxide for 30 mins then rinsed three times in DEPC-treated water before drying at 80°C. All solutions were prepared using RNase free glassware, DEPC-treated water and where possible chemicals reserved for work with RNA.

2.3.5.2 Labelling of Oligonucleotide Probes with Digoxigenin

The oligonucleotide cocktails were labelled at the 3' end with digoxigenein-11-dUTP using terminal deoxynucleotidyl transferase (Deng and Wu, 1981). To enhance efficiency of labelling, excess hapten is included in the reaction and dATP was also included to act as a linker arm, allowing enhanced incorporation of digoxigenin labelled nucleotides by minimising stearic hindrance. A range of concentrations of dATP was initially evaluated for their influence on labelling efficiency, but no discernible difference was perceived beyond the lowest concentration employed, which was therefore used for all further labelling.

The labelling reaction was set up by mixing 0.5 μg of the probe cocktail with 12 μl 1mM digoxigenin-12-dUTP, 3.3 μl 5 mM dATP, 4 μl 5 x Boehringer Mannheim Buffer, 4 μl 25

mM Cobalt Chloride, 3 µl Tdt (15 U/µl), made up to a total volume of 20 µl with PCR grade water. The mixture was incubated at 37°C for 2hrs in a water bath, and the enzyme activity halted by addition of EDTA.

2.3.5.3 Evaluation of Probe Labelling Efficiency on Test Strips

Test strips allow some estimation of the efficiency of probe labelling. A dilution series of the labelled cocktail from 0.1 pg/µl to 1 ng/µl was prepared in Diluent Buffer. The samples were boiled at 100°C for 5 min, quenched on ice then dotted onto a nitrocellulose membrane which was placed between sheets of 3MM Whatmann paper and baked at 80°C for 2 hrs. Following rehydration in sterile UP water, the membrane was incubated in Blocking Solution at 42°C for 30 mins. For detection of the labelled probe, the membrane was then incubated in a solution of anti-digoxigenin-alkaline phosphatase (anti-dig-AP) diluted 1:300 in Blocking Solution for 1 hr, followed by 2 x 5 min washes in Blocking Solution and a further 1 hr incubation in a fresh solution of anti-dig-AP. After two further washes in Blocking Solution and 1 x 5 min wash in Buffer 3, the membrane was incubated in freshly prepared substrate of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and checked every 15 mins until a signal developed.

2.3.5.4 Validation of Probe Specificity by Northern Blotting

The specificity of each probe cocktail to the target RNA sequences was assessed by Northern analysis on mRNA extracted from HT 1080 cells. The method of mRNA extraction employed is detailed in Chapter 3, section 3.3.3.1.

Samples were denatured at 65°C for 15 mins then quenched on ice and 3 µg mRNA in 5 x formaldehyde Gel Running Buffer (total volume of 20 µl) was loaded onto a 1.2% denaturing

agarose gel (1.2% w/v agarose, 40 mM 3-(n-morpholino) propanesulphonic acid, 10 mM

sodium acetate, 1 mM EDTA, 0.74% v/v formaldehyde). The samples were loaded together

with RNA markers and the gel run at 70 V in 1 x Formaldehyde Gel Running Buffer for 3

hours. The gel was then rinsed several times in DEPC-water to remove the formaldehyde and

equilibrated in 20 x SSC. Fractionated RNA was then transferred onto positively charged nylon

membrane with 20 x SSC as the transfer buffer. The filter was then soaked in 6 x SSC for 5

mins and dried for 30 mins followed by 30 sec exposure to ultraviolet light to enhance cross-

linking.

The filter was then incubated in prewarmed prehybridisation solution (5 x SSC, 10

mg/ml denatured ssDNA, 0.1% sarcosyl, 0.02% SDS, 30% formamide, 2 x Blocking reagent)

at 37°C for 1 hour in a Hybaid oven. Digoxigenin-labelled probes were added to the pre-

hybridisation solution at a concentration of 5 ng/ml, and incubated at 37°C for 12 hrs. The

filters were subjected to 2 x 10 min posthybridisation washes in prewarmed 2 x SSC/0.1% SDS

at 37°C, then after 1 min in Washing Solution and 30 min in Blocking Solution, they were

incubated in anti-dig-AP diluted 1:20 000 in Blocking Solution for 30 mins. The filters were

washed 2 x 15 min in Washing Solution, equilibrated in Substrate Buffer for 5 min then

exposed to the CPD-Star substrate, diluted 1:250 in Substrate Buffer, for 5 mins. The substrate

was drained from the filter which was then covered in Saranwrap and exposed to Hyperfilm

ECL for varying periods of time.

2.3.5.5 In-situ Hybridisation (ISH)

The probes were initially tested out on HT 1080 cells grown on glass coverslips prior to

optimisation of conditions for their use on paraffin embedded formalin fixed material.

- ISH on HT 1080 cells

Cells were grown on poly-d-lysine coated glass coverslips and fixed in 4% paraformaldehyde

for 20 mins at 4°C. After 2 x 5 min washes in cold sterile PBS and 1 x 5 min in DEPC water,

the cells were incubated in 2 x SSC at 70°C for 10 mins. Following a brief wash in DEPC water

at room temperature, the cells were either directly incubated in prehybridisation solution for 1

hr at 37°C, or prior to this incubated for 1 hr at 37°C in 2 μg/ml Proteinase K (PK). Following

prehybridisation, an equal volume of hybridisation solution was added and the coverslips

incubated overnight (10 hrs). In initial studies the probe concentration was varied as shown in

Table 2.4. Posthybridisation washes were carried out for 2 x 10 min at 37°C at varying

stringency, by altering the formamide concentration, as detailed in Table 2.4. After washing in

Blocking Solution for 5 mins, detection of bound probe was achieved by incubation in anti-dig-

AP diluted 1:600 in Blocking Solution for 30 mins, 2 x 15 min washes in TBS, followed by

equilibration in Substrate Buffer for 5 min prior to incubation in substrate made up as detailed

in *2.3.5.3*. The coverslips were incubated in the dark and checked periodically to assess

development of signal. In parallel to the test cells, control samples were set up including cells

treated with 100 μg/ml RNase A at 37°C for 1 hr prior to the prehybridisation stage, and a 'no

hybridisation' control, in which the probe was omitted. In some experiments an acetylation step

was introduced, in which after fixation, cells were immersed in 0.05% acetic anhydride in

xylene for 5 mins before completing the protocol. To control for RNA integrity, each case was

hybridised with a probe to a housekeeping gene. In this study a probe to mitochondrial mRNA

was used, designed in house by Dr. Howard Pringle.

- ISH on Paraffin Embedded Fixed Tissue

This was carried out as described for HT 1080 cells with several additional steps necessary because of the tissue fixation. Sections were dewaxed and rehydrated to DEPC-water then following the wash in 2 x SSC, the sections were incubated in a prewarmed solution of Proteinase K for 1 hr at 37°C. Varying concentrations of Proteinase K were assessed in initial studies as shown in Table 2.4. Following digestion, the tissue was then fixed in 0.4% paraformaldehyde at 4°C for 20 mins, and after washing in DEPC-water at room temperature for 5 min, the prehybridisation step was carried out, and the method was then followed as described for HT 1080 cells.

For each case, parallel tissue sections were (i) treated with 100 μg/ml RNase A at 37°C for 1 hr prior to the prehybridisation step, (ii) incubated as the test section but without addition of probe, and (iii) hybridised with a probe to mitochondrial mRNA to control for RNA integrity. When optimal signal had developed, the sections were washed in tap water and mounted onto glass coverslips using Apathys aqueous mounting media, and the distribution of signal was recorded for each case.

Table 2.4: Conditions Tested for In-Situ Hybridisation on HT 1080 Cells and on Primary Tissue

VARIABLE	HT 1080	TISSUE
Probe Concentration (ng/ml)	100, 200, 400, 800	100, 200, 400, 800
Stringency (% Formamide)	20, 30, 50	20, 30, 50
Protease K Concentration (μg/ml)	None, 2	2, 5, 10

2.4 Results

2.4.1 Western Blotting

2.4.1.1 Optimisation of Conditions

To confirm the spectrum of reactivity of the non-commercial antibodies, Western blot analysis was performed on conditioned media (CM) of HT 1080 fibrosarcoma cells, which are known to secrete MMP-2, MMP-9, TIMP-1 and TIMP-2 (Ramos-DeSimone et al, 1993), and on protein extracted from Concanavalin A-treated cells, for MT1-MMP. Since the synthetic peptide that was used to raise the MT1-MMP antibody was available, Western blotting was also performed following absorption of the antibody with the peptide.

A range of primary antibody dilutions was evaluated, and the final antibody concentration employed, which gave maximum signal with minimum background reactivity, is shown in Table 2.5. Optimum reactivity was seen with secondary antibody used at 1:2000 dilution.

Table 2.5: Concentration of Primary Antibodies used for Western Blotting

ANTIBODY	CONCENTRATION
MMP-2 (ss)	1:2500
MMP-9 (ss)	1:5000
TIMP-1 (ss)	1:7500
TIMP-2 (ss)	1:5000
MT1-MMP	1:500

ss : Antibodies from Dr. Stetler-Stevenson, N.I.H., Bethesda

2.4.1.2 Reactivity of Antibodies

The antibody against MMP-2 identified a band at 72 kDa, and a second band separated from

this by approximately 10 kDa, indicating latent and active enzyme species, respectively. A third

band at approximately 68 kDa indicated the intermediate form of MMP-2 (fig. 2.1).

MMP-2 ← 72 kDa
← 68 kDa
← 62 kDa

MMP-9 ← 92 kDa
← 82 kDa

MT1-MMP ← 63 kDa
← 58 kDa
← 43 kDa

Absorbed MT1-MMP

TIMP-1 ← 28 kDa

TIMP-2 ← 21 kDa
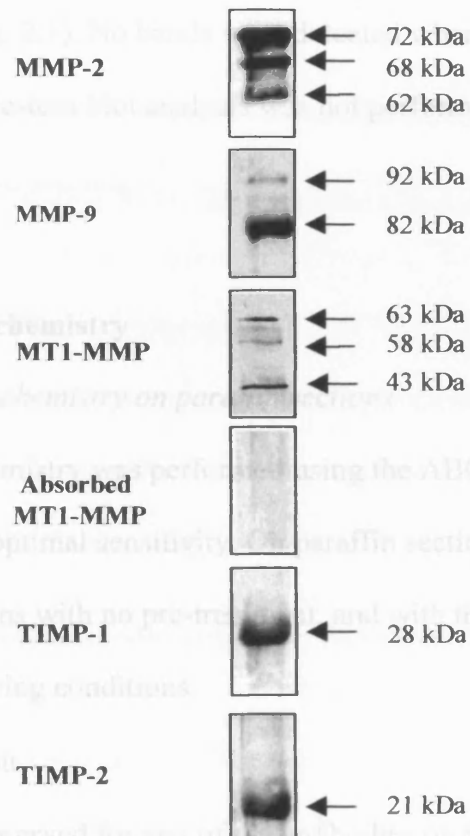
Fig. 2.1: Western Blot Analysis of MMP/TIMP Antibodies.
Western blotting was performed on CM from HT 1080 cells for MMP-2, MMP-9, TIMP-1 and TIMP-2.
Blotting for MT1-MMP was performed on protein extracted from Concanavalin-A-treated HT 1080 cells
with a control blot following preabsorption of the antibody with synthetic peptide.

The antibody against MMP-9 labelled a doublet at ~92 kDA and 82 kDA, indicating

latent and active enzyme species, respectively. A strong band was identified at 28 kDA

for TIMP-1 and 21 kDa for TIMP-2, corresponding to the expected sizes for unbound TIMP.

The MT1-MMP antibody detected three major bands at 63 kDa, 58 kDa and 43 kDa, with a

weaker band at ~60 kDa. These correspond to the MT1-MMP proform, the active membrane

bound protein and a further N-terminally processed form of MT1-MMP, respectively

(Lohi *et al*, 1996; fig. 2.1). No bands were detected when the antibody was pre-absorbed with

synthetic peptide. Western blot analysis was not performed for monoclonal antibodies obtained

commercially.

2.4.2 Immunohistochemistry

2.4.2.1 Immunohistochemistry on paraffin sections

All immunohistochemistry was performed using the ABC indirect immunoperoxidase

technique to afford optimal sensitivity. On paraffin sections, immunohistochemistry was

carried out on sections with no pre-treatment, and with the use of two different antigen retrieval

techniques with varying conditions.

- No Pre-Treatment

No reactivity was observed for any of the antibodies on formalin fixed paraffin embedded

tissue with no pre-treatment. Cytoplasmic reactivity was seen in monensin-treated HT 1080

cells with MMP, TIMP and MT1-MMP antibodies, as was expected. Anti-HMFG antibodies

were used as a positive control on fixed tissue, and this gave strong reactivity. Cryostat sections

of normal skin was used as a positive control for integrin and E-Cadherin antibodies, since this

has previously been shown to give good reactivity, and strong, well defined reactivity was

observed for all cell adhesion molecule antibodies. Due to the lack of reactivity, a number of

antigen retrieval techniques were tested.

- Protease Digestion

Tissue sections were pre-treated in a 0.05% Type XIV protease solution for 5, 10 or 15 mins prior to application of antibodies, however, no reactivity was detected for any of the antibodies under these conditions.

- Microwave Pre-Treatment

Microwave antigen retrieval was performed with a 0.01M solution of citric acid. Subsequent immunohistochemical staining by the ABC method failed to demonstrate staining for any of the cell adhesion antibodies or for MMP-9, TIMP-1 and TIMP-2. With MMP-2 (ss) and MT1-MMP antibodies, a diffuse homogeneous 'blush' was observed throughout the breast tissue, of equal intensity in the epithelial and stromal compartments. This staining was not thought to be specific and therefore further efforts for immunohistochemical detection of these proteins were directed towards use of frozen tissue sections.

2.4.2.2 Immunohistochemistry on Frozen Tissue

Optimal staining combined with adequate morphological preservation was achieved with frozen tissue sections, briefly fixed in cold acetone. The paraformaldehyde/ methanol/acetone method gave similar results, therefore for convenience, the former shorter fixation method was employed for all studies. The optimal antibody dilution was established in initial experiments and then used throughout the study (see Table 2.6)

The expression pattern will be considered for MMPs and their inhibitors first, and in the discussion of the tumours, will be grouped according to their enzyme systems, that is, MMP-2 with its inhibitor and activator, and MMP-9 with its inhibitor. The expression of adhesion molecules will then be described and finally the relationship between the two will be presented.

Table 2.6: Concentration of Primary Antibodies used for Immunohistochemistry

ANTIBODY	DILUTION
α2, α3, α5, α6 integrins	1:200
β1, β4 integrins	1:500
E-Cadherin	1:50
MMP-2(os), MT1-MMP, TIMP-1(os), TIMP-2(os)	1:50
MMP-2(ss), MMP-9(ss), TIMP-1(ss)	1:1000 (4 μg/ml)
TIMP-1(ss)	1:1500 (10 μg/ml)

2.4.2.3 Expression of MMPs and TIMPs in Breast Tissue

• Expression of MMPs and TIMPs in Normal/Benign Tissue

Normal breast tissue taken from reduction mammoplasty specimens, and benign breast tissue

was examined for expression of MMPs, TIMPs and MT1-MMP. In all cases, a band of staining

was observed for MMP-2, TIMP-1, TIMP-2 and MT1-MMP in the basement membrane zone

around ducts and some acini (Fig.2.2). A similar pattern of reactivity was also observed in

relation to blood vessel basement membrane. Occasionally, weak reactivity was seen for MMP-

9 around ducts, but this was inconstant and seen in only 2 of 15 cases. No stromal staining

(other than that related to blood vessels) was seen with any of the antibodies.

• Expression of MMP-2, TIMP-2 and MT1-MMP in Malignant Breast

Each component of this enzyme system was widely expressed in breast carcinomas, with

only10 of 114 cases lacking reactivity for either MMP-2 or MT1-MMP, and 22 tumours

lacking significant expression of TIMP-2 (Table 2.7). The pattern of reactivity consistently

observed for MMP-2 was of diffuse cytoplasmic staining of the tumour cell groups (fig.2.3): all

90

cases positive for MMP-2 exhibited this type of staining, though to a variable degree and with some differences between the two antibodies used. With the monoclonal antibody (MMP-2(os)), 91% of the tumours showed cytoplasmic MMP-2 compared to 97% with the polyclonal antibody (MMP-2(ss)). The degree of cytoplasmic reactivity showed no correlation with tumour type, grade or lymph node status. In all cases exhibiting reactivity, staining was also localised to the stroma, most prominently around individual tumour groups. In addition, staining was also present at the tumour cell membrane, in 34% of cases with MMP-2(os) and 55% of cases with the MMP-2(ss) (Fig.2.3). This staining pattern was present to a variable extent; using MMP-2(os), 46% of positive cases contained <20% of tumour cells with membrane reactivity compared to 38% with the MMP-2(ss). A greater number of cases showed membrane staining using the MMP-2(ss) and in some cases this was more extensive, 59% of positive tumours displaying 20-50% reactivity compared to 53% with the MMP-2(os). The presence and extent of membrane reactivity showed no correlation with tumour grade, however, there was a significant positive association with the presence of lymph node metastasis (p=0.001 for MMP-2(os) and p=0.008 for MMP-2(ss); fig.2.4).

The staining pattern seen for MT1-MMP was similar to that of MMP-2 in that all positive cases revealed a variable extent of diffuse cytoplasmic reactivity in the tumour cell population, staining of peri-tumoural stromal cells and in 68% of cases distinct tumour cell membrane staining (Fig.2.3). Of these positive cases, 35% showed membrane staining in <20% of tumour cells, with 20-50% reactivity in 59% of cases. As with MMP-2, membrane staining for MT1-MMP was also significantly associated with a positive lymph node status (p=0.01; Fig.2.4). There was no association with tumour grade. There was a significant association between those cases showing membrane reactivity for MT1-MMP and membrane reactivity for MMP-2(os) (p=0.02; fig. 2.5), but this relationship was not significant in the case of MMP-
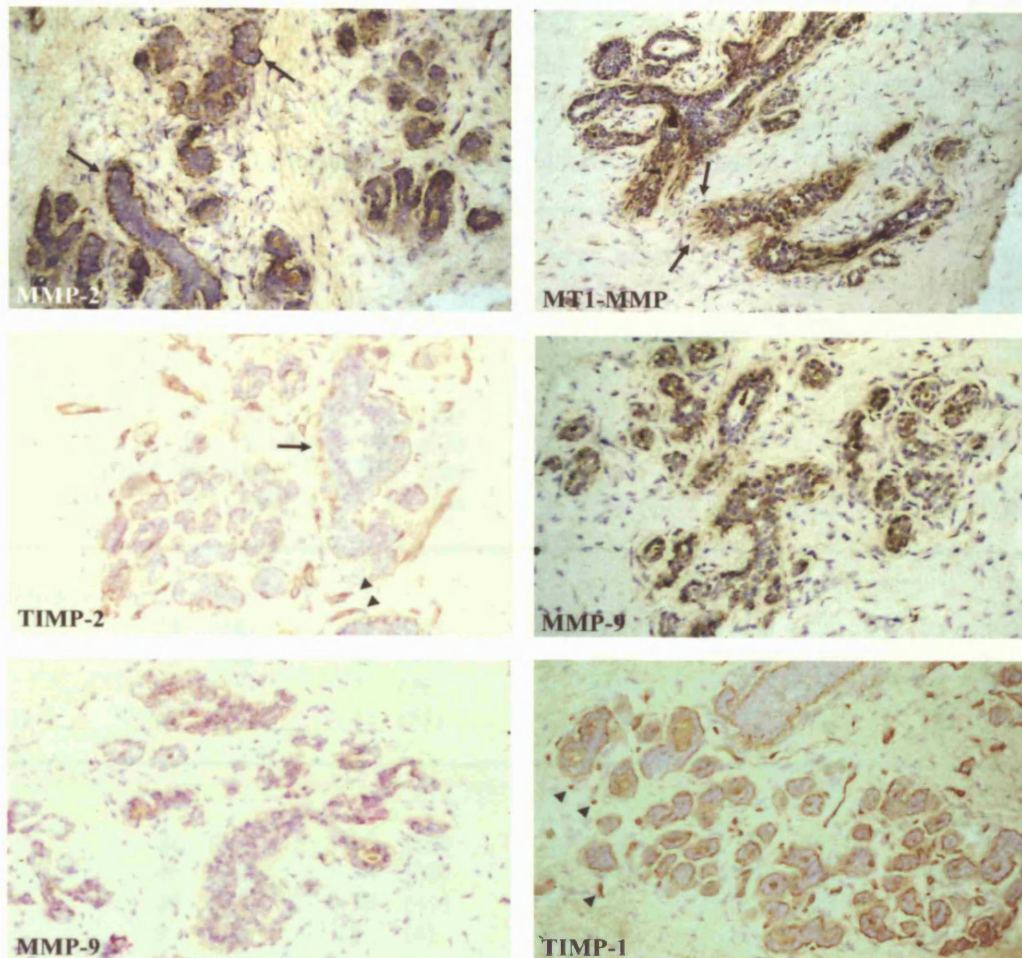
Fig 2.2 : Expression of MMPs and TIMPs in Normal Breast

A band of staining for MMP-2, MT1-MMP, TIMP-1 and TIMP-2 is present around ducts and acini in relation to the basement membrane (arrows). Small blood vessels show reactivity for TIMP-1 and TIMP-2 (arrow heads). Staining for MMP-9 is less consistent but in some cases weak reactivity is present in the basement membrane zone.

2(ss) (fig. 2.6), where membrane reactivity was seen in 16% of cases which did not show

membrane MT1-MMP. This is in contrast to MMP-2(os) which showed membrane staining in

only 7% of cases not exhibiting membrane MT1-MMP. When tumours expressing both

Table 2.7: Summary of extent and pattern of staining for MMP-2, MT1-MMP and TIMP-2 in Carcinomas

Staining Pattern	MMP-2 (os) % (no.)	MMP-2 (ss) % (no.)	MT1-MMP % (no.)	TIMP-2 (os) % (no.)	TIMP-2 (ss) % (no.)
Tumour Cytoplasm					
<20%	10 (12)	7 (8)	2 (2)	3 (3)	1 (1)
20-50%	51 (57)	46 (52)	63 (72)	10 (11)	4 (5)
>50%	30 (35)	44 (50)	33 (38)	9 (11)	0
Neg.	9 (10)	3 (4)	2 (2)	78 (89)	95 (108)
Tumour Membrane					
<20%	16 (18)	21 (24)	24 (27)	3 (3)	0
20-50%	17 (20)	32 (37)	40 (46)	15 (17)	7 (8)
>50%	1 (1)	2 (2)	4 (5)	9 (11)	5 (5)
Neg.	66 (75)	45 (51)	32 (36)	73 (83)	89 (101)
Stroma					
1	22 (25)	25 (29)	24 (28)	30 (34)	35 (40)
2	45 (51)	34 (38)	45 (51)	41 (47)	36 (41)
3	24 (28)	36 (41)	30 (34)	21 (24)	10 (11)
Neg	9 (10)	5 (6)	1 (1)	8 (9)	19 (22)
Blood vessels					
Pos	95 (108)	96 (110)	92 (102)	0	0
Neg	5 (6)	4 (4)	8 (12)	100 (114)	100 (n114)

ss : Antibodies from Dr. Stetler-Stevenson, N.I.H., Bethesda
os : Antibodies from Oncogene Science

membrane MMP-2 and MT1-MMP were analysed for their relationship with lymph node

status, there was a highly significant correlation with the presence of metastasis (p=<0.001 for

MMP-2(os) and for MMP-2(ss)).

The predominant pattern of reactivity seen for TIMP-2 was of diffuse stromal staining

with 92% and 81% of cases staining with TIMP-2(os) and TIMP-2(ss) respectively (fig.2.3).
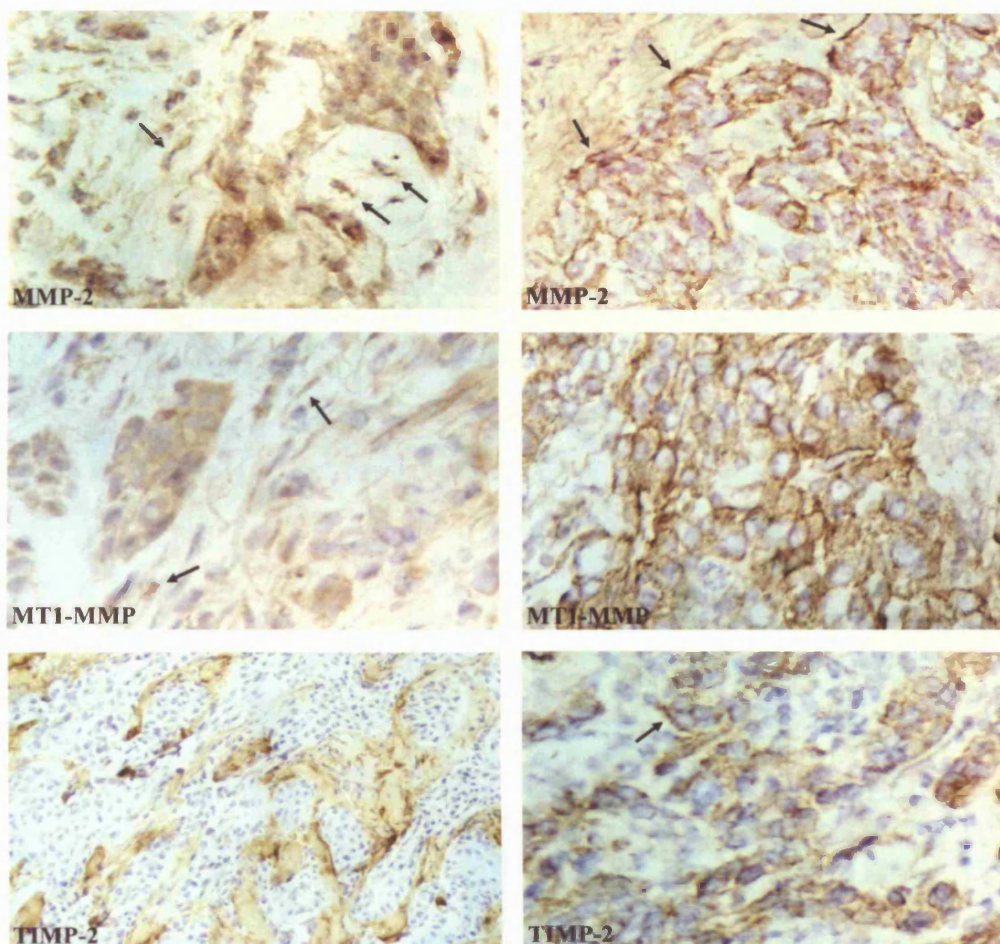
Fig 2.3 : Expression of MMP-2, MT1-MMP and TIMP-2 in Breast Carcinoma

Diffuse cytoplasmic staining of tumour cells is seen for MMP-2 and MT1-MMP in the majority of cases, together with staining of stromal fibroblasts (arrows, left panel top and middle). Tumour cell membrane staining for MMP-2 and MT1-MMP is evident in some cases (right panel, top and middle). Staining for TIMP-2 is localised predominantly within the stroma around tumour groups (lower left), though focal tumour cell membrane and cytoplasmic staining is present in some tumours (lower right).

(i)
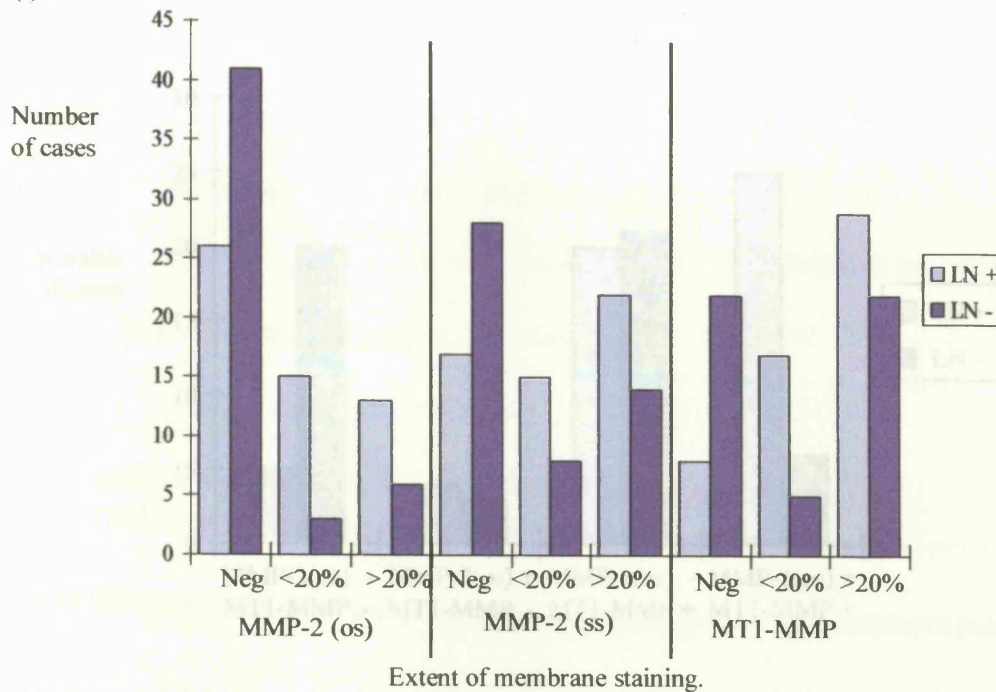
(ii)
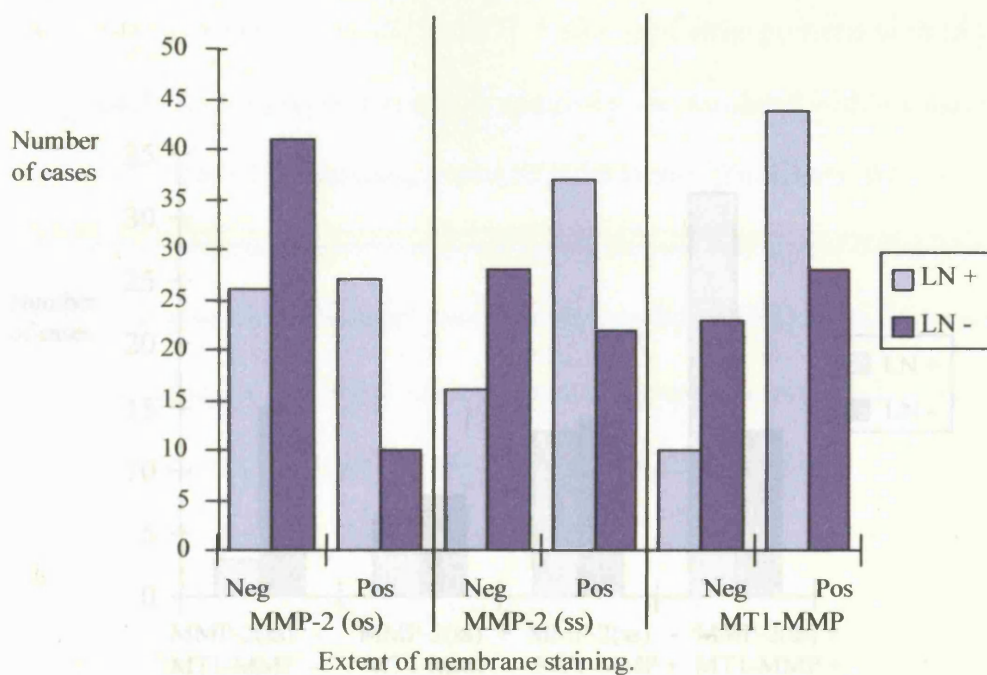
Fig. 2.4: Relationship between membrane MMP-2 and membrane MT1-MMP staining and lymph node status. (i) categorising extent of reactivity into 3 groups and (ii) showing distribution of cases according to presence or absence of staining. A significant association is demonstrated between the presence of membrane reactivity and positive lymph node status (p= 0.001, 0.008 and 0.01 for MMP-2 (os), MMP-2 (ss) and MT1-MMP, respectively.
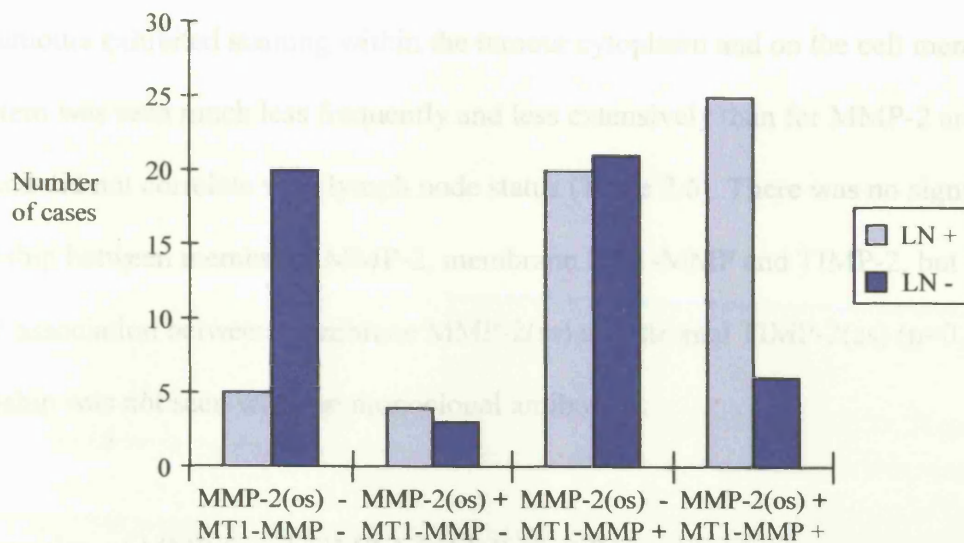
Fig. 2.5: Relationship between membrane staining for MT1-MMP and MMP-2(os). There is a significant association between membrane reactivity for both antibodies (p=0.03), and a highly significant association between membrane reactivity with both antibodies and the presence of lymph node metastases (p=<0.001).
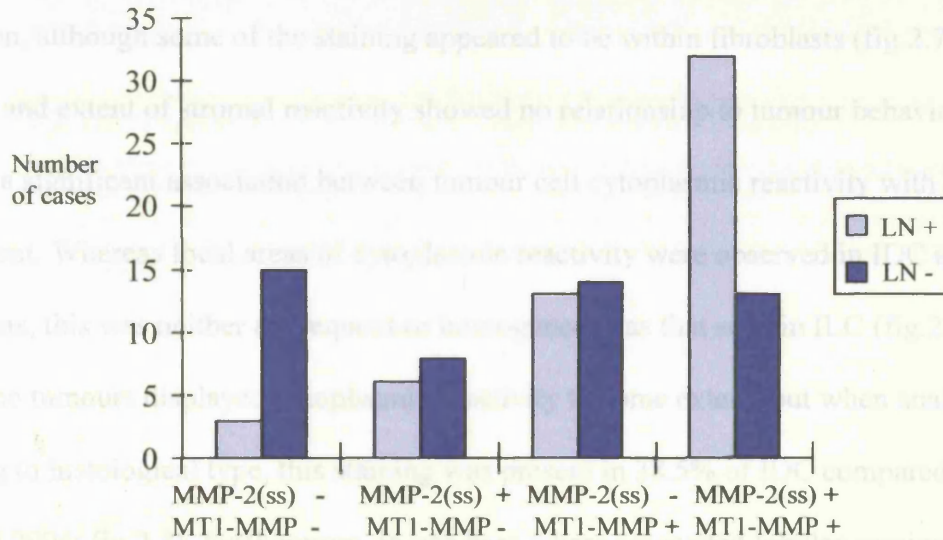
Fig. 2.6: Relationship between membrane MT1-MMP and membrane MMP-2 (ss). There is no significant association between membrane reactivity for these antibodies, but membrane staining with both antibodies is associated with the presence of lymph node metastases (p=0.001).

96

Some tumours exhibited staining within the tumour cytoplasm and on the cell membrane but this pattern was seen much less frequently and less extensively than for MMP-2 and MT1-MMP and did not correlate with lymph node status (Table 2.6). There was no significant relationship between membrane MMP-2, membrane MT1-MMP and TIMP-2, but there was a positive association between membrane MMP-2(ss) and stromal TIMP-2(ss) ($p=0.03$). This relationship was not seen with the monoclonal antibodies.

- Expression of MMP-9 and TIMP-1 in Malignant Breast

Reactivity for MMP-9 was less ubiquitous compared with that for MMP-2 and MT1-MMP, with only 68% of carcinomas exhibiting staining (Table 2.8). In 47% of cases staining was evident within the stromal compartment (fig. 2.7). Staining of serial sections with anti-CD68 revealed that in many cases a proportion of this reactivity was localised within a macrophage population, although some of the staining appeared to be within fibroblasts (fig.2.7). The presence and extent of stromal reactivity showed no relationship to tumour behaviour. In contrast, a significant association between tumour cell cytoplasmic reactivity with tumour type was evident. Whereas focal areas of cytoplasmic reactivity were observed in IDC and tubular carcinomas, this was neither as frequent or homogeneous as that seen in ILC (fig.2.7). Overall, 49% of the tumours displayed cytoplasmic reactivity to some extent, but when analysed according to histological type, this staining was present in 38.5% of IDC compared to 81.5% of ILC ($p=0.0004$; fig.2.8). Furthermore, in one case where associated lobular carcinoma in-situ was present, these cells also displayed cytoplasmic reactivity for MMP-9 (fig.2.7). There was no association of this staining pattern with grade or lymph node status.

Predominantly stromal reactivity was present for TIMP-1 being localised either in the wall of small blood vessels, as confirmed by staining of serial sections with QBEND10
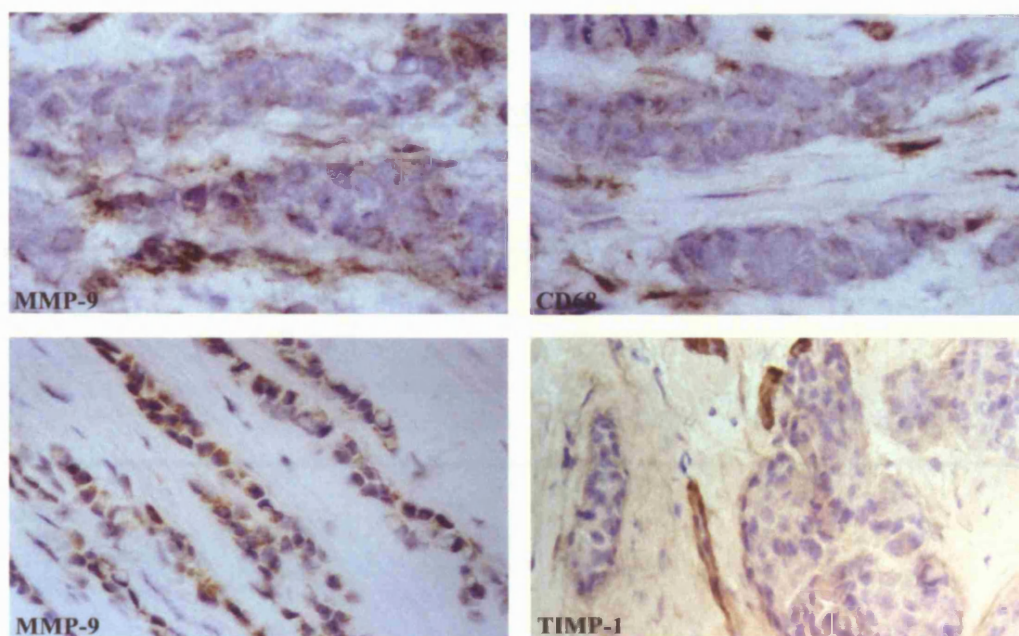
Fig 2.7 : Expression of MMP-9 and TIMP-1 in Breast Carcinoma

In IDC, MMP-9 is localised predominantly in the stromal compartment (top left) and staining of serial sections with CD68 suggests that at least a proportion of this reactivity is present in macro--phages (top right). In ILC, staining for MMP-9 is consistently seen in the tumour cell cytoplasm (lower left). TIMP-1 is present diffusely in the stroma and also decorates small blood vessels (lower right).

antibody, or diffusely in fibroblasts and the extracellular matrix (fig.2.7). Reactivity within the

tumour cell compartment was rare and showed no association with tumour type or behaviour.

There was no significant relationship between staining for MMP-9 and reactivity of TIMP-1.

Table 2.8: Summary of Extent and Pattern of Staining for MMP-9 and TIMP-1 in Carcinomas

Staining Pattern	MMP-9 (ss) % (no.)	TIMP-1 (os) % (no.)	TIMP-1 (ss) % (no.)
Tumour Cytoplasm			
<20%	6 (7)	0	1 (1)
20-50%	30 (34)	6 (7)	4 (4)
>50%	13 (15)	3 (3)	0
Neg.	51 (58)	91 (104)	95 (109)
Tumour Membrane			
<20%	0	1 (1)	2 (2)
20-50%	0	4 (5)	3 (3)
>50%	0	5 (5)	0
Neg.	100 (114)	90 (103)	95 (109)
Stroma			
1	26 (30)	30 (34)	38 (43)
2	17 (20)	19 (22)	16 (18)
3	4 (4)	0	3 (4)
Neg	53 (60)	51 (58)	43 (49)
Blood vessels			
Pos	0	60 (68)	95 (108)
Neg	100 (114)	40 (46)	5 (6)

ss : Antibodies from Dr. Stetler-Stevenson, N.I.H., Bethesda
os : Antibodies from Oncogene Science
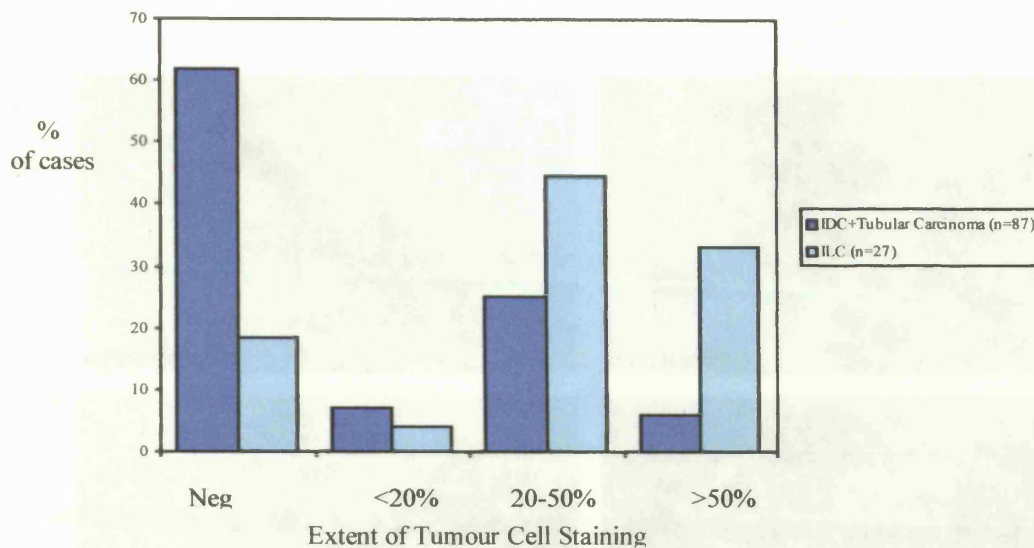
Fig. 2.8: Relationship Between Tumour Cell Staining for MMP-9 and Tumour Type.
A highly significant relationship is identified between the presence of tumour cell cytoplasmic
MMP-9 and Infiltrating Lobular Carcinoma (p=0.0004).

2.4.2.4 Expression of Cell Adhesion Molecules in Breast Tissue

• **Expression of Cell Adhesion Molecules in Normal/Benign Breast**

Strong reactivity for α2-, α3- and α6 integrins was present in normal breast. Staining was most

prominent on the membrane of myoepithelial cells, particularly in relation to the basement

membrane, but also at the lateral cell borders (fig. 2.9). Weak staining for α2- and α3-integrin

was seen on the lateral cell membranes of luminal epithelial cells (fig. 2.9). A similar pattern of

reactivity was observed for β1-integrin again with strongest staining present on the

myoepithelial component, together with staining of stromal fibroblasts and small blood vessels

(fig. 2.9). Staining for β4-integrin was confined to the basal aspect of myoepithelial cells at the

cell-stromal interface (fig. 2.9), with focal staining of small blood vessels. There was strong

reactivity for E-Cadherin in normal breast ducts and acini, localised to the intercellular borders,

both between epithelial cells and at the epithelial-myoepithelial junction. Staining was not

99
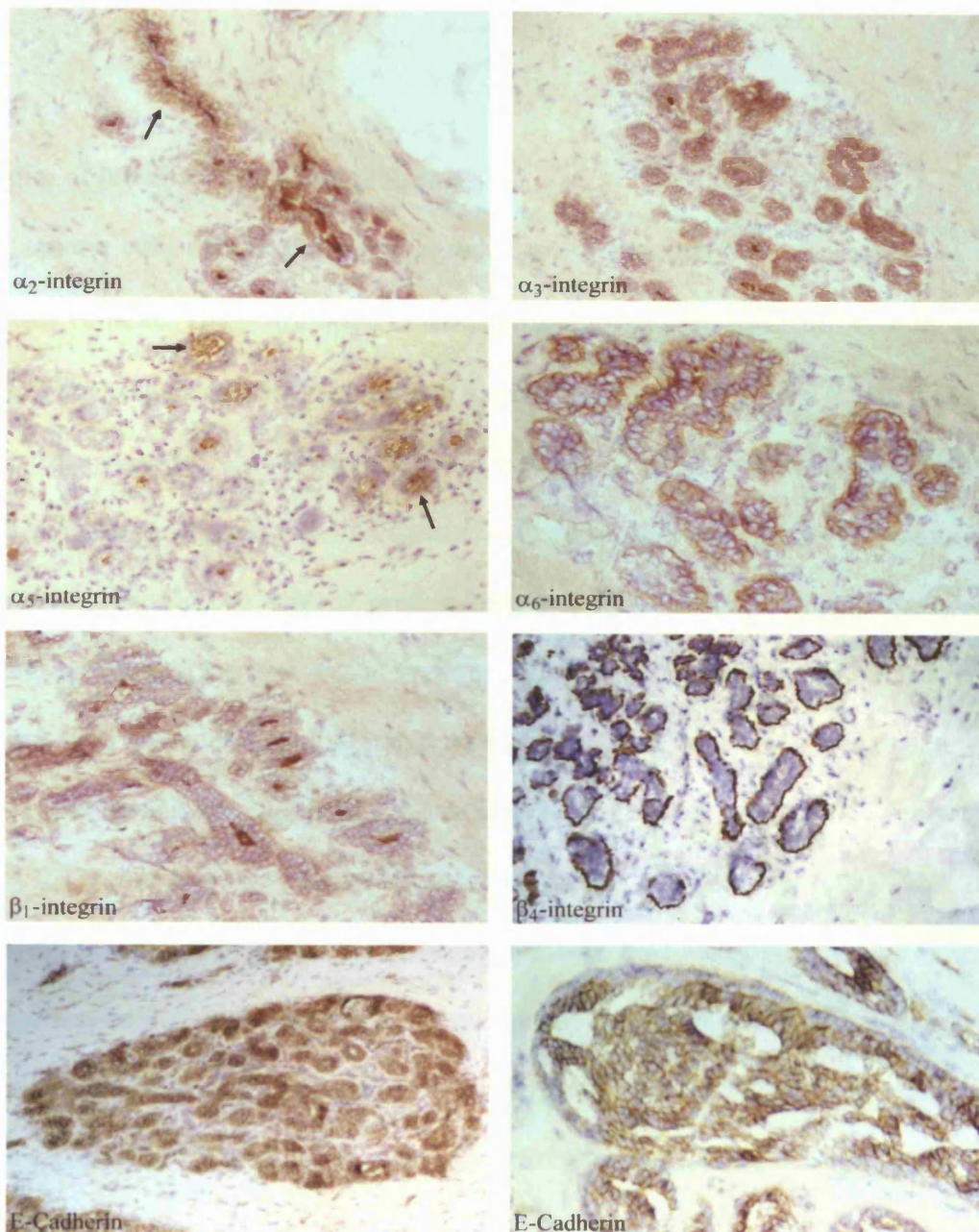
Fig 2.9 : Expression of Cell Adhesion Molecules in Normal Breast

Staining for α_2- and α_3-integrin is present at the cell-basement membrane junction (arrows top left) and at cell-cell borders. No convincing membrane reactivity is observed for α_5-integrin. The arrows indicate non-specific staining of proteinaceous material in gland lumens. Staining for α_6-integrin is seen at cell-cell borders with accentuation of reactivity at the cell-basement membrane interface. β_1-integrin has a similar distribution to that of α_2- and α_3-integrins, whilst reactivity for β_4-integrin is limited to the cell-basement membrane interface. E-Cadherin reactivity is observed at cell-cell borders in all ducts and acini. Staining is particularly strong in luminal epithelial cells compared to myoepithelial cells (lower left).

evident at the cell-matrix junction, and there was no reactivity within the stromal compartment (fig. 2.9).

• Expression of Integrins in Malignant Breast

In all cases, tumour cells exhibited some integrin expression though the extent and distribution of staining was variable. The distribution of α2-, α3- and β1 integrin was similar, with staining both at the tumour cell-stromal interface and at tumour cell-cell borders (fig. 2.10). Reactivity for these integrins was also seen in a cytoplasmic localisation in some tumour cells. Some degree of β1-integrin expression was maintained in all cases, however in 24% of tumours only patchy membrane staining was evident, predominantly at the tumour cell-stroma interface. For all three integrin subunits, the extent of membrane staining correlated with tumour differentiation, with a greater degree of membrane staining seen in better differentiated tumours; this relationship was highly significant for α2 and β1 integrin (p=0.0001 for both subunits; fig. 2.11), but only just reached significance for the α3 subunit (p=0.05; Table 2.8b; fig. 2.11). There was also a significant relationship between the levels of membrane expression of these integrin subunits, with high levels of one subunit being associated with high levels of another, particularly for α2 and β1 integrins (p=0.0001). An interesting pattern of reactivity was observed for α5 integrin: this subunit was not expressed by tumour cells, but in all but 3.5% of tumours, staining was present in the peri-tumoural fibroblasts (fig. 2.10). In 24% of cases just occasional groups of tumour cells were surrounded by reactive fibroblasts, however, in 43% of cases there was extensive fibroblast reactivity. The extent of α5 staining showed a significant correlation to tumour grade (p=0.01), but was not related to lymph node status or ER status. Staining for β1 integrin was also evident in the stromal compartment, but the stromal reactivity was diffuse and not limited to the peri-tumoural fibroblast population, and was also seen in
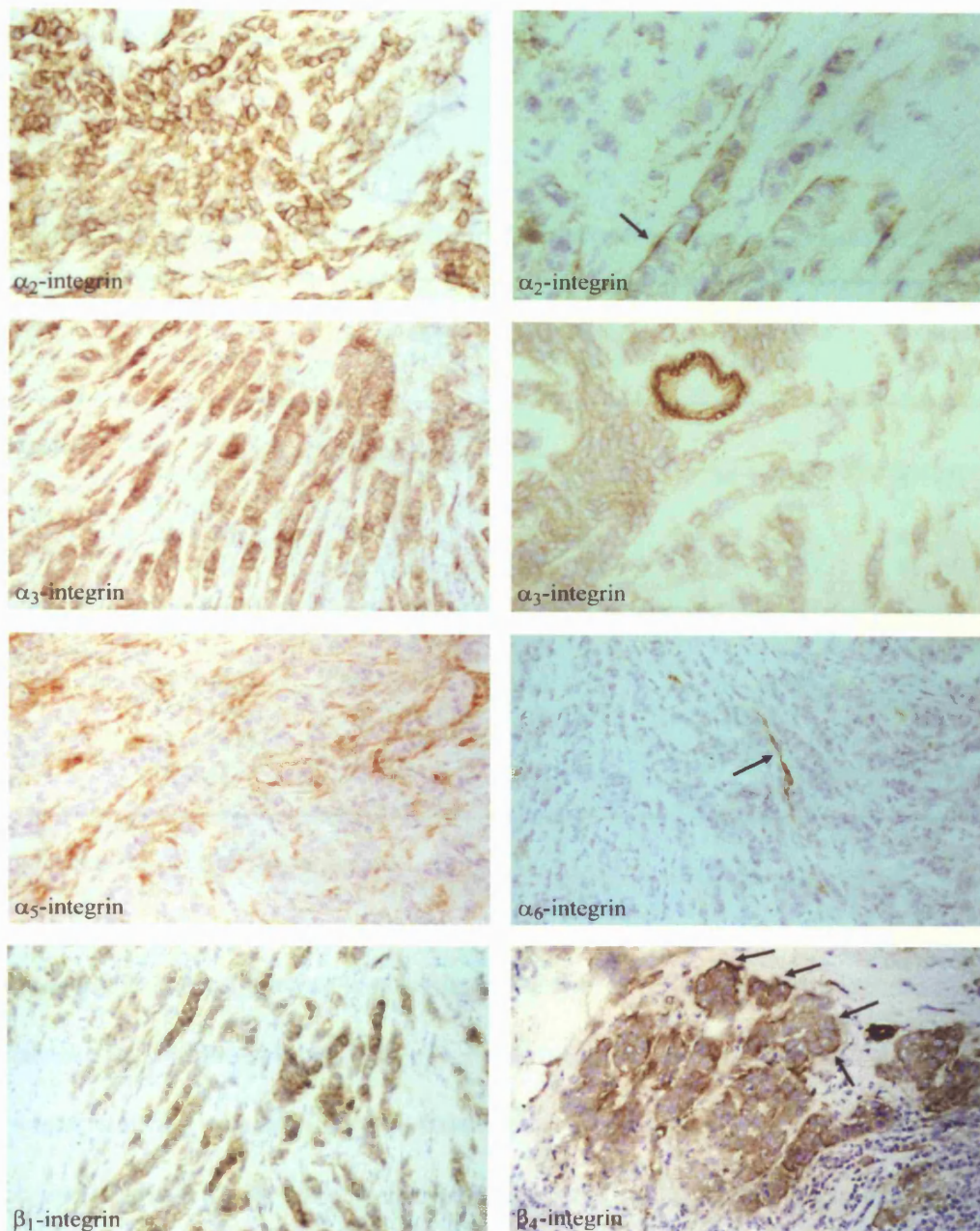
Fig 2.10 : Expression of Integrins in Breast Carcinoma

Some carcinomas exhibit high levels of membrane reactivity for α_2- and α_3-integrins (top left panel), though in other tumours staining is more focal and localised predominantly to the cell-stromal interface (top right). Whilst some tumours exhibit staining for integrins on the majority of tumour cells, this is reduced in intensity compared to residual normal glands (α_3-integrin, right panel). Tumour cells do not stain for α_5-integrin, but reactivity is observed in stromal fibroblasts around tumour groups. Many tumours lack α_6-integrin, staining being observed only in small blood vessels (arrow). Reactivity for β_1 is similar to that seen for α_2- and α_3-integrins, with staining of tumour cell membranes. In those tumours displaying reactivity for β_4-integrin, this is distributed both within the tumour cell cytoplasm and to the tumour cell membrane.

normal/benign breast tissue (fig. 2.10). Membrane expression of α2 integrin was inversely

related to stromal reactivity for α5 integrin (p=0.04).

Table 2.8b Summary of Extent and Pattern of Staining for Integrins in Carcinomas*

Staining Pattern	α2 integrin % (no)	α3 integrin % (no)	α6 integrin % (no)	β1 integrin % (no)	β4 integrin % (no)
Tumour Cytoplasm					
<20%	16 (18)	15 (17)	6 (7)	16.5 (19)	6 (7)
20-50%	7 (8)	7 (8)	11 (13)	8 (9)	7 (8)
>50%	21 (24)	15 (17)	7 (8)	3.5 (4)	6 (7)
Neg.	56 (64)	63 (72)	76 (86)	72 (82)	81 (92)
Tumour Membrane					
<20%	31.5 (36)	20 (23)	16 (18)	24 (27)	15 (17)
20-50%	17 (19)	26 (30)	11 (13)	28 (32)	10.5 (12)
>50%	41 (47)	50 (57)	6 (7)	48 (55)	3.5 (4)
Neg.	10.5 (12)	3.5 (4)	67 (76)	0	72 (82)

* α5 integrin limited to the stromal compartment (see text).

Both α6 and β4 integrins were less frequently expressed than the other subunits with

only 33% staining for α6 integrin and 29% of tumours staining for β4 integrin (Table 2.8b). In

most cases where staining was seen for one subunit, the other subunit was also expressed; only

1 case which was positive for β4 integrin lacked α6 integrin, and 6 cases were positive for

α6 integrin but did not express the β4 subunit. For both subunits, staining was seen either in a

diffuse cytoplasmic pattern, diffuse membrane reactivity or a combination of the two (fig.

2.10). There was no association between expression of these integrins and tumour type or

lymph node status, but reactivity for both integrins correlated with high tumour grade (p=0.01

and 0.001 for membrane and cytoplasmic α6; p= 0.02 and 0.01 for membrane and cytoplasmic
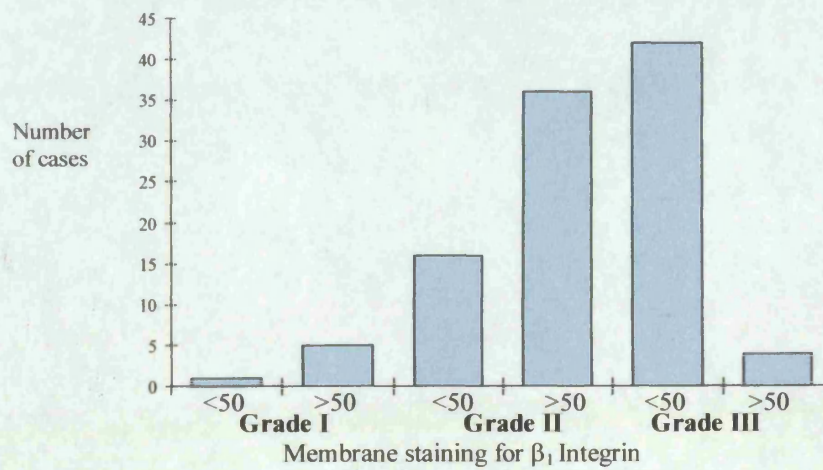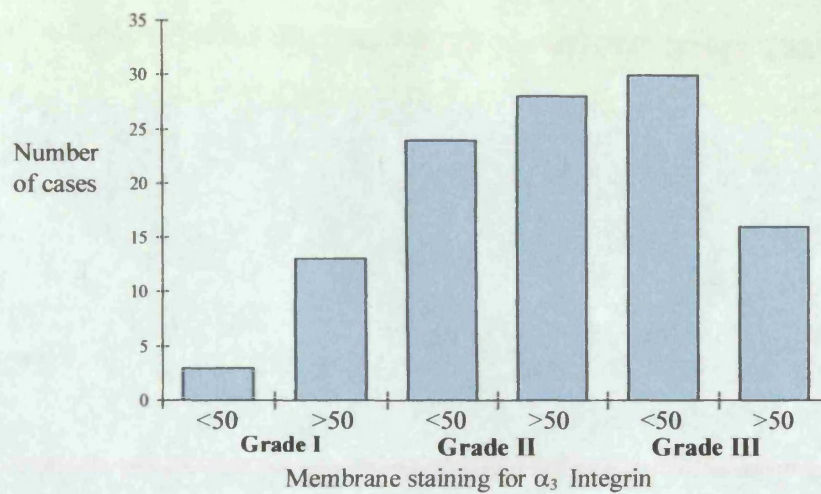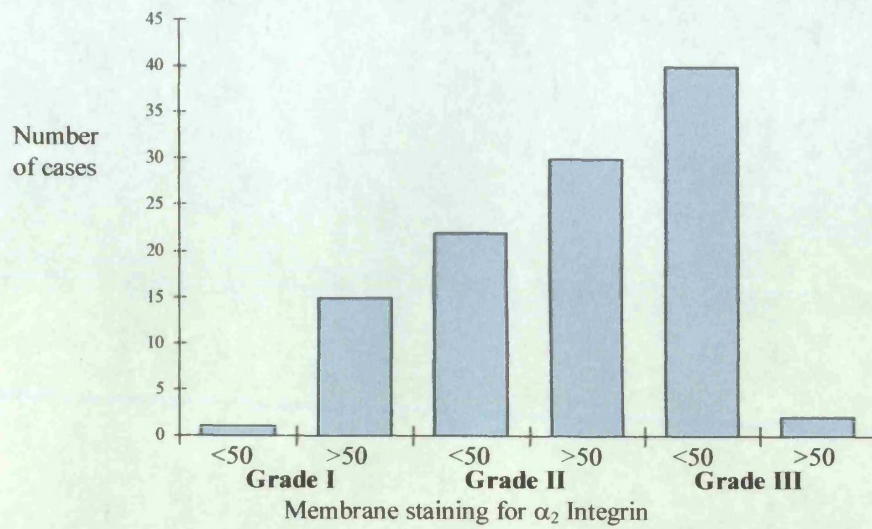
Fig. 2.11: Relationship Between Membrane Reactivity for α2, α3, and β1 Integrins and Tumour Grade. A highly significant association is demonstrated between membrane α2 (p=0.0001) and β1 (p=0.0001) and low tumour grade. A similar but weaker relationship is identified for α3 integrin (p=0.05)
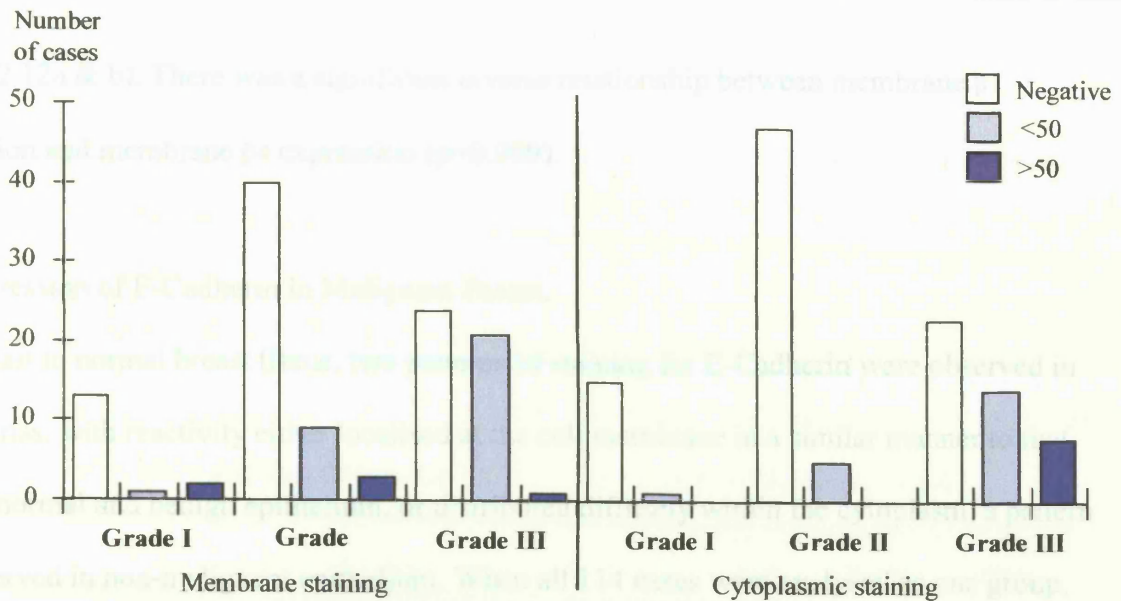
Fig.12a: The relationship between membrane and cytoplasmic α_6 integrin and tumour grade. A significant association is demonstrated between membrane α_6 (p= 0.01) and cytoplasmic α_6 (p= 0.001) and high tumour grade.
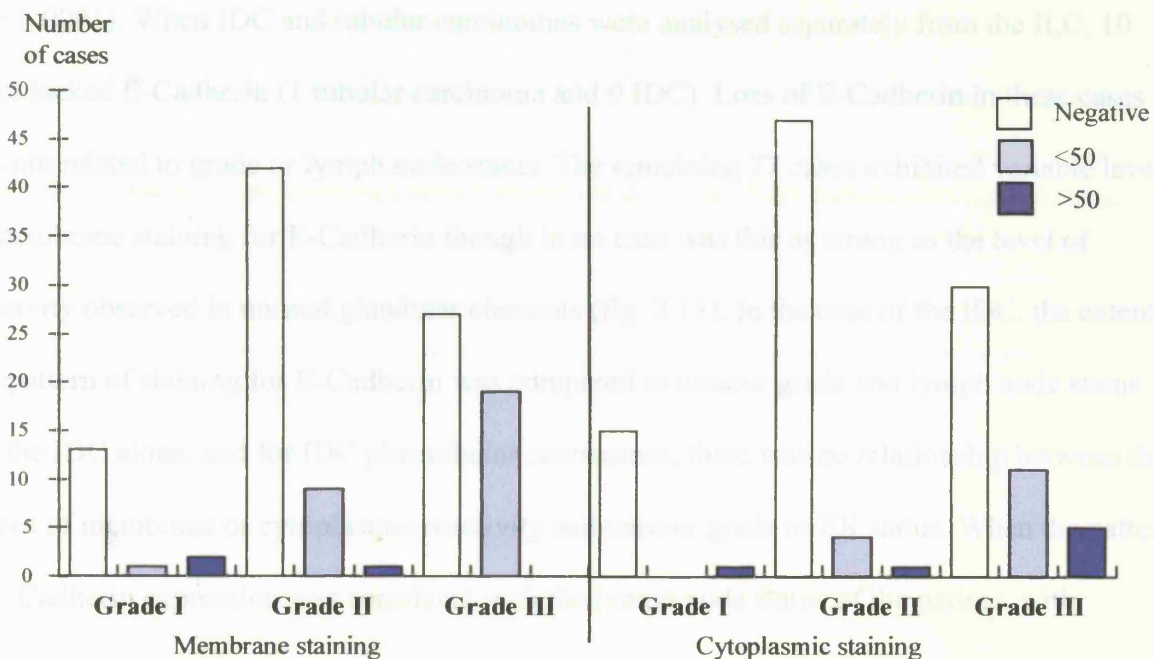
Fig. 12b: The relationship between membrane and cytoplasmic β_4 integrin and tumour grade. A significant association is demonstrated between presence of membrane β_4 (p=0.02) and cytoplasmic β_4 (p=0.01) and high tumour grade.

β4; fig. 2.12a & b). There was a significant inverse relationship between membrane β1

expression and membrane β4 expression (p=0.009).

- Expression of E-Cadherin in Malignant Breast.

In contrast to normal breast tissue, two patterns of staining for E-Cadherin were observed in

carcinomas, with reactivity either localised at the cell membrane in a similar manner to that

seen in normal and benign epithelium, or distributed diffusely within the cytoplasm, a pattern

not observed in non-malignant epithelium. When all 114 cases were analysed as one group,

31% (no.=35) showed no evidence of staining for E-Cadherin, either membrane or cytoplasmic.

Loss of reactivity for E-Cadherin was strongly related to tumour type, with 26 of 27 ILC

lacking E-Cadherin expression entirely, and 1 case displaying diffuse cytoplasmic staining

(p=<0.0001). When IDC and tubular carcinomas were analysed separately from the ILC, 10

cases lacked E-Cadherin (1 tubular carcinoma and 9 IDC). Loss of E-Cadherin in these cases

was not related to grade or lymph node status. The remaining 77 cases exhibited variable levels

of membrane staining for E-Cadherin though in no case was this as strong as the level of

reactivity observed in normal glandular elements (fig. 2.13). In the case of the IDC, the extent

and pattern of staining for E-Cadherin was compared to tumour grade and lymph node status.

For the IDC alone, and for IDC plus tubular carcinomas, there was no relationship between the

degree of membrane or cytoplasmic reactivity and tumour grade or ER status. When the pattern

of E-Cadherin expression was correlated with the lymph node status of the patient, with

tumours categorised into one of two groups, either low level reactivity (<50% staining) or high

level reactivity (>50% staining), there was a significant association between low level

membrane reactivity and the presence of lymph node metastasis (0.005>p>0.001) and a highly

significant association was demonstrated between the presence of cytoplasmic reactivity for E-

Cadherin in the tumour cells and lymph node metastasis (p<0.001) (Table 2.9). This

relationship was maintained when the analysis was carried out on a larger group of cases (Jones

et al, 1996).

Table 2.10: Summary of Reactivity for E-Cadherin in Relation to Tumour Type, Grade* and Lymph Node Status*

	MEMBRANE %(no)				CYTOPLASMIC %(no)			
	Neg.	<20%	20-50%	>50%	Neg.	<20%	20-50%	>50%
Tubular	1 (1)	0	2 (2)	4 (5)	1 (1)	0 (0)	3 (3)	3 (4)
IDC	8 (9)	7 (8)	18 (21)	36 (41)	7 (8)	7 (8)	18 (21)	37 (42)
ILC	24 (27)	0	0	0	23 (26)	0	1 (1)	0
Grade I**	1 (1)	3 (3)	3 (3)	11 (9)	1 (1)	3 (3)	6 (5)	8 (7)
Grade II	5 (4)	3 (3)	9 (8)	13 (11)	5 (4)	3 (3)	10 (8)	13 (11)
Grade III	6 (5)	2 (2)	14 (12)	30 (26)	4 (4)	2 (2)	13 (11)	32 (28)
LN +ve	9 (8)	6 (5)	16 (14)	16 (14)	2 (1)	2 (1)	23 (9)	73 (30)
LN -ve	1 (1)	1 (1)	9 (8)	33 (29)	18 (7)	18 (7)	36 (14)	28 (11)

*Not including ILC; ** Includes grade I IDC and Tubular carcinomas

The only relationship identified between staining for E-Cadherin and integrin receptors

was an inverse correlation between membrane E-Cadherin and the presence of cytoplasmic β4

integrin staining, though not membrane reactivity (r=0.195; p=0.02).
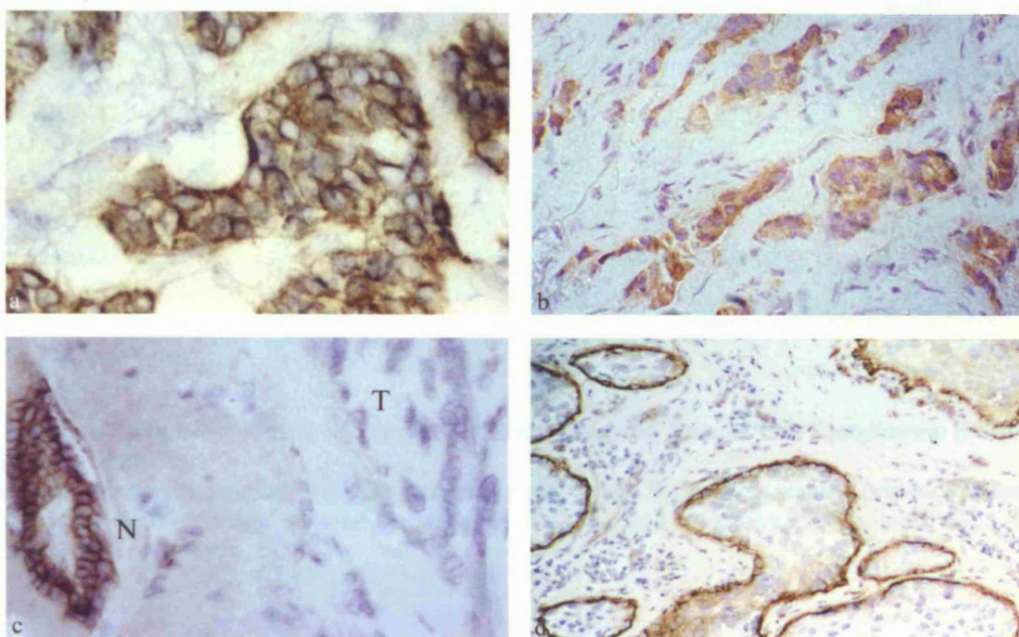
Fig 2.13 : Expression of E-Cadherin in Breast Carcinoma

Many tumours retain membrane expression of E-Cadherin (top left), whereas some carcinomas exhibit predominantly cytoplasmic staining (top right). ILC lack E-Cadherin (lower left, T) whilst strong membrane staining is observed in residual normal ducts and acini (N). Loss of reactivity for E-Cadherin is also evident in foci of lobular carcinoma in-situ (lower right).
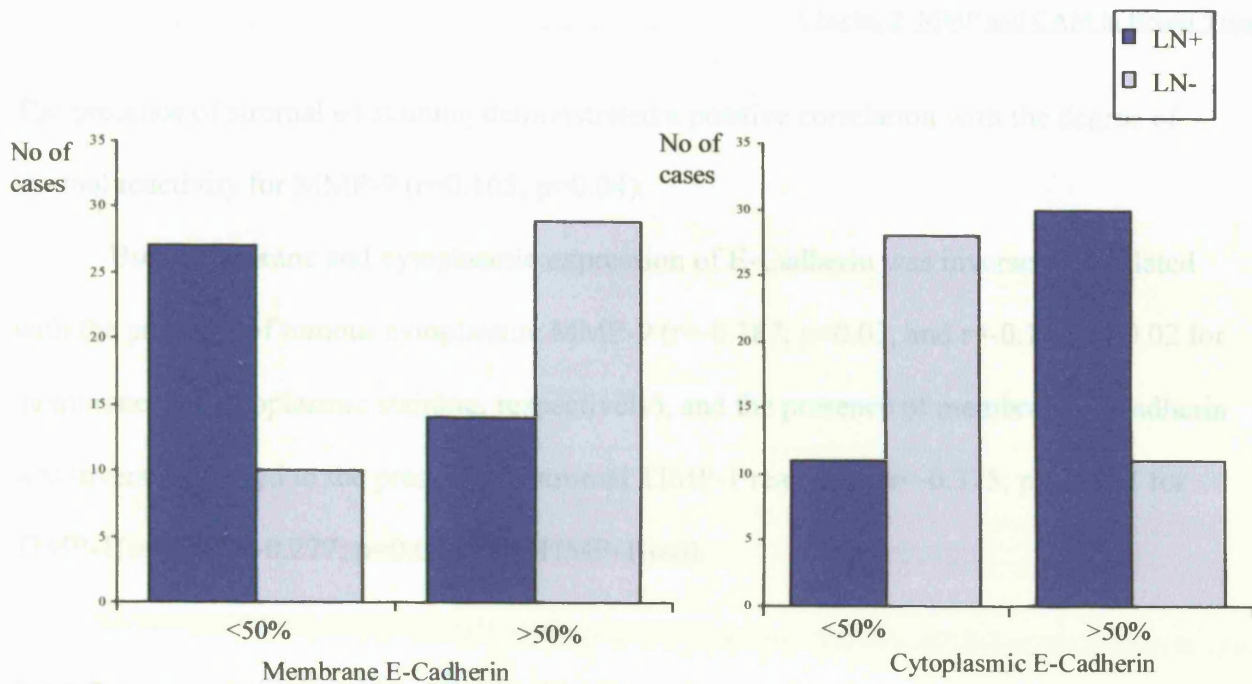
Fig 2.14: Relationship Between Extent of Tumour Cell Membrane and Tumour Cell Cytoplasmic E-Cadherin and Lymph Node Status.
 A significant association is demonstrated between loss of membrane E-Cadherin and the presence of lymph node metastases (p = <0.005), and between the presence of tumour cell cytoplasmic E-Cadherin and a positive lymph node status (p = <0.001).

2.4.2.5 Relationship between Cell Adhesion Molecule Expression, and Expression of MMPs and TIMPs

Kendalls tau correlation was calculated to assess any associations between the expression

pattern of cell adhesion molecules and of MMPs and TIMPs. The presence of membrane

reactivity for α6 integrin showed a positive correlation with the presence of membrane MMP-

2(os) (r=0.211; p=0.01), but not with MMP-2(ss) or with membrane MT1-MMP. Cytoplasmic,

but not membrane, β4 integrin reactivity also exhibited a positive correlation with membrane

MMP-2(os), and with membrane reactivity for TIMP-2, both (os) and (ss) (r=0.273; p=0.002).

The presence of stromal α5 staining demonstrated a positive correlation with the degree of stromal reactivity for MMP-9 (r=0.165; p=0.04).

Both membrane and cytoplasmic expression of E-Cadherin was inversely correlated with the presence of tumour cytoplasmic MMP-9 (r=-0.187; p=0.03, and r=-0.180; p=0.02 for membrane and cytoplasmic staining, respectively), and the presence of membrane E-Cadherin was inversely related to the presence of stromal TIMP-1 reactivity (r=-0.375; p=0.0001 for TIMP-1(ss) and r=-0.277; p=0.0001 for TIMP-1(os)).

2.4.3 Zymographic Analysis of Breast Tissue

2.4.3.1 Determination of Linear Range of Assay

Dilution series of recombinant pro-MMP-2 and pro-MMP-9 were applied to gelatin substrate gels and subjected to zymography followed by densitometric analysis, in order to establish the linear range of the assay. The ID (i.e. density multiplied by cross-sectional area) of the inverted image was measured for concentrations ranging from 0.1 ng to 200 ng of gelatinase fig. 2.15).

Since pro-MMP-2 autoactivates at higher concentrations, the measurement for pro-MMP-2 was taken as the sum of active and latent bands. For both enzymes, zymography was shown to be highly sensitive, detecting enzyme concentrations down to 0.1 ng. Higher concentrations of enzyme resulted in larger and more intense bands of gel lysis. A linear correlation between integrated density and MMP concentration was demonstrated between concentrations of 0.1 - 50 ng of MMP-2 (r=0.918) and 0.1 - 75 ng of MMP-9 (r= 0.897; fig. 2.15).
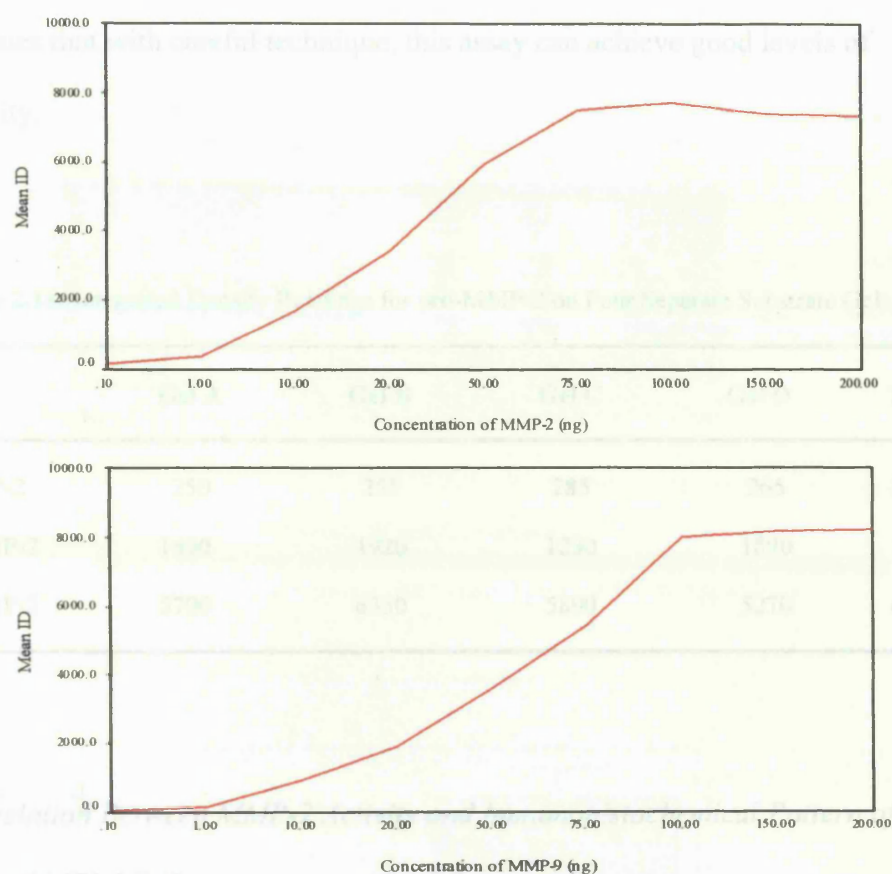
Fig. 2.15: Relationship between Integrated Density (ID) Readings for (i) MMP-2 and (ii) MMP-9 and Concentration of Purified Enzyme.

2.4.3.2 Assessment of Reproducibility

To assess the reproducibility of zymography, samples of pro-MMP-2 of 1 ng, 10 ng and 50 ng

concentration were subjected to analysis on four separate substrate gels, on the same day (to

eliminate any effect of storage of sample on the results). The ID for each sample together with

the standard deviation is shown in Table 2.10. For each sample the SD is < 10% of the mean,

which indicates that with careful technique, this assay can achieve good levels of

reproducibility.

Table 2.11: Integrated Density Readings for pro-MMP-2 on Four Separate Substrate Gels

	Gel A	Gel B	Gel C	Gel D	MEAN	S.D.
1 ng pro-MMP-2	250	255	285	265	265	15.5
10 ng pro-MMP-2	1600	1920	1290	1890	1675	294.4
50 ng pro-MMP-2	5700	6350	5890	5270	6052	309.0

2.4.3.3 Correlation Between MMP-2 Activity and Immunohistochemical Pattern of Expression of MMP-2 and MT1-MMP

A series of 22 breast carcinomas which had exhibited either low or high levels of membrane

MMP-2 and MT1-MMP on immunohistochemistry were selected for zymographic analysis.

Substrate gels were loaded with equal protein concentrations of each sample, together with 1ng

of pro-MMP-2 as an internal standard for each gel. The ID was measured for lytic bands at 72

kDa (pro-MMP-2) and 62 kDa (active MMP-2) and an activation ratio was calculated (ID

active MMP-2 : ID pro-MMP-2 plus active MMP-2). Where a band at ~ 68 kDa was detected,

representing intermediate MMP-2, this was measured with pro-MMP-2. The relationship of

MMP-2 activation ratio to level of membrane reactivity for the MMP-2/MT1-MMP system was

then analysed using the Wilcoxen Mann-Whitney U test. All densitometric readings fell within

the linear range of analysis and thus were taken to be proportional to gelatinase concentration

(Table 2.11). A representative zymogram is shown in figure 2.16 and raw data of all cases

analysed is shown in Appendix 1. A significant positive correlation was identified between a

high MMP-2 activation ratio and high levels of membrane MMP-2 and MT1-MMP (Chi

Square=15.8; df=1; p=<0.001; fig. 2.17).
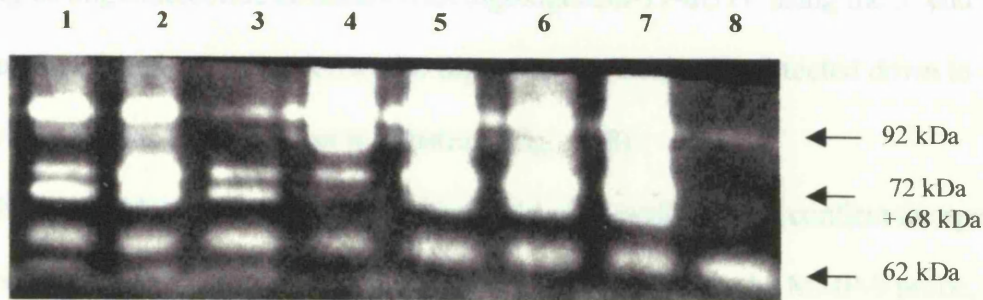
Fig. 2.16: Substrate Gel Zymogram of Primary Breast Carcinomas.
In cases where individual bands for both latent MMP-2 and intermediate MMP-2 can be detected, these were
measured as a single band. Lane numbers correspond to case figures listed in Appendix 1.
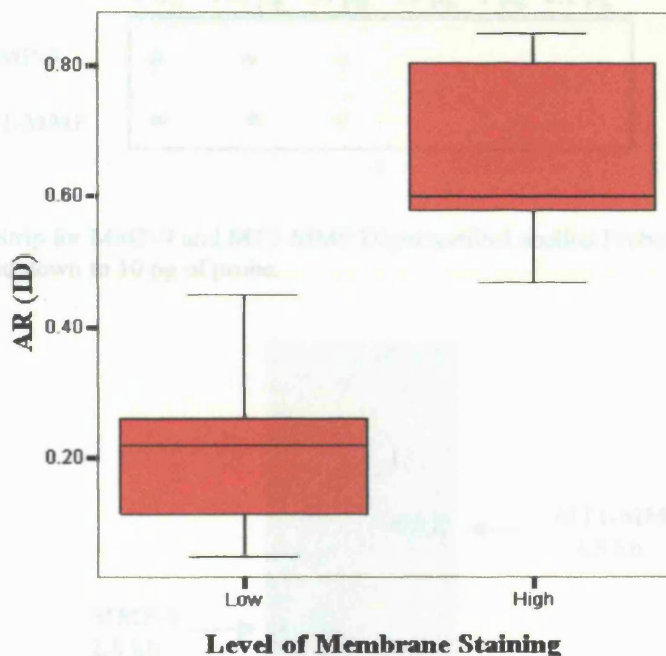
Fig. 2.17: Relationship Between MMP-2 Activity and Extent of Membrane Reactivity for MMP-2 and MT1-MMP.
Cases were classified into one of two groups – those showing low level of membrane staining for both MMP-2 and
MT1-MMP and those with high level membrane staining for both enzyme components. A significant positive
correlation is identified between the level of reactivity and the activation ratio of MMP-2, as assessed on
zymography (p<0.001). The boxes indicate cases falling between the upper and lower quartiles and the bar
represents the median. The whiskers connect the largest and smallest values.

2.4.4. In-situ Hybridisation for MMP-9 and MT1-MMP

2.4.4.1 Labelling of Probes and Northern Blot Analysis

Labelling of oligonucleotide cocktails with digoxigenein-11-dUTP using the 3' end labelling technique was found to be very efficient. Signal on test strips was detected down to 10 pg of probe within 5 mins of incubation in substrate (fig. 2.18).

Northern blot analysis on HT 1080 mRNA was performed to confirm the specificity of the probes. A single band estimated at 2.8 kb was detected using the MMP-9 probe, however, the band was quite weak even after 10 mins exposure to film (fig. 2.19). A single band at 4.5 kb was shown using the MT1-MMP probe (fig. 2.19).
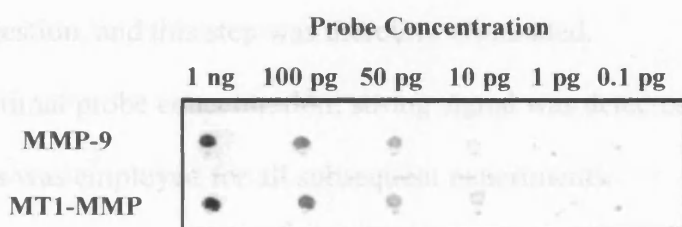
Fig. 2.18: Test Strip for MMP-9 and MT1-MMP Digoxigenin-Labelled Probes. Signal is detected down to 10 pg of probe.
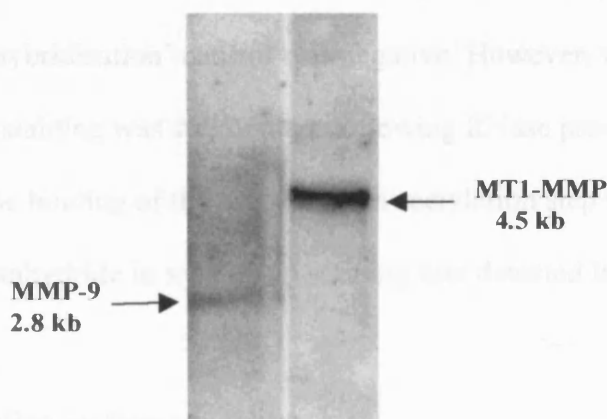
Fig. 2.19: Northern Blot on HT 1080 cells for using Probes to MMP-9 and MT1-MMP. 3 μg of mRNA was loaded in each lane and the blot was subjected to chemiluminescent detection. Blots illustrated were exposed to 10 mins (for MMP-9) and 3 mins (for MT1-MMP).

2.4.4.2 In-Situ Hybridisation

- In-Situ Hybridisation on HT 1080 Cells

Since HT 1080 cells are known to synthesise both MMP-9 and MT1-MMP, the methodology was worked up on these cells grown on coverslips prior to working on tissues. Intense cytoplasmic staining was detected for both MMP-9 and MT1-MMP, as well as the mitochondrial probes. A number of parameters were tested to optimise detection:

(i)   Effect of Proteinase K digestion: strong signal was evident for MMP-9, MT1-MMP and the mitochondrial probe in HT 1080 cells both with and without PK treatment, however, the level of background and the preservation of the cells was suboptimal following PK digestion, and this step was therefore eliminated.

(ii)   Optimal probe concentration: strong signal was detected using 200 ng/ml of probe, so this was employed for all subsequent experiments.

(iii)   Stringency: strong signal, which was eliminated by RNase pre-treatment, was detected using washes containing 30% formamide. The signal intensity was weaker using 50% formamide washes therefore, 30% was employed for subsequent experiments.

In all cases, the 'no-hybridisation' control was negative. However, with all probes, weak but definite cytoplasmic staining was still evident following RNase pre-treatment. This suggested a degree of non-specific binding of the probes, so an acetylation step was introduced. Following immersion in acetic anhydride in xylene, no staining was detected in the RNase-treated cells (fig. 2.20).

- In-Situ Hybridisation on Primary Tissue

Having established that the probes to MMP-9 and MT1-MMP yield specific hybridisation in HT 1080 cells, they were then applied to a series of 10 ILC and 12IDC, on which

immunohistochemistry had previously performed. Sections were also hybridised with the

mitochondrial probe to control for RNA integrity. Several parameters were evaluated:

(i)   Proteinase K concentration: no signal with any of the probes was detected in the

absence of PK pre-treatment. The optimal concentration of PK digestion varied between

cases, and as a standard procedure each case was subjected to digestion with 5 µg/ml

and 10 µg/ml PK.

(ii)   Probe concentration: no signal was detected using the lowest probe concentration. The

intensity of signal increased with higher concentrations but there was also an increase in

the level of background staining. A concentration of 400 ng/ml was considered

optimum for both probes.

(iii)   Stringency: as with HT 1080 cells, little or no signal was evident when the formamide

concentration was increased to 50%, therefore washes using 30% formamide were

employed.

'No hybridisation' controls and sections pre-treated with RNase A showed no signal for

any of the probes.

The pattern and extent of signal detected for each probe varied between cases. All of the

ILC displayed some positivity with the MMP-9 probe, and in all cases where tumour

cytoplasmic reactivity was seen for the protein, there was some evidence of mRNA in the

tumour cell population (Table 2.11; fig. 2.10), though this was consistently less extensive than

the level of protein. Stromal cell positivity was also observed in the majority of cases that

demonstrated stromal reactivity on immunohistochemistry (IHC). Tumour cell positivity for

MMP-9 mRNA was less frequently seen in IDC, and when present was focal in nature, in

keeping with the IHC findings.

Table 2.12: Comparison of Immunohistochemistry and In-Situ Hybridisation for MMP-9 and MT1-MMP

No	RW No	TYPE	GRDE	MMP-9 IHC	MMP-9 ISH	MT1-MMP IHC	MT1-MMP ISH
1	628	IDC	II	Neg	Neg	TM 4 ; S++	T 1; S +++
2	653	IDC	III	Tcyt 1; S -ve	Neg	TM 2; S ++	Occ T +; S +++
3	738	TUB	I	Neg	Neg	TM 1; S +	T -ve; S +
4	812	IDC	III	Neg	Neg	TM 1; S +++	T -ve; S ++
5	824	IDC	II	Neg	Neg	TM 2; S +++	Occ T +; S ++
6	831	IDC	III	Tcyt 2; S +/-	Neg T; S +/-	TM 1; S +++	T -ve; S +++
7	840	IDC	III	Tcyt 2; S +++	Neg T; S ++	TM 2; S ++	T 1; S ++
8	845	IDC	I	Tcyt 1; S +	Neg T; S +	TM 2; S +	Occ T +; S +
9	867	IDC	III	Tcyt 2; S -	Occ T +	T -ve; S +++	T -ve; S +++
10	903	IDC	II	Tcyt 2; S +/-	Occ T +; S -	TM -ve; S +	T -ve; S +
11	941	IDC	III	Neg	Neg	TM -ve; S ++	T -ve; S ++
12	961	IDC	III	Neg	Neg	TM -ve; S +++	T -ve; S +++
13	861	ILC	II	Tcyt 2; S +	T 1; S +	TM -ve; S +	T -ve; S +
14	892	ILC	II	Tcyt 3; S +	T 3; S +/-	TM 3; S +++	T 2; S +++
15	894	ILC	II	Tcyt 2; S +	T 1; S +/-	TM 3; S +++	T 1; S ++
16	1022	ILC	II	Tcyt 4; S +/-	T 2; S -	TM -ve; S +	T -ve; S +/-
17	1046	ILC	II	Tcyt 2; S -	Occ T +; S -	TM -ve; S +/-	T -ve; S -
18	1181	ILC	III	Tcyt 3; S -	T 1; S -	TM 1; S +	T -ve; S +/-
19	1302	ILC	II	Tcyt 4; S +	T 2; S +/-	TM -ve; S +	T -ve; S +/-
20	1589	ILC	II	Tcyt 3; S +	T 1; S +	TM 1; S ++	T -ve; S +
21	1614	ILC	II	Tcyt 3; S +/-	Occ T +; S -	TM 1; S +++	T -ve; S ++
22	1637	ILC	II	T cyt Neg; S ++	T -ve; S ++	TM 1; S ++	T -ve; S ++
23	1654	ILC	II	Tcyt 4; S ++	T 2; S +	TM-ve; S +++	T -ve; S ++

Tcyt = Tumour cell cytoplasmic staining; TM= Tumour membrane staining; S = Stroma
1: 1-25% staining; 2: 25-50% staining; 3: 50-75% staining; 4: >75% staining
+/- : occasional positive cells; + : few positive cells; ++ : moderate staining; +++ : abundant staining
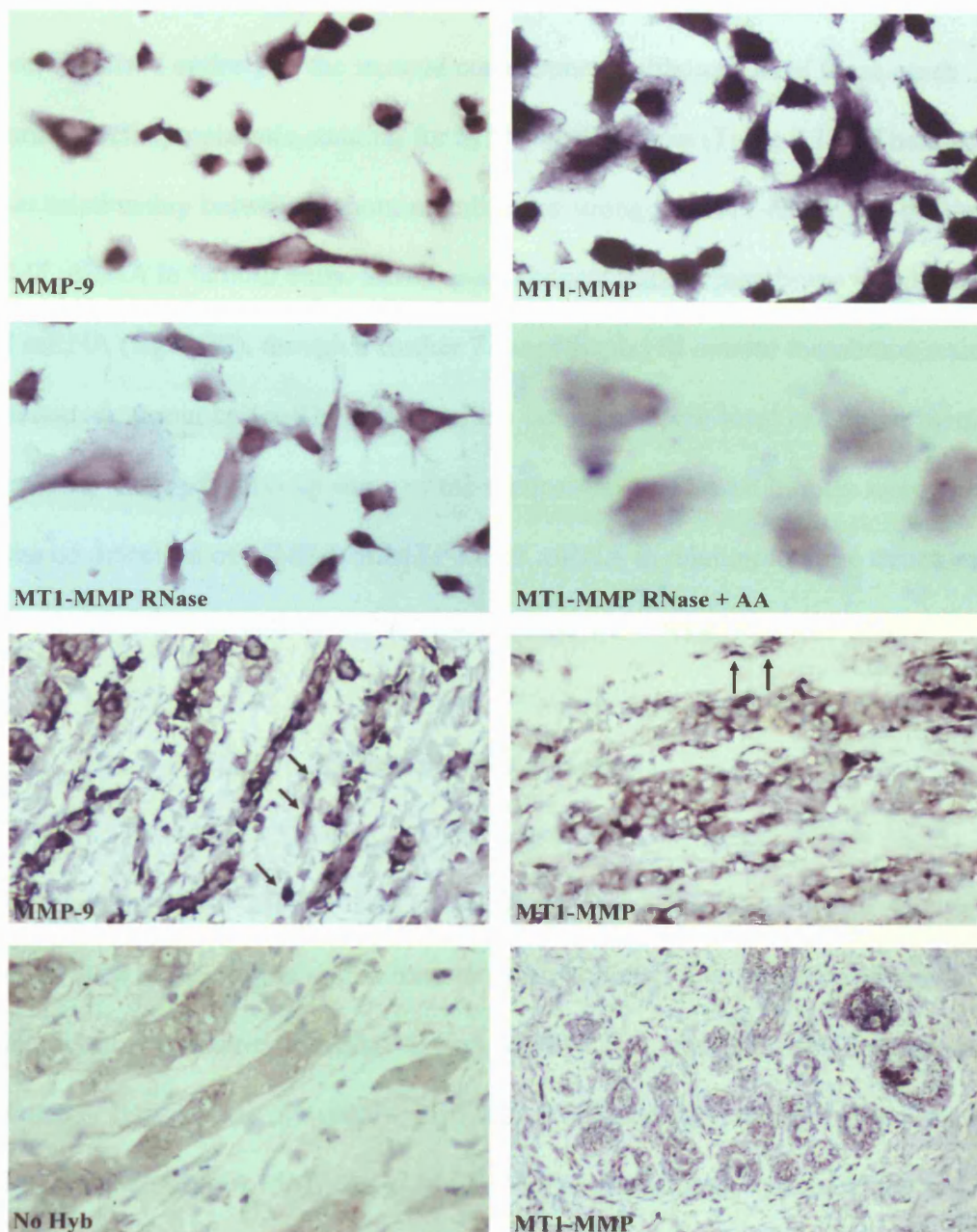
Fig 2.20 : In-Situ Hybridisation for MMP-9 and MT1-MMP on HT 1080 Cells and Primary Breast Tissue.

The top four images show HT 1080 cells hybridised with probes to MMP-9 and MT1-MMP. Intense signal is generated, particularly with MT1-MMP. Signal is not completely eradicated with RNase pre-treatment, but no signal is seen with introduction of an acetylation step (AA). The lower four images are of primary breast tissue. Signal for MMP-9 is seen in tumour cells of ILC, and focal positivity for MT1-MMP is present in tumour cells of the IDC. Stromal positivity is seen with both probes (arrows). No signal is seen in the 'no hybridisation' control, and normal breast ducts and acini do not exhibit staining.

Signal for MT1-MMP mRNA was present in all but one case. In 16 of the 23 cases,

positivity was localised entirely to the stromal compartment, although all of these cases

exhibited tumour cell cytoplasmic staining for MT1-MMP protein (Table 2.11). There appears

to be a closer relationship between tumour membrane staining for MT1-MMP and the detection

of MT1-MMP mRNA in tumour cells. Seven cases showed tumour membrane staining and

tumour cell mRNA (fig. 2.20), though a further 7 cases displayed tumour membrane staining

and no evidence of tumour cell mRNA. In all of the latter cases, the level of tumour membrane

staining was low (<25% of cells). In some of the sections normal breast lobules were present

and there was no detection of MMP-9 or MT1-MMP mRNA in relation to these structures (Fig.

2.20). In all cases, 'no hybridisation' and RNase controls were negative.

2.5 Summary of Results

- Components of the MMP-2/TIMP-2/MT1-MMP system are widely expressed in breast

  carcinomas, both in the stroma and in tumour cell cytoplasm. The presence of tumour cell

  membrane immunoreactivity for MMP-2 (os), MMP-2 (ss), and MT1-MMP correlated with

  the presence of lymph node metastases (p=0.001; 0.008 and 0.01, respectively). The level

  of membrane reactivity for MMP-2 and MT1-MMP was positively related to the activation

  ratio of MMP-2 on zymography. In situ hybridisation demonstrated the predominant site of

  MT1-MMP synthesis within the stromal compartment. There was no correlation between

  the presence of tumour cytoplasmic reactivity for MT1-MMP and localisation of mRNA to

  the tumour population, however, all cases exhibiting >50% tumour membrane staining also

  showed positivity for MT1-MMP mRNA, though this was consistently less extensive than

  the protein.

- MMP-9 was detected in 68% of carcinomas and the pattern of reactivity varied in relation to tumour type. Both IDC and ILC showed staining of stromal cells and macrophages, but staining in the cytoplasm of tumour cells was most frequent in ILC ($p=0.0004$). In-situ hybridisation confirmed mRNA expression in some tumour cells of ILC, though the extent of message detected was less then observed for the MMP-9 protein.

- Reduced expression of $\alpha2$, $\alpha3$ and $\beta1$ integrins was seen in carcinomas, the extent of which was associated with tumour grade ($p=0.0001$ for $\alpha2$ and $\beta1$, 0.05 for $\alpha3$). Whereas all tumours retained some level of expression of these integrins, $\alpha6$ and $\beta4$ integrin subunits were expressed by only ~30% of tumours and those that did stain for these integrins were poorly differentiated ($p=0.01$ and 0.02, respectively). Staining for $\alpha5$ integrin was observed in peri-tumoural fibroblasts of carcinomas, with more extensive reactivity present in higher grade tumours ($p=0.01$). The expression pattern of integrin receptors did not relate to lymph node status.

- E-Cadherin expression was maintained in 93% of IDC but was lost in ILC. In some IDC, reactivity was localised diffusely within the cytoplasm in contrast to the normal pattern of membrane staining. Reduced membrane and increased cytoplasmic staining both independently correlated with a positive lymph node status ($p<0.05$ and <0.001, respectively).

- Expression of E-Cadherin was inversely correlated with MMP-9 staining of tumour cytoplasm. Stromal $\alpha5$ integrin was positively correlated with stromal MMP-9, and membrane $\alpha6$ and cytoplasmic $\beta4$ correlated with membrane staining for MMP-2 (os).

2.6 Discussion

2.6.1 Discussion of Techniques

2.6.1.1 Antigen Retrieval

None of the integrin or MMP/TIMP antibodies used in this study showed convincing reactivity on formalin fixed tissue. Both MMP-2 and MT1-MMP exhibited a generalised, weak staining pattern on sections following microwave irradiation, however, the universal nature of the staining questioned the specificity of the reaction. Whilst heat based antigen retrieval (HBAR) techniques are now used routinely in many pathology laboratories, it is evident that both differences in the nature of the target epitope, e.g. nuclear versus cytoplasmic (Pileri et al, 1997), and differences in antibodies can influence the success of such techniques. It has been reported that different clones of antibodies may show variability in staining following AR methods (Norton et al, 1993), and this may explain why one study has successfully applied β4-integrin antibodies to formalin fixed tissue (Allen et al, 1998). The only other study, to my knowledge, that has detected reactivity for integrins on fixed tissue was reported by Hanby et al (1994), who found that a monoclonal β4-integrin antibody gave good staining on Carnoy's fixed tissue but not on formalin fixed tissue.

In contrast to the lack of success with integrin antibodies, a number of monoclonal antibodies to MMPs and their inhibitors have now become available commercially which show reactivity on formalin fixed tissue following HBAR techniques (Murray et al, 1998). This will undoubtedly promote further studies on expression and distribution of these enzymes in archival material. The better morphology and wider range of tissue specimens afforded by routine fixation and processing will greatly facilitate interpretation of immunohistochemistry, but it will be important to compare the staining patterns achieved on fixed and frozen tissue.

Unfortunately, these antibodies became available towards the end of this project so it has not yet been possible to carry out such a comparative study.

2.6.1.2 Substrate Gel Zymography

Substrate gel zymography is an electrophoretic technique in which enzymes are separated in a polyacrylamide gel containing the substrate of choice (Heussen and Dowdle, 1980). The enzymes are separated under denaturing but non-reducing conditions, then refolded in washes that remove the SDS. The gel is then incubated in a suitable buffer system at 37°C to allow the enzymes to act and degrade the protein substrate. Coomassie blue staining of the gel will reveal clear, lytic bands at the site of protease activity. The major advantage of substrate gel zymography over other techniques for the detection of proteolytic enzymes is the ability of this method to distinguish between active and latent enzymes (Springman *et al*, 1990). Furthermore, there is no difference in the efficiency of substrate digestion by the active and latent enzymes, so quantitation of both forms is possible (Kleiner *et al*, 1994) and providing the samples fall within the linear range of the assay, this allows the calculation of an activation ratio for a particular enzyme, which has been shown to correlate more closely with prognosis (Davies et al, 1993b; Onisto et al, 1995). This study has demonstrated the technique to be highly sensitive, giving readings on densitometric analysis down to 0.1 ng concentration of protein. It also offers good reproducibility - in this study the SD between gels was less than 10% of the mean ID, which means that different gels can be compared with reasonable confidence, particularly if known standards are included in each run.

2.6.1.3 In-situ Hybridisation

The synthesis of specific RNA species and their spatial distribution within a tissue can provide important information on cell function. In-situ hybridisation has become a powerful and versatile tool for the detection and localisation of nucleic acid sequences in cell and tissue preparations (Coghlan et al, 1985; Cox et al, 1984). Early studies used predominantly radiolabelled probes, but the development of a range of non-radioactive labels has simplified the technique and affords accurate localisation of message within tissue sections (Burns et al, 1986; Pringle et al, 1987; Pringle et al, 1989).

Either DNA or RNA probes can be used for the detection of RNA sequences, and both single stranded (ss) and double stranded (ds) probes are available with each having different advantages and disadvantages. Most of the published studies on localisation of MMP mRNA have used single stranded RNA probes called riboprobes (Basset et al, 1990; Gilles et al, 1996; Heppner et al, 1996). These can be produced directly from the cDNA of the gene of interest by a transcription reaction in-vitro; they are highly specific since they form a perfect RNA/RNA hybrid, however, they are more difficult to use because of their susceptibility to degradation by nucleases. Oligonucleotide probes were employed in this study. Oligonucleotides are short nucleotide sequences, usually between 15-50 bp long, produced using a DNA synthesiser by phosphoramidite chemistry (Beaucage and Caruthers, 1981). There are a number of advantages to using oligonucleotides for RNA in-situ. A cDNA is not needed, only the published sequence is required; since the probes are designed from the gene sequence, areas of secondary structure in the target and sequences common to other unrelated genes can be avoided. They are easy to label and use, and because they are small single stranded molecules, they penetrate cells on a tissue section more readily (Cook et al, 1988; Lewis et al, 1985). With use of multiple oligonucleotide probes to the same target in a 'cocktail' enhanced sensitivity of detection is also allowed.

The probe cocktails to MMP-9 and MT1-MMP labelled appropriate-sized bands on Northern

blot analysis of HT 1080 cells. Strong signal was generated on in-situ hybridisation with these cells,

which was largely abolished, though not completely, by pre-treatment of the cells with RNase A.

This persistence of staining suggested non-specific probe binding, which was improved by

quenching of charged groups in the cells using acetic anhydride (Hayashi et al, 1978; Leisi et al,

1986), and the absence of staining in these cells, and in cells incubated without probe, confirmed the

specificity of the signal. The protocol was successfully transferred to tissue sections, where the

controlled proteolytic cleavage of some of the cross-links induced by fixation was achieved using

Proteinase K. The problems with non-specific binding of probes was not evident in the tissue

sections and the complete abolition of signal on RNase A-treated sections and in sections incubated

without probe confirmed the specificity of hybridisation.

2.6.2 Discussion of Results

2.6.2.1 Expression of MMPs and TIMPs in Normal Breast Tissue

An early clue that MMPs were a functional component of the breast came from the observation that Type IV collagenase was markedly up-regulated in the involuting mammary gland and was likely to play a role in removal of basement membrane (Martinez-Hernandez et al, 1976). Since then, a wide range of MMPs have been demonstrated in the mammary gland, and have been shown to be important in development of the breast (Witty et al, 1995), and to be up-regulated at times of remodelling, most noteably in postlactational involution (Talhouk et al, 1992). In this study, MMP-2, MT1-MMP, TIMP-1 and TIMP-2 were all detected in the basement membrane zone of normal breast ducts and acini, with the presence of MMP-9 being an inconstant feature. This suggests a role for these proteolytic enzyme systems in the normal physiological turnover of the breast, as proposed by previous workers (Wicha et al, 1980; Monteagudo et al, 1990). It is interesting to speculate that the variability of reactivity for MMP-9 may relate to cyclical variation in the cases; unfortunately LMP data was not available on these cases. Whilst MMP and TIMP protein is readily detectable in normal breast tissue, previous in situ hybridisation studies have not detected signals for MMP in normal breast (Heppner et al, 1996), suggesting that the rate of synthesis is very low.

2.6.2.2 Expression of MMP-2, MT1-MMP and TIMP-2 in Breast Carcinomas

This study has demonstrated the expression of MMP-2, MT1-MMP and TIMP-2 in the majority of invasive breast carcinomas, regardless of type or grade. A significant correlation was identified between tumour cell membrane reactivity both for MMP-2 and MT1-MMP with the presence of lymph node metastases. The association with positive lymph node status was particularly strong for tumours exhibiting membrane reactivity for both enzyme components. A

number of studies, both in animal models and on human tissue (Garbisa et al, 1987; Alvarez et al, 1990; Davies et al, 1993) have revealed an association between type IV collagenase expression and invasive or metastatic behaviour. However, in many cases it is an increase in the level of *active* MMP-2 that correlates with disease progression to a greater degree than total MMP-2 expression (Azzam et al, 1993; Brown et al, 1993; Onisto et al, 1995). In contrast to these functional studies, immunohistochemistry has resulted in inconsistent results in terms of association with disease progression. This may in large part be due to the inability of antibodies generated to date to distinguish between active and latent forms of the enzymes. In keeping with the findings of the present study, Hoyhtya et al (1994) and Iwata et al (1996) found the majority of invasive breast carcinomas to express MMP-2, but whilst tumour membrane staining is commented on in both studies, this was not assessed independently. However, with the current understanding of the role of the MT-MMPs in localising and activating MMP-2 at the cell membrane (Sato et al, 1994; Strongin et al, 1995; Kolkenbrock et al, 1997), membrane-associated MMP-2 may well be a stronger indicator of enzyme activity. The demonstration in this study of a stronger correlation between membrane reactivity for MMP-2 and MT1-MMP with tumour spread as opposed to total reactivity for these enzymes would be consistent with this hypothesis. In keeping with this, Ueno et al (1997) have recently shown that the level of expression of MT1-MMP correlated with increased levels of active MMP-2, and they also demonstrated a significant correlation between expression levels of MT1-MMP in breast carcinomas and the presence of lymph node and distant metastasis. Whilst membrane reactivity was observed for TIMP-2, this did not correlate with metastatic status.

In addition to membrane reactivity, the majority of tumours displayed diffuse cytoplasmic staining of the tumour cells and stromal reactivity for both enzyme and activator. It is possible that this indicates tumour cell synthesis of these enzymes, or alternatively, that this

represents internalisation of MMPs following their activation at the tumour cell membrane, with resulting accumulation of immunoreactive product within the tumour cell cytoplasm. A number of reports have indicated that the major source of MMP in breast carcinomas is the stromal cell (Poulsom et al, 1993; Heppner et al, 1996), which favours the latter explanantion. A further possibility is that the tumour cells may be exhibiting a difference between the rate of mRNA translation and capacity for intracellular storage of the protein. This may be particularly relevant for MT1-MMP, where intracellular storage may occur prior to the intracellular activation of the proform, after which the protein is transported to the cell surface. Evidence from cell culture studies indicate that at least some breast cancer cell lines can synthesise components of this enzyme system (Alessandro et al, 1993; Yu et al, 1995).

2.6.2.3 Expression of MMP-9 and TIMP-1 in Breast Carcinomas

In contrast to the ubiquitous distribution of MMP-2 and MT1-MMP in carcinomas, reactivity for MMP-9 was observed in only 68% of breast carcinomas. Staining was localised either within the stromal compartment or in tumour cell cytoplasm. In keeping with other reports, a proportion of this reactivity was localised to infiltrating macrophages (Davies et al, 1993; Heppner et al, 1996). In 49% of carcinomas, cytoplasmic reactivity for MMP-9 was present within tumour cells. Breast tumour cell synthesis of MMP-9 has previously been reported (Soini et al, 1994; Himmelstein & Muschel, 1996) and tumour cell staining has been shown to be associated with the presence of lymph node metastases (Iwata et al, 1996), though no correlation with node status, nor, in contrast to Davies et al (1993), with tumour grade was found in this study. The most striking observation was the association of tumour cell reactivity with tumour type, being much more frequent and more homogeneous in ILC. This is of particular interest in that a recent report has implicated the cell-cell adhesion molecule E-

Cadherin in the control of MMP-9 expression in mouse skin tumour cell lines (Llorens et al,

1998). A number of studies have reported loss of E-Cadherin expression in ILC (Moll et al,

1993; Jones et al, 1996), raising the possibility that enhanced expression of MMP-9 in these

tumours may implicate E-Cadherin in the control of enzyme production.

Stromal reactivity for TIMP-1 was detected in 49% and 57% of carcinomas (with (os)

and (ss) antibodies, respectively), but showed no relationship to tumour grade or lymph node

status. In apparent contradiction to the known inhibitory effect of TIMP-1 in in-vitro systems

(Schultz et al, 1988), a number of studies have reported an association between elevated TIMP-

1 expression and the malignant phenotype. Lu et al (1991) demonstrated higher levels of

TIMP-1 in colonic carcinomas compared to adjacent normal mucosa, whilst Li et al (1996)

found elevated TIMP-1 levels in undifferentiated, metastatic mouse mammary tumours, with

non-detectable levels in well differentiated non-metastatic cases. Yoshiji et al (1996) reported

enhanced expression of TIMP-1 mRNA in primary breast carcinomas compared to non-

neoplastic breast tissue. It has been suggested that TIMP-1 may contribute to tumour

progression via growth promoting activity, since it is known to possess erythroid-potentiating

activity (Gasson et al, 1985). The present study has revealed strong expression of TIMP-1 in

small blood vessels in a majority of invasive carcinomas, and the increased vascularity

associated with most malignancies may explain, at least in part, the source of elevated TIMP-1.

2.6.2.4 Relationship between Immunohistochemical Expression of MMP-2 and MT1-MMP, and

Enzyme Activity

A positive correlation between the amount of active MMP-2 and level of expression of MT1-

MMP, measured either by mRNA quantitation or by immunohistochemistry, has been

demonstrated in breast (Ueno et al, 1998), gastric (Nomura et al, 1995), lung (Tokuraku et al,

1995), brain (Yamamoto et al, 1996) and head and neck carcinomas (Yoshizaki et al, 1997) indicating that MT1-MMP may be a major activator of MMP-2 in-vivo. The present study found that only membrane staining for MMP-2 and MT1-MMP exhibited a correlation with tumour behaviour, suggesting that this pattern of reactivity may be a better indicator of enzyme activation than the total extent of staining observed within the tumour and stroma. To investigate this hypothesis, substrate gel zymography was carried out on a series of cases characterised as showing either high or low levels of membrane MMP-2 and MT1-MMP. The results demonstrated a significant positive correlation between a membrane distribution of the proteins on immunohistochemistry and a high activation ratio of MMP-2. It is of interest that membrane reactivity for both MMP-2 and MT1-MMP has been commented on in a number of studies (Bramhall et al, 1996; Nomura et al, 1996; Ueno et al, 1997), but in only one study was this analysed separately, where it showed a strong correlation with the presence of vascular invasion (Nomura et al, 1996).

2.6.2.5 Localisation of MMP-9 and MT1-MMP Synthesis by In-Situ Hybridisation

The pattern that has emerged from a number of studies on different human malignancies including breast (Basset et al, 1990; Polette et al, 1994; Heppner et al, 1996; Tetu et al, 1998), colon (Poulsom et al, 1992; Pyke et al, 1993) and skin tumours (Pyke et al, 1992), is that most MMPs are synthesised in the stromal compartment probably in response to tumour-derived factors. The expression of MMPs by infiltrating macrophages and lymphocytes has also been reported (Canete-Soler et al, 1994; Nielsen et al, 1996).

A specific question to be addressed by this study was whether there was evidence of MMP-9 synthesis in the tumour cells of ILC. The results demonstrated definite signal for MMP-9 in a proportion of the tumour cells in these cases, though this was not as homogeneous

129

or extensive as staining for the protein. This suggests that either the level of synthesis in many of the tumour cells is low, beyond the level of sensitivity of the technique, or alternatively that only a proportion of cells produce this enzyme, and the remainder of the cytoplasmic staining may represent internalisation of exogenous protein. Focal staining was also observed in tumour cells of IDC, but as with the immunohistochemical findings, this was less frequently seen than in ILC. In contrast to MMP-2, tumour cell synthesis of MMP-9 has been reported in skin (Pyke et al, 1992) and lung carcinomas (Canete-Soler et al, 1994) as well as in breast tumours (Soini et al, 1994; Himmelstein et al, 1995; Lindsay et al, 1997). In the studies on breast, where tumour type is indicated, no comment is made on a difference between ILC and IDC, and the extent of reaction in any given tumour is unclear, so it is difficult to comment on whether the greater frequency and extent of MMP-9 mRNA expression in tumour cells of ILC observed in this study was also demonstrated in these previous studies.

There has been controversy in the literature regarding the cellular source of MT1-MMP, some reports indicating a stromal source in tumours of the colon, breast and head and neck cancers (Okada et al, 1995; Heppner et al, 1996), whilst others have demonstrated mRNA in the tumour cell population in cervical (Gilles et al, 1996), colon (Ohtani et al, 1996), lung (Polette et al, 1996) and malignant brain tumours (Forsyth et al, 1999). The present study supports the suggestion that fibroblasts are the major source of MT1-MMP in breast cancers, however, in a proportion of cases, groups of tumour cells were shown to express MT1-MMP mRNA. These tumours all demonstrated membrane reactivity for MT1-MMP protein in >50% of tumour cells. Two recent studies using cell lines have indicated that breast cancer cells may synthesise MMPs, including MT1-MMP, but this is characteristic of tumour cells which exhibit some 'mesenchymal' features, such as loss of Cadherin expression or gain of vimentin expression (Gilles et al, 1997; Mortorana et al, 1998). It may be that expression of MT1-MMP

by tumour cells is evidence of a tumour showing an epithelial-to-mesenchymal transition, and this may be associated with more aggressive tumour behaviour. The numbers examined in this study are too small to analyse these relationships, but a larger in-situ hybridisation study, combined with immunohistochemical characterisation of tumours for markers such as vimentin would enable this to be addressed.

Whilst a relationship was identified between the presence of tumour membrane staining and tumour cell synthesis of MT1-MMP, there was no correlation between cytoplasmic reactivity for the protein and detection of mRNA. It is possible that cytoplasmic staining indicates internalisation of the MT1-MMP/MMP-2 complex following proteolysis. Alternatively, there may be differences in the rates of mRNA synthesis or translation between tumour cells and fibroblasts, or variation in the capacity for intracellular storage of the protein. In contrast to most MMPs, the MT-MMPs are activated intracellularly by a family of precursor-processing endopeptidases known as pro-protein convertases (Pei and Weiss, 1995; Sato et al, 1996). It is possible that membrane expression of MT1-MMP by tumour cells is regulated at the level of pro-MT1-MMP activation, rather than primarily at the level of gene transcription, which could explain the presence of stored immunoreactive protein in tumour cells without detectable levels of mRNA. Okada et al (1995) suggested that MT1-MMP produced by stromal cells may be cleaved from the cell surface and bind to tumour cells by a receptor-mediated mechanism, although to date there is no evidence for such a mechanism.

2.6.2.6 Expression of Cell Adhesion Molecules in Normal/Benign Breast

Strong reactivity for α2, α3, α6, β1 and β4 integrins was seen in normal breast, particularly in relation to the myoepithelial cell, with β4 integrin being limited to the cell-basement membrane interface. Weak staining for α2, α3 and β1 integrin was observed at luminal epithelial cell-cell

borders. These findings are in agreement with a number of studies (Pignatelli et al, 1991; Koukoulis et al, 1991; Glukhova et al, 1995), although Koukoulis et al (1991) also reported expression of $\alpha 1$ integrin, which has not been reported by other groups and was not examined in this study. In addition, Zutter et al (1990) reported basal expression of $\alpha 5$ integrin in normal breast. Again, expression of this subunit has not been shown by other groups, and there was no evidence of reactivity for $\alpha 5$ integrin in this study. This discrepancy between studies is difficult to explain: each study has used different (though all monoclonal) antibodies to $\alpha 5$ integrin, but all the studies have been carried out on frozen, acetone fixed tissue. The antigenicity of the integrin receptors appears to be very labile, since it was noted in this study that storage of sections for more than 5 days resulted in reduced reactivity, but this would not explain the lack of reactivity in normal tissue, since staining was achieved for other integrins and for $\alpha 5$ in adjacent malignant tissue.

E-Cadherin was strongly expressed at luminal epithelial cell-cell borders and at the site of epithelial-myoepithelial interactions in keeping with its role in adherens junctions.

2.6.2.7 Expression of Integrins in Breast Carcinomas

In carcinomas, a reduction in $\alpha 2$, $\alpha 3$ and $\beta 1$ integrin subunits was observed, with greater loss of reactivity in poorly differentiated tumours, a relationship that was particularly strong for $\alpha 2$ and $\beta 1$ integrins. A similar relationship between $\alpha 2 \beta 1$ reactivity and breast tumour grade has been reported by other workers (Zutter et al, 1990; Koukoulis et al, 1991; Pignatelli et al, 1991), and also in other adenocarcinomas, such as colorectal (Koretz et al, 1991; Pignatelli et al, 1992; Koukoulis et al, 1993), renal (Korhonen et al, 1992), pancreatic (Weinal et al, 1992) and lung carcinomas (Damjanovich et al, 1992). No reduction in $\alpha 2$ or $\alpha 3$ was recorded by D'Ardenne et al (1992), however, they did not distinguish between membrane and cytoplasmic staining

which may have influenced the results since other studies, including the present study, refer specifically to loss of membrane reactivity. In-vitro studies have supported a direct role for $\alpha2\beta1$ in the induction of breast epithelial differentiation. Transfection of a poorly differentiated mouse mammary tumour cell line with $\alpha2$ integrin was shown to promote glandular morphogenesis (Zutter et al, 1995), whilst expression of anti-sense $\alpha2$ mRNA into a well-differentiated $\alpha2$-expressing breast cancer cell line inhibited gland formation in three-dimensional collagen matrices (Keely et al, 1995). Reduced $\alpha3$ expression in parallel with $\alpha2$ and $\beta1$ has been described by Pignatelli et al (1992), but has not been confirmed by other groups. However, the role of $\alpha3\beta1$ in breast appears to be distinct from that of $\alpha2\beta1$, since antibodies to $\alpha2$ integrin block branching morphogenesis in three-dimensional gels but this is not seen with blocking antibodies to $\alpha3$ integrin (Berdichevsky et al, 1992).

In this study whilst $\alpha5$ integrin was not detected in normal breast, expression by peri-tumoural fibroblasts was observed in the majority of carcinomas, the extent of which correlated with grade. Little attention in the literature has been paid to changes in stromal integrin reactivity, although $\alpha5$ reactivity is commented on in one study (Pignatelli et al, 1992). The $\alpha5$ integrin has been implicated in the regulation of cell growth and tumourigenicity; overexpression of $\alpha5$ integrin in CHO cells resulted in reduced tumourigenicity (Giancotti & Ruoslahti, 1990), as well as reduced tumour cell proliferation. The latter has recently been demonstrated to be a result of $\alpha5\beta1$-mediated suppression of MAP kinase activity (Gong et al, 1998). In fibroblasts in-vivo, however, signalling by $\alpha5\beta1$ integrin has been shown to induce expression of MMP-1, 2 and 3 (Huhtala et al, 1995). This is of interest in relation to the potential role of $\alpha5$ integrin neo-expression in tumour-associated fibroblasts, as identified in this study. The limitation of fibroblast-associated $\alpha5$ integrin expression to the peri-tumoural

fibroblasts, rather than a diffuse reactivity of all fibroblasts suggests that tumour cell-stromal cell interactions could be involved in the induction of this integrin.

A significant correlation was demonstrated between staining for $\alpha6$ integrin and $\beta4$ integrin, however, the most striking observation was the lack of reactivity for these integrin subunits in >65% of tumours. Reduction or loss of reactivity for $\beta4$ integrin has been a consistent finding in studies on breast carcinomas (Koukoulis et al, 1991; Natali et al, 1992; Hanby et al, 1993; Gui et al, 1995), though in other malignancies, particularly squamous carcinomas, either of skin, cervix or head and neck, $\alpha6\beta4$ is consistently up-regulated (Savoia et al, 1993; Van Waes et al, 1991; Carico et al, 1993). This suggests that integrins may play very different roles in different types. Interestingly, studies on prostate carcinoma, which bear more similarities to breast tumours, report loss or down-regulation of $\alpha6\beta4$ in a similar manner to that seen in breast carcinoma (Nagle et al, 1995; Allen et al, 1998). Although the majority of breast tumours lacked $\alpha6\beta4$, its expression was associated with the more aggressive, high grade tumours (p=0.01 for $\alpha6$ integrin and p=0.02 for $\beta4$ integrin). Friedrichs et al (1995) found expression of $\alpha6$ was associated with the presence of lymph node metastases and a reduced patient survival compared to $\alpha6$-negative tumours. The present study showed no relationship between expression of any of the integrin receptors and lymph node status. A recent study found that expression of $\alpha6\beta4$ was associated with poor patient prognosis, and this was independent of grade and lymph node status (Tagliabue et al, 1998). Furthermore this group revealed that patient outcome was poorest for those whose tumours expressed both $\alpha6\beta4$ integrin and laminin, suggesting that $\alpha6\beta4$ plays an important role in tumour progression particularly when it interacts with its ligand. In apparent contrast to this tumour-promoting role for $\alpha6\beta4$, using an approach of RT-PCR differential display, Sager et al (1993) found $\alpha6$

integrin to be differentially expressed in normal breast compared to tumours, and suggested that

α6 may function as a tumour suppressor protein.

In attempting to understand the potential role of α6β4 (or any other molecule) in breast

cancer it is important to appreciate its role in the normal breast. The α6β4 heterodimer is limited

to the cell-basement membrane interface in normal breast ducts and acini, in keeping with its

incorporation into hemidesmosomes (HD). Whereas occasional luminal cells extend from the

glandular lumen to make contact with the basement membrane, this interface is formed

primarily by myoepithelial cells, which are rich in HD. Myoepithelial cells are lost in invasive

carcinomas and therefore the lack of α6β4 integrin in the majority of invasive breast carcinomas

may simply be a reflection of lack of this cell population. A parallel situation exists in the

prostate gland, where the cell-basement membrane interface is formed by the basal cell, which

is rich in HD and expresses high levels of α6β4 integrin whereas the majority of prostatic

adenocarcinomas lack HD and α6β4 (Allen et al, 1998). It is generally accepted that the

majority of breast carcinomas exhibit a luminal epithelial phenotype, on the basis of cytokeratin

profile, expression of polymorphic epithelial mucin (PEM) and oestrogen receptor (ER)

expression in a proportion of cases (Bartek et al, 1985; Taylor-Papadimitriou et al, 1993).

However, from a number of studies it has become evident that a subclass of tumours show

some features of a basal or myoepithelial phenotype, expressing markers more typical of these

cells such as vimentin, cytokeratin 14, high levels of EGFR, and lacking ER (Wetzels et al,

1990; Cattoretti et al, 1988; Domagala et al, 1990a). These markers are frequently associated

with a poor prognosis (Domagala et al, 1990b). Thus, one possible interpretation of α6β4

expression in a breast carcinoma is that this represents a further marker of the

basal/myoepithelial phenotype. Taylor-Papadimitriou et al (1993) suggested that such 'basal'

tumours are a more aggressive group of tumours since they may contain the stem cell

population of the breast. This would be supported by the recent data indicating that $\alpha6\beta4$

integrin expression in breast carcinomas is a poor prognostic marker (Tagliabue et al, 1998).

Whereas in the normal breast, $\alpha6\beta4$ is incorporated into the HD of myoepithelial cells,

HD are not seen in invasive breast carcinomas (Bergstraesser et al, 1995). It seems likely

therefore that the $\alpha6\beta4$ heterodimer plays a different role in tumour cells compared to its

anchoring role in normal cells. In keeping with this, Rabinovitz and Mercurio (1997) have

shown that $\alpha6\beta4$ expression in a colon and breast cancer cell line promoted cell migration, and

was localised in complexes distinct from HD. In contrast, other studies have indicated that up-

regulation of $\alpha6\beta4$ integrin in breast cancer cells reverses some features of the malignant

phenotype, leading to enhanced glandular morphogenesis (Weaver et al, 1998) and restoration

of contact inhibition of growth (Sun et al, 1998). Both of these studies also emphasise the

interplay between different integrin receptors, and Weaver et al (1998) suggests that it is the

balance of signals transduced by $\beta1$ and $\beta4$-integrins that modulates tissue organisation and

function. The present study has demonstrated an inverse relationship between membrane levels

of $\beta1$ and $\beta4$ integrins supporting the suggestion that it is likely to be the integration of a series

of changes in adhesion molecules that determines tumour behaviour.

2.6.2.8 Expression of E-Cadherin in Breast Carcinomas

In vitro studies have established an invasion suppressor role for E-Cadherin (Frixen et al, 1991;

Vleminckz et al, 1991), suggesting that loss of E-Cadherin may be a pre-requisite for tumour

invasion. In contrast, tissue studies have shown that many epithelial tumours retain E-Cadherin

expression, albeit at a lower level than normal tissue (Schipper et al, 1991; Kinsella et al,

1993). In the breast, one of the most consistent findings is loss of E-Cadherin in ILC (Moll et

al, 1993; Lipponen et al, 1994; Gonzalez et al, 1999) which was confirmed by the findings in

this study. Loss of expression in ILC has been attributed to truncation mutations in the extracellular domain of E-Cadherin, with associated loss of heterozygosity of wild-type allele (Berx et al, 1996; Vos et al, 1997). Other studies have not found a high frequency of gene mutation as a cause for loss of E-Cadherin and hypermethylation of the promotor has been implicated as the mechanism of gene silencing (Graff et al, 1995; Yoshiura et al, 1995; Herman et al, 1996). The lack of E-Cadherin in ILC undoubtedly gives rise to the classical morphology of these tumours, with cells infiltrating singly or as strands with no attempt at gland formation. Interestingly, mutations in E-Cadherin have been discovered in diffuse type gastric carcinomas, which have a similar morphology to ILC (Becker et al, 1994). The presence of homogeneous cytoplasmic staining in one case of ILC in this study may be a result of demethylation of the gene, as suggested be De Leeuw et al (1997). It is evident that loss of E-Cadherin alone is not sufficient for invasion, however, since foci of LCIS also demonstrate absence of this molecule.

In contrast to the loss of reactivity in ILC, 88% of IDC retained E-Cadherin expression, though at a variable level. Reduced membrane E-Cadherin in IDC has been associated with high tumour grade (Moll et al, 1993; Oka et al, 1993; Gonzalez et al, 1999), and Oka et al (1993) also demonstrated a significant relationship between reduced membrane E-Cadherin and the presence of lymph node metastases, though Lipponen et al (1994) showed no relationship with grade or lymph node status. The present study demonstrated a significant association between reduced membrane E-Cadherin in IDC and the presence of lymph node metastases, but no correlation with tumour grade was identified. A second pattern of staining for E-Cadherin was observed in many carcinomas, that of a diffuse cytoplasmic distribution. This pattern of staining has been commented on in certain tumours (Pignatelli et al, 1994), though the significance of this distribution has not been addressed. The present study has demonstrated a highly significant association between tumour cytoplasmic reactivity for E-Cadherin and the

presence of lymph node metastasis. There are a number of possible explanations for this

finding. One is that the cytoplasmic reactivity indicates a reversible change in E-Cadherin

expression by the cell in response to the local environment. It has been demonstrated that E-

Cadherin expression can be easily altered in vitro in response to the culture environment

(Mareel et al 1991) and that highly metastatic ovarian tumour cells have unstable E-Cadherin

expression (Hashimoto et al 1989). Cytoplasmic reactivity for E-Cadherin and the catenins has

been reported in bronchopulmonary carcinomas, and by immunoprecipitation experiments this

redistribution was shown to correlate with changes in tyrosine phosphorylation of E-Cadherin

(Nawrocki et al, 1998). Growth factors such as Hepatocyte Growth Factor and EGF lead to

dissociation of E-Cadherin/catenin complexes from the cytoskeleton and loss of E-cadherin

from the cell membrane (Shiozaki et al, 1995). In keeping with this, an extended investigation

based on the current study demonstrated a significant correlation between the expression of

EGF-R by tumour cells and the presence of cytoplasmic reactivity for E-Cadherin (Jones et al,

1996). This may illustrate one mechanism whereby local environmental influences could

modulate both tumour cell growth and invasive behaviour. Such a temporal and reversible

disruption of E-Cadherin mediated adhesion would allow tumour cell detachment whilst re-

expression could favour colonisation at a distant site. In support of this, there are reports of

increased E-Cadherin expression in metastatic lesions compared to their primary tumour

(Mayer et al 1993).

For functional E-Cadherin mediated adhesion, the adhesion molecule must be

incorporated into complexes linked to the actin cytoskeleton, via α-catenin, to either β-catenin

or plakoglobin (Nagafuchi et al, 1989; Ozawa & Kemler, 1992; Hinck et al 1994). Deletion of

the intracellular catenin-binding domain of E-Cadherin or alterations in the catenins results in

loss of adhesive function (Nagafuchi & Takeichi, 1989; Ozawa et al, 1989; Hulsken et al,

1994). A number of studies have reported abnormalities of the catenins, both in breast

carcinomas (Rimm et al, 1995; Bukholm et al, 1998; Gonzalez et al, 1999) and in other

tumours (Shiozaki et al, 1994; Kadowaki et al, 1994; Takayama et al, 1996). Whilst mutations

in the β-catenin gene have been identified in colorectal neoplasia (Morin et al, 1997), there is

no evidence of mutations in breast carcinomas (Candidus et al, 1996) although altered tyrosine

phosphorylation of β-catenin and low levels of plakoglobin have been reported, with

consequent failure to incorporate E-Cadherin into cell membrane associated complexes

(Sommers et al, 1994). Bukholm et al (1998) found that whereas in breast carcinomas changes

in E-Cadherin alone did not correlate with metastatic status, a significant correlation was

evident between the presence of metastasis and alteration of one or more of the E-

Cadherin/catenin system, suggesting this is a better indicator of E-Cadherin adhesive function.

The detection of cytoplasmic reactivity for E-Cadherin in this study may be indicative of non-

functioning E-Cadherin, reflecting an abnormality in other components of the system. Analysis

of catenin expression was not undertaken in this study (although the work is currently being

undertaken in the laboratory), and it will be of great interest to establish whether this will be a

better predictor of disease behaviour.

These findings indicate distinct pathways of development in different tumours.

Whilst in ILC loss of E-Cadherin appears to be an early event in tumour development, in IDC

altered E-Cadherin expression appears to be more directly related to the metastatic process, and

may reflect changes in post-translational regulatory mechanisms. The association of

cytoplasmic E-Cadherin with lymph node metastasis suggests a sophisticated control over cell-

cell adhesion possibly in response to local growth factors such as EGF, and this link between

E-Cadherin expression and signalling pathways may be an important stage in understanding

tumour progression.

2.6.2.9 Relationship between Expression of Cell Adhesion Molecules and MMP/TIMPs

There is increasing evidence that synthesis and release of proteolytic enzymes may be regulated at least in some situations by integrin-matrix interactions. Classical studies on rabbit synovial fibroblasts demonstrated that expression of a number of MMPs was regulated by $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin receptors (Huhtala et al, 1995). MMP synthesis is induced in wound-associated keratinocytes in an $\alpha 5\beta 1$-dependent manner (Saarialho-Kere et al, 1993), and macrophage interaction with fibronectin induces MMP-9 expression (Xie et al, 1998). In tumour cells also, integrin-dependent mechanisms have been implicated in regulation of MMP-2 (Seftor et al, 1993; Teti et al, 1998; Bafetti et al, 1998) and MMP-9 (Niu et al, 1998; Chintala et al, 1998). In this study, changes in E-Cadherin and integrin receptor expression were correlated with patterns of MMP expression in an attempt to demonstrate potential relationships between the two. A positive correlation was identified between membrane $\alpha 6$ reactivity and the presence of membrane MMP-2 (ss), and with cytoplasmic, but not membrane, $\beta 4$ integrin and membrane MMP-2. This may simply reflect an association between two molecules independently related to tumour differentiation, or alternatively could imply a modulatory link between the integrin and MMP, although if this were the case one would expect to see a relationship for membrane $\beta 4$ as well as the $\alpha 6$ integrin. A further relationship was identified between stromal reactivity for $\alpha 5$ integrin and stromal MMP-9 expression. Activation of a MAP-kinase signalling pathway following $\alpha 5\beta 1$-matrix interactions has been shown to regulate MMP expression in-vitro (Gong et al, 1998), and the identification of this relationship in primary breast tumours suggests that such a mechanism for control of MMP-9 could exist in-vivo.

Interpretation of immunohistochemical expression of different proteins and the relationships between them can provide correlative data only, and in a system as dynamic as cell-matrix interactions, such an approach has many limitations. A major limitation is that

140

whilst integrin-matrix interactions have been implicated in regulating MMP synthesis, such

interactions may not depend on de-novo expression of integrin receptors, but rather on

reorganisation of receptors in the cell membrane; such reorganisation would not be detected on

immunohistochemistry. Another possibility is that receptors not examined in this study may be

expressed de-novo and be involved in initiating signals from the matrix. The situation is further

complicated by recent studies which indicate that proteolytically-cleaved matrix proteins can

alter interactions with integrins, for example, collagenase-3 (MMP-13) cleavage of collagen I

prevents $\alpha 2\beta 1$ ligation to this matrix protein (Messent et al, 1998), and MMP-2 cleavage of

laminin-5 exposes a cryptic site on the ECM molecule which then supports binding to $\alpha 3\beta 1$

(Gianelli et al, 1997). Thus, the nature of interaction of a cell with the matrix is itself modified

by matrix remodelling, and without investigating changes in matrix composition, interpretation

of whether integrin receptors are bound or unbound cannot be known. In spite of these

apparently overwhelming limitations, such 'snapshot' analysis of the static state may still

provide valuable pointers towards significant changes in the tumour environment.

An interesting relationship demonstrated in this analysis was the inverse correlation

between E-Cadherin expression (both membrane and cytoplasmic) and the presence of MMP-9

staining within tumour cells. Whereas the role of integrin signalling in control of gene

expression is well recognised, a signalling role for E-Cadherin is less well established. Several

studies have reported an inverse correlation between E-Cadherin expression and type IV

collagenase expression, both MMP-2 and MMP-9, in tumours other than breast (Anzai et al,

1996; Kuniyasu et al, 1999), however, despite using an in-situ hybridisation approach, these

studies do not indicate whether it is the tumour or stromal compartment that is the source of the

enzyme, and since all are tissue-based studies they are unable to address whether enhanced

MMP expression is a direct consequence of reduced E-Cadherin. One study which has

demonstrated a direct relationship between E-Cadherin and MMP-9 expression used E-Cadherin-negative (E-CD-) and E-Cadherin-expressing (E-CD+) clones of a mouse skin cancer cell line. They found E-CD- clones produced high levels of MMP-9 whereas levels were low in E-CD+ clones. Transfection of E-CD+ clones with E-CD antisense cDNA resulted in increased MMP-9 expression (Llorens et al, 1998). Further evidence for a link between MMPs and the Cadherin/catenin system comes from identification of MMP-7/matrilysin as a downstream target gene of β-catenin signalling in a mouse model (Fingleton et al, 1999). Whether such a direct causal link between E-Cadherin and MMP-9 exists in breast merits further study (see Chapter 3).

2.7 Conclusions

- The MMP-2/MT1-MMP/TIMP-2 system is widely expressed in the stroma and in tumour cell cytoplasm of breast carcinomas. The presence of tumour cell membrane reactivity for MMP-2 and MT1-MMP correlates with the presence of lymph node metastases and with the activity of MMP-2 on zymography, suggesting that this distribution is indicative of MMP-2 activation. Tumour cell cytoplasmic staining for MT1-MMP is not reflected by the presence of MT1-MMP mRNA in tumour cells, however, tumours with >50% of cells displaying membrane staining do show evidence of MT1-MMP mRNA synthesis. The lower level of mRNA detected compared to protein within the tumour cells may be due to lower rates of mRNA synthesis, possibly due to intracellular storage of the protein.

- Changes in integrin receptor expression are present in many breast carcinomas when compared to normal breast; downregulation of $\alpha2$, $\alpha3$ and $\beta1$ integrin is related to loss of differentiation whereas presence of $\alpha6$ and $\beta4$ integrin is associated with high tumour grade, and an inverse relationship is seen between $\beta1$ and $\beta4$ integrins. A positive correlation exists between membrane expression of $\alpha6$ integrin and both membrane and cytoplasmic MMP-2, but this relationship is not seen for $\beta4$ integrin, and its significance is not clear. These findings support a role for integrins in modulating differentiation. The presence of $\alpha6\beta4$ in a proportion of cases may indicate a group of tumours that demonstrate a myoepithelial phenotype.

- The most striking relationship observed for the MMP-9/TIMP-1 system is the association with tumour type, with MMP-9 being more frequently and more homogeneously localised

to tumour cell cytoplasm in ILC compared to IDC. IDC and ILC also exhibit different patterns of expression of E-Cadherin: ILC (and LCIS) show homogeneous loss of E-Cadherin whilst IDC exhibit a variable reduction in membrane E-Cadherin and increase in cytoplasmic staining, the extent of which relates to the presence of lymph node metastases. For all carcinomas an inverse relationship exists between tumour cytoplasmic MMP-9 and loss of membrane E-Cadherin. In-situ hybridisation reveals that MMP-9 is localised predominantly to the stromal compartment, but in ILC a proportion of tumour cells exhibit mRNA expression. These results raise the possibility of a direct link between E-Cadherin and the control of MMP-9 expression, which would facilitate the invasive process.

- These results suggest that distinct pathways may exist for the control of MMP/TIMP expression, which relate to tumour behaviour and in some situations may be specific to different tumour types. In vitro systems are essential to directly address the nature and relevance of these interactions.

Chapter 3

In-Vitro Studies of the Relationship Between MMP Expression, Invasion and Cell Adhesion Molecule Expression

AIM: To develop an in-vitro model system to quantify breast cancer cell invasion and to relate the level of invasion to (i) the level of MMP and TIMP expression and (ii) the expression profile of Integrins and E-Cadherin. To manipulate E-Cadherin and Integrin function in these cell lines and analyse the effect on invasion and on MMP/TIMP expression.

3.1 Introduction

There is abundant evidence from tissue studies and in-vitro models that tumour cell invasion is associated with changes in cell-cell and cell-matrix interactions (Pignatelli et al, 1991; Koukoulis et al, 1993; van Duinen et al, 1994; Fukushima et al, 1998). Studies have also demonstrated that increased expression of proteolytic enzymes or reduced levels of their inhibitors promotes tumour invasion (Garbisa et al, 1987; DeClerk et al, 1991; DeClerck et al, 1992). There is now increasing evidence to indicate a functional link between the processes of proteolysis and signalling via cell adhesion molecules (Seftor et al, 1993; Huhtala et al, 1995; Irigoyen et al, 1997; Stanton et al, 1998; Crawford et al, 1999).

The first part of this thesis has identified changes in E-Cadherin and integrin expression in many breast carcinomas compared to normal breast. Enhanced expression of gelatinases, MT1-MMP and TIMPs was also identified in the majority of invasive tumours. Whether these changes are related is not clear, since tissue-based studies allow only static associations to be determined. Therefore, in order to address more directly the inter-relationship between cell adhesion molecule expression, MMP/TIMP expression and tumour invasion, an in-vitro approach is necessary.

The aim of this part of the study was to develop an in-vitro model system to quantify breast cancer cell invasion, and to use this system to relate invasive ability to MMP/TIMP expression and activity and to the expression profile of cell adhesion molecules. Established breast cancer cell lines were used since these are simple to grow and can be used in multiple experiments. An inverse relationship between E-Cadherin and tumour cell expression of MMP-9 has been identified (Chapter 2) and the hypothesis that loss of E-Cadherin leads to MMP-9 expression will be addressed by blocking E-Cadherin-mediated adhesion and analysing the effect on MMP-9 expression. My previous work in this laboratory has demonstrated up-regulation of $\alpha6\beta4$ integrin in MDA-MB 231 cells following treatment with TGF-$\beta1$ with or without hydrocortisone (Jones et al, 1997). This system will be used to investigate the effect of such altered integrin expression on invasion and on MMP/TIMP expression.

A variety of in-vivo and in-vitro systems have been developed to assess the invasiveness of tumour cells. In-vivo models usually involve subcutaneous, intramuscular or intravascular injection of tumour cells into syngeneic animals (van Larnsweerde et al, 1983; Tsuruo et al, 1983). These models have the advantage of not only allowing assessment of invasive ability but also of metastatic potential, and it could be argued that the complex physiological environment provided by the in-vivo situation is superior to a non-physiological culture environment. However, this complexity and the inevitable variability of the environment may affect the growth and behaviour of the implanted tumour cells and influence experimental findings (Kyriazis & Kyriazis, 1980; Kozlowski et al, 1984). In contrast, in-vitro systems allow much greater control over the environment although they are undoubtedly a less accurate reflection of the in-vivo situation. A further advantage of in-vitro systems is that tumour cells and conditioned media can be retrieved from the experiments for analysis. A two-compartment in-vitro invasion assay, based on that described by Albini et al (1987) was the

147

technique of choice in this study since this has been shown to be relatively simple and give

high levels of reproducibility (Hendrix et al, 1987; Hendrix et al, 1989; Saiki et al, 1994).

MMP/TIMP expression will be measured at the mRNA level using Reverse Transcriptase-

Polymerase Chain Reaction (RT-PCR), and net proteolytic activity will be analysed by substrate gel

zymography. In some situations knowledge of the presence or not of MMP gene expression is

adequate, however, in other experiments information on changes in the level of MMP/TIMP

expression are required. A number of approaches have been developed to quantitate mRNA

following PCR amplification, which are all based on the inclusion of internal or external standards

against which the target gene can be measured. Those techniques using internal standards are more

commonly employed because they are regarded as being simpler and more reproducible than those

employing external standards. Quantitative PCR techniques using internal standards can be divided

into two categories: those using an exogenous mRNA or DNA standard added in known

concentration to the reaction mixture (competitive RT-PCR and RT-competitive PCR, respectively),

and those using endogenous housekeeping gene transcripts. Whilst competitive techniques can

provide absolute quantitation of PCR products, the goal of many studies, including my own study, is

to determine the relative change in mRNA levels rather than the exact number of molecules in a

sample. One method for analysing levels of mRNA is to compare levels of the amplified target gene

against an endogenous housekeeping gene. A housekeeping gene is used on the assumption that it is

transcribed constantly and independently from the extracellular environment and typical examples

include β-actin and glyceraldehyde -3 phosphate-dehydrogenase (GAPDH) (Lonn et al, 1992;

Pannetier et al, 1993; Dukas et al, 1993; Wong et al, 1994). To measure the amount of RT-PCR

product, an Enzyme Linked Oligonucleotide Assay (ELOSA) will be adopted. The method is a four-

step technique which involves:

(i)   immobilisation of PCR product in a microwell plate

(ii)   denaturation of double stranded DNA to yield single stranded bound DNA

(iii)   hybridisation of specific probe to the complementary single strand, and

(iv)   colorimetric detection of probe/DNA hybrids.

ELOSA combines both sensitivity and specificity of a probe hybridisation technique with rapid and

simple methods of detection and quantification (Alard et al 1993).

3.2 Materials

3.2.1 Cell Lines

Breast cancer cell lines were originally obtained from American Type Culture Collection (ATCC). The fibroblast cell line was a gift from Department of Surgery, University of Leicester, and was originally also obtained from ATCC.

- MCF-7: Derived from malignant pleural effusion secondary to breast carcinoma. It retains characteristics of differentiated mammary epithelium, including expression of oestrogen receptor (Soule et al, 1973).

- T-47D: This cell line was isolated from a pleural effusion from a 54 yr old lady with an infiltrating ductal carcinoma. It shows epithelial differentiation and expresses oestrogen receptor (Freake et al, 1981).

- MDA-MB 231: Derived from a malignant pleural effusion secondary to breast carcinoma. It forms poorly differentiated adenocarcinoma, consistent with a grade III tumour, in nude mice (Cailleau et al, 1974). The tumour cells are oestrogen receptor negative.

- MDA-MB 468: Human breast adenocarcinoma derived from a pleural effusion. Tumour cells are oestrogen receptor negative (Cailleau et al, 1978)

- F518: Human skin fibroblasts derived from a normal individual. Finite life expectancy of approximately 35 passages. For this study cells were used within the first 15 passages.

3.2.2 Tissue Culture Reagents

Dulbeccos modified Eagles medium (DMEM), Fetal Bovine Serum (FBS), L-Glutamine, insulin from bovine pancreas, hydrocortisone and poly-d-lysine were all from Sigma. Sterile Phosphate Buffered Saline (PBS) and Trypsin/EDTA were from Gibco. Ultrapure natural

human Transforming Growth Factor β1 (TGF- β1) derived from human platelets was obtained

from Genzyme Diagnostics (Cambridge, MA). Matrigel, a basement membrane matrix

extracted from the Engelbroth-Holm-Swarm (EHS) mouse sarcoma was from Becton

Dickinson. Its major component is laminin, with collagen IV, heparan sulfate proteoglycans,

entactin and nidogen (Kleinman et al, 1982). Tissue culture plastics including 25 cm^2 flasks,

pipettes, 6- and 12-well multiwell plates, 1.0 μm and 8.0 μm pore cell culture inserts by Falcon

from Becton Dickinson.

3.2.3 Antibodies

MMP, TIMP, MT1-MMP, Integrin and E-Cadherin antibodies as detailed in section 2.2.2.

Vinculin

Mouse monoclonal antibody to vinculin, received as a gift from Prof. D.R.Critchley,

Department of Biochemistry, University of Leicester.

HD1/Plectin

Mouse monoclonal antibody recognising the rod domain of the 300 kDa band of plectin, and

inhibits association of plectin/HD1 with vimentin, obtained from Sigma.

Vimentin

Mouse monoclonal antibody to the intermediate filament vimentin, obtained from Dako.

Anti-Epithelial Membrane Antigen

Mouse monoclonal antibody to Epithelial Membrane Antigen, which belongs to a heterogenous

group of highly glycosylated proteins known as Human Milk Fat Globule Membrane (HMFG)

or MUC1 proteins, obtained from Dako. This antibody displays a similar pattern of reactivity to

anti-HMFG, though the specific epitope recognised by EMA is not yet known.

Anti-Oestrogen Receptor

Mouse monoclonal anti-human oestrogen receptor (clone ID5), from Dako.

Anti-Epidermal Growth Factor Receptor (EGFR)

Mouse monoclonal anti-human Epidermal Growth Factor Receptor-1 from Amersham. Raised

against membrane fraction of A431 cells, it recognises the extracellular domain of the receptor.

Rhodamine (TRITC)-labelled phalloidin was obtained from Sigma. This toxin is derived from

Amanita phalloides and binds irreversibly to polymeric actin.

Fluoro-isothiocyanate (FITC) - labelled rabbit anti-mouse IgG from Dako was used as the

secondary antibody in all immunofluorescence.

3.2.4 Oligonucleotide Primers and Probes

Oligonucleotide primers and probes were designed in-house with the help of Dr. Howard

Pringle, using the Molecular Biology Suite 'gcg' primer design software PRIMER, probes

being selected from the reverse primer sequences. Where possible, primers were designed to

amplify across exon boundaries. Specificity of primers to the target gene was checked against

the European Molecular Biology Library (EMBL) and GENBANK databases using

FINDPATTERNS on the 'gcg' system. The oligonucleotides were synthesised by Gibco BRL,

Oswell and Genosys, and the forward primer of each primer set was synthesised biotinylated.

The sequence of the primers and probes are detailed in Table 3.1.

Table 3.1: Oligonucleotide Primer and Probe Sequences

GENE	PRIMERS (5' - 3')	SIZE	PROBE (5' - 3')
MMP-2	F: ATT GAT GCG GTA TAC GAG GC R: GGC ACC CTT GAA GAA GTA GC	350 bp	CTC CAG AAT TTG TCT CCA GC
MMP-9	F: TTC TAC GGG CCA CTA CTG TGC R: CGC CCA GAG AAG AAG AAA AG	250 bp	GTT GCA GGC ATC GTC CAC CGG ACT CAA AGG
MT1-MMP	F: TCG CCA ATG GAA AGA CCT AC R: TGA TGA TCA CCT CCG TCT CC	315 bp	CAT CCA GAA GAG AGC AGC ATC AAT CTT GTC
TIMP-1	F: TGG GGA CAC CAG AAG TCA AC R: CAG GGG ATG GAT AAA CAG GG	350 bp	GTA GTG ATG TGC AAG AGT CC
TIMP-2	F: AAC GAC ATT TAT GGC AAC CC R: ACC TGT GGT TCA GGC TCT TC	250 bp	TCT ATA TCC TTC TCA GGC CC
GAPDH	F: AGA ACA TCA TCC CTG CCT C R: GCC AAA TTC GTT TGC ATA CC	350 bp	GTT GAA GTC AGA GGA GAC C
β4 INTEGRIN	F: AGG AGA ACC TGA ACG AGG TC R: ATC AGC ACT GTG TCC ACA ATG	125 bp	*

*No probe produced for β4-integrin since this was not used in quantitative assays.

3.2.5 Chemicals

In addition to those detailed in Chapter 3, other chemicals - all of analar grade - were purchased as follows: diethanolamine (DEA), polyoxyethylene sorbitan monolaurate (Tween 20), lithium chloride (LiCl), dithiothreitol (DTT), biotin-NHS, dimethyl sulphoxide (DMSO), avidin, β-2-mercaptoethanol, p-nitrophenyl phosphate (PNPP), p-aminophenylmercuric acid (APMA) were from Sigma (Poole, UK). Magnesium sulphate anhydrate ($MgSO_4.7H_2O$), disodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) were from Fisons (Loughborough, UK). Potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium hydroxide (NaOH) were from Fisher Scientific UK (Loughborough, UK). Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) was from

Hopkins & Williams (Essex, UK). Rapid hybridisation buffer was from Amersham Life

Sciences Ltd (Buckinghamshire, UK). Ultrapure bovine serum albumin (BSA) and Agarose

MP were from Boehringer Mannheim UK Ltd (East Sussex, UK). BSA and dinucleotide

triphosphates (dNTPs) were from Advance Protein Products (Brierley Hill West Midlands,

UK).

3.2.6 Molecular Biology Enzymes

Avian myeloblastosis virus reverse transcriptase (AMV RT) and buffer, RNasin ribonuclease

inhibitor and Tdt were from Promega (Southampton, UK). *Thermus aquaticus* DNA

polymerase (Taq) was from Advanced Biotechnologies Ltd (Surrey, UK).

3.2.7 Miscellaneous Reagents

Oligo (dT)$_{25}$ Dynabeads were obtained from Dynal UK Ltd. DNA molecular weight markers -

ϕX174/Hae III and 100 bp ladder - were from Promega (Southampton, UK). PCR grade water,

RNase free, was obtained from Sigma.

3.3 Methods

3.3.1 Cell Culture

3.3.1.1 Cell Line Banking

At the time of receipt of cell lines, the original ampoule was expanded to yield a stock of 'seed' cultures. Expansion of selected seed cultures then generated 'working' cultures which were used for experimental work.

3.3.1.2 Maintenance of Cell Lines

Established cell lines were routinely maintained in 25 cm^2 culture flasks containing phenol red-free DMEM supplemented with 10% FBS and 2mM L-Glutamine, and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Complete media change was carried out every 2/3 days.

3.3.1.3 Passage of Cell Lines

At subconfluence (80-90%), culture media was aspirated, cell monolayers washed in pre-warmed sterile PBS and cells detached from the flask by incubation with trypsin/EDTA at 37°C for 2-3 mins. The cells were removed from the flask by addition of warm PBS and the suspension centrifuged at 300g for 5 mins. The cell pellet was then resuspended in an appropriate volume of pre-warmed culture medium and seeded into fresh culture flasks according to the optimum split ratio of the cell line (a ratio of 1:3 was optimal for MCF-7, T47D and MDA-MB 231 cells, and a ratio of 1:4 for MDA-MB 468 and HT1080 cells).

3.3.1.4 Cell Freezing

Subconfluent cultures were removed from flasks by trypsinisation, centrifuged to yield a cell

pellet and resuspended in freezing medium (50% DMEM, 2 mM L-Glutamine, 40% FBS, 10%

DMSO). Freezing was carried out gradually by transferring the ampoules to a polystyrene box

and cooling at -70°C for 10 hrs then transferring to a liquid nitrogen bank for longer term

storage.

3.3.1.5 Culture of Cells under Serum-Free Conditions

Growth of the cell line was established routinely in serum-supplemented conditions in culture

vessels or on glass coverslips coated with poly-d-lysine (5 μg/ml). Prior to the experiment

(determined by the degree of confluence of the culture), the medium was removed and the

cultures washed twice, gently, in serum-free DMEM. The cells were then maintained in serum-

free DMEM for 24 hrs at which point the medium was again changed and the required

conditions for the experiment instituted.

3.3.2 Invasion Assays

The invasion assay used was based on the method described by Albini et al (1987). The basic

assay involves construction of a barrier using 12 mm cell culture inserts supporting an 8μm

pore polyethylene terephthalate track-etched (PET) membrane. First the lower surface of the

membrane was coated with 10μg/ml fibronectin in PBS for 30 mins, and the inserts allowed to

dry in a laminar flow hood for 1 hr. The upper surface of the membrane was coated with

Matrigel using pre-cooled pipettes and tubes, having pre-cooled the inserts. The Matrigel

barrier was then set by incubating the inserts in their multiwell plates at 37°C for 2 hours. Cells

grown under different conditions were removed from the culture plates by scraping, and 5×10^5

cells were resuspended in 500μl DMEM plus L-Glutamine (with or without any additive to be examined). The cells were seeded onto the Matrigel membrane and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. The assay was terminated by removing the cells on the upper surface of the membrane under direct observation using a cotton-tipped swab. The inserts were then fixed in 10% formalin, stained with haematoxylin and eosin and the membranes removed from the insert using a scalpel and mounted onto a glass slide. Cells from 10 high power fields, each covering $0.25mm^2$ area, were counted. In order to take account of any differences in rates of cell growth during the experiment, the invasion assays were always set up in duplicate, and for every case, one membrane was processed to maintain the cells on the upper surface (i.e. the inoculated cells) and cell counts were performed on these in an identical manner. Cells adherent to the lower well chamber were also counted in 10 random high power fields. This allowed calculation of an invasion index on the basis of the following formula:

$$\frac{\text{no. cells lower membrane + no. cells in lower well}}{\text{total no. cells (upper membrane + lower membrane + lower well)}} \times 100\ \%$$

Initial studies were carried out to optimise the conditions of the assay using the normal fibroblast cell line F518 as a negative control (i.e. a non-malignant cell line that should not invade in this assay), and the human fibrosarcoma cell line HT 1080, which is reported to be highly invasive in similar in-vitro invasion assays (Kramer et al, 1986; Noel at al, 1991). Several parameters were tested:

(i)   *chemotactic gradient:* to facilitate movement of cells across the Matrigel barrier, the

rate of invasion observed with different concentrations of fibroblast CM (derived from growth

of F518 fibroblasts in serum-free DMEM) versus DMEM alone was analysed.

(ii)   *concentration of Matrigel barrier:* the efficiency of invasion was assessed using

Matrigel barriers of 200 µl of final protein concentrations 1µg, 5 µg, 10 µg, 20 µg, 50 µg and

100 µg.

(iii)   *length of invasion assay:* to determine the optimal time period, assays were terminated

after 24 hrs, 48 hrs and 72 hrs.

Each assay was repeated, in duplicate, at least three times. For antibody inhibition

assays, the cells were pre-incubated with excess monoclonal antibody (25 µg/ml), or with

mouse IgG as a control, in DMEM at room temperature for 1 hour with rotation. The invasion

assay was then carried out as described above, with addition of the appropriate antibody to the

medium in the assay.

3.3.3 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

3.3.3.1 mRNA Extraction

mRNA was extracted from cells using oligo (dT) Dynabeads. The cells were harvested by

trypsinisation and following centrifugation a cell count was performed. For each extraction, 5 x

10^5 cells were resuspended in lysis/binding buffer, mixed by repetitive pipetting and incubated

on ice for 10 mins. To prepare the oligo (dT) beads, 30 µl of beads were removed from the

storage buffer, pelletted in the MPC and resuspended in 30 µl lysis/binding buffer. Lysates

were then mixed gently with the beads and allowed to anneal for 10 mins on ice. The beads

with bound mRNA were collected in the Magnetic particle Concentrator (MPC) and washed

twice in washing buffer with SDS and three times in washing buffer without SDS. The final

bead pellet was resuspended in 20 μl of RNase-free sterile water.

3.3.3.2 Reverse Transcription -Polymerase Chain Reaction

The 20 μl of bead-bound mRNA was divided equally into two sterile eppendorfs, one to be

reverse transcribed, the other to act as a negative RT control. Reverse transcription reactions

were carried out at 42°C for 1 hr in a total volume of 25 μl containing 1 x AMV RT buffer, 10

mM each of dNTPs, 28 U RNasin and, in the positive RT reactions, 5 U of AMV RT enzyme.

Following reverse transcription the cDNA can be stored at 4°C, however, in most cases in this

study it was used immediately for PCR.

PCR amplification was carried out using 2 μl of the cDNA as a template in each

reaction. The reaction mixture contained in a final volume of 50 μl: 2 μl of template, 1 x AJ

PCR buffer, 10 pmoles each of forward and reverse primers. The reactions were overlaid with

sterile mineral oil and after an initial denaturation step of 5 mins at 95°C, Taq polymerase was

added at 1 U per reaction. In each case a parallel negative RT reaction was set up, and a

positive control (HT 1080 cells) was included in each run. For each set of primers, initial

reactions were carried out at a range of annealing temperatures (AT) using cDNA from

HT1080 cells to establish the optimal AT, and to confirm amplification of the appropriately

sized product. Thereafter, reactions were carried out at the optimal AT, which was 59°C for

MMP-2, TIMP-1 and TIMP-2, and 60°C for all other primer sets. The following amplification

programme was employed for all reactions:

Denaturation: 5 mins at 95°C followed by a hold at AT as Taq polymerase was added

Amplification: annealing at optimal AT for 1 min; extension at 72°C for 1 min (1 cycle)

denaturation at 95 °C for 1 min; annealing at optimal AT for 1 min;

extension at 72°C for 1 min (n cycles).

Final Extension: a final extension stage at 72°C for 7 mins.

All PCR in this study was performed on a Hybaid thermal cycler.

3.3.3.3 Agarose Gel Electrophoresis

The RT-PCR products were run on a 2% Agarose MP gel (2% w/v Agarose in 1 x TAE buffer)

to check for the presence or absence of specific product, and also to assess for any

contaminating products. Samples of PCR product were mixed with 5 x GLB and loaded onto

the gel which was run at a constant voltage of 75V. A DNA size marker was run on each gel.

Following electrophoresis the gel was stained with 2 μg/ml ethidium bromide for 30 mins and

visualised on a UV transilluminator.

3.3.3.4 ELOSA Quantitation of PCR Products

In experiments where information on the relative change in level of gene expression was

required ELOSA quantitation of the PCR products was performed, as outlined in fig. 3.1.

- Probe preparation: oligonucleotide probes designed to detect specific PCR products were

  labelled with digoxigenin according to the method described in section 2.3.5.2.

- Sample preparation: quantitation of PCR product must be done over the linear phase of the

  reaction, therefore to determine this stage, during the PCR 5 μl aliquots of product were

  removed from each reaction at 20, 25, 30 and 35 cycles, and each of these samples was

analysed in the ELOSA assay. Once this had been established, future quantitation was performed on the appropriate cycle-time sample.

- ELOSA: CovaLink multiwell plates were coated with biotin by incubation overnight at room temperature with 20µg/ml n-hydroxy-succinyl-biotin in sterile PBS. The plates were then washed three times in Cova buffer using a primed Wellwash 4 Mk 2. The wells were then incubated with avidin (50µg/ml in Cova buffer) for 30 mins with agitation. The plates were aspirated and washed three times in Buffer 2. To block non-specific binding of DNA, the plates were incubated in 3% PBS/BSA for 15 minutes at room temperature, with agitation. The PBS/BSA was aspirated and 1 µl of each sample, diluted 1:10 in sterile water, was added to the appropriate well followed by 90 µl of 3% PBS/BSA. The samples were incubated for 1 hr at room temperature with agitation, allowing the biotinylated product to bind to the avidin-coated plate. The non-biotinylated, reverse strand PCR-product was denatured from the biotinylated forward stand by addition of 100µl 0.25 M NaOH to each well. Incubation for 10 minutes at room temperature was followed by aspiration and washing with Buffer 2. The plates were then incubated with 100µl digoxigenin-labelled oligonucleotide probe diluted to a final concentration of 0.2 pmol/100µl Rapid Hybridisation Buffer for 1.5 hours at 42°C. After hybridisation, the plates were again aspirated and washed in Buffer 2, and incubated with alkaline phosphatase-labelled anti-digoxigenin diluted 1:500 in PBS/BSA for 30 minutes at room temperature with agitation. After a final wash the plates were incubated in the chromogen para-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine pH 9.8, for 2.5 hrs at 37°C. The OD was read at 405 nm with a differential of 630 nm on a multiwell plate reader (Denley Instruments Ltd., Billingshurst, UK).

Amplification

Adsorption

Denaturation

Hybridisation

DIG

Detection

DIG

Absorbance
at 450 nm
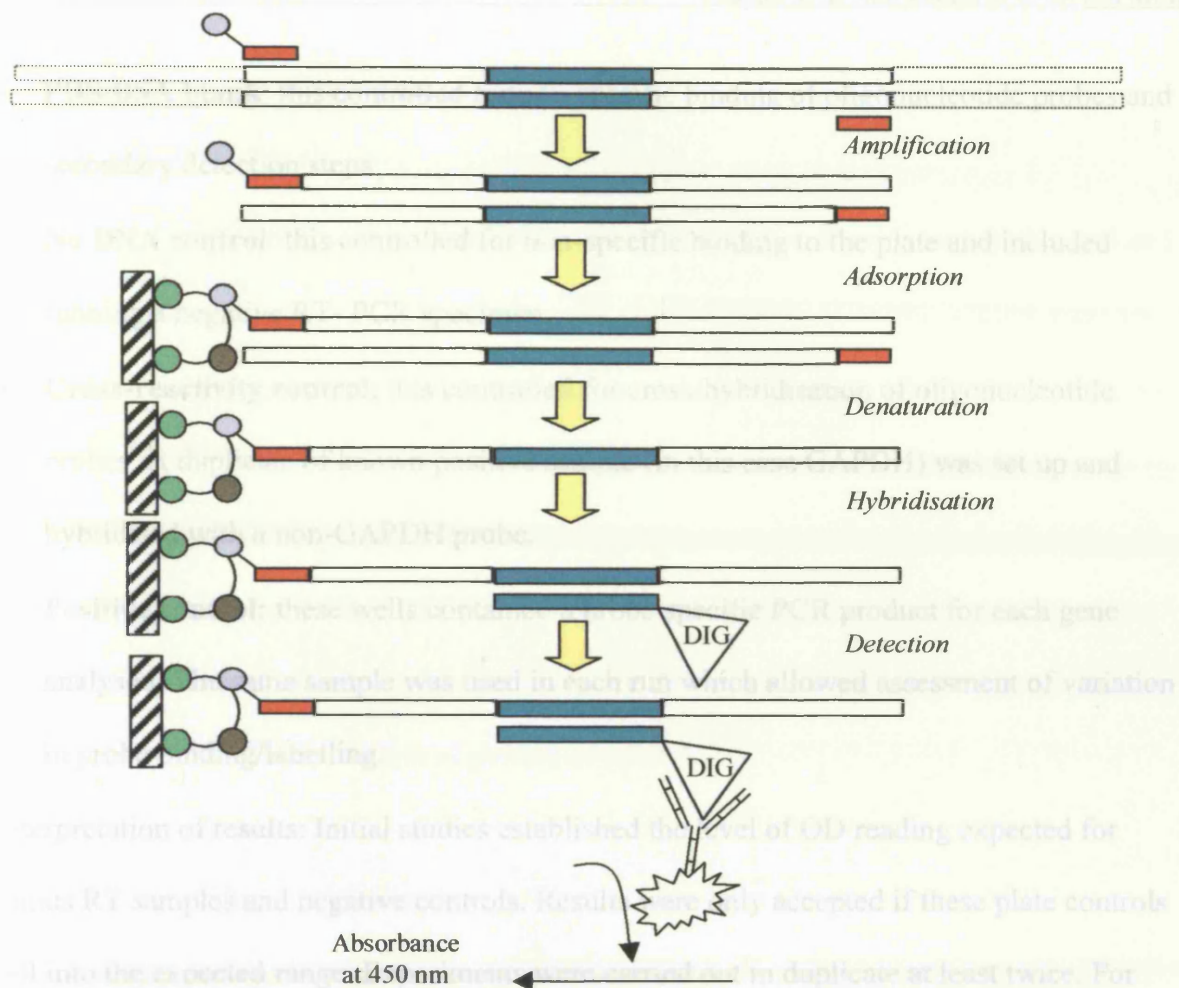
Fig.3.1: Schematic Outline of PCR-ELOSA.
PCR product is generated using biotinylated forward primer and captured on a streptavidin-coated plate.
Single-stranded DNA fragments are achieved by denaturation with NaOH. A labelled probe is hybridised
to the complementary sequence followed by colorimetric detection.

- Controls: each sample was set up in duplicate, and the following controls were included in each run:
    - **Substrate only blank (SOB):** this controlled for level of background chromogen binding. The wells were coated with biotin-NHS and avidin, but no sample was added, no detection steps were carried out; only chromogen development was performed.

- **PBS/BSA blank**: this controlled for non-specific binding of oligonucleotide probes and secondary detection steps.

- **No DNA control**: this controlled for non-specific binding to the plate and included running a negative RT- PCR specimen.

- **Cross-reactivity control**: this controlled for cross-hybridisation of oligonucleotide probes. A duplicate of known positive sample (in this case GAPDH) was set up and hybridised with a non-GAPDH probe.

- **Positive control:** these wells contained a probe specific PCR product for each gene analysed. The same sample was used in each run which allowed assessment of variation in probe binding/labelling.

- Interpretation of results: Initial studies established the level of OD reading expected for minus RT samples and negative controls. Results were only accepted if these plate controls fell into the expected range. Experiments were carried out in duplicate at least twice. For each sample, the mean of 4 OD readings for each gene, corrected for minus RT readings, was calculated. The ratio of this to the mean OD reading for GAPDH in that sample was taken as a measure of the *relative* amount of the different mRNA species (measured in arbitrary units).

Initial studies were carried out to test the reproducibility of this technique using mRNA extracted from two separate cultures of HT 1080 cells. RT-PCR was performed on both cell populations for GAPDH and MMP-2 and ELOSA quantitation was carried out for each sample in triplicate. This allowed assessment of reproducibility both within the same sample and between samples.

3.3.4 Culture of Cells Under Different Conditions

3.3.4.1 Addition of TGF-β1

After establishment of cultures in serum-free conditions for 24 hrs, the media was changed and

cultures set up in the presence of 10 ng/ml TGF-β1 plus 0.1% BSA. Control cultures were set

up in which 0.1% BSA in an equal volume of serum-free DMEM in place of TGF-β1. Cultures

were incubated for 48 hrs at which time the media was removed, centrifuged to remove any

debris and frozen in 1 ml aliquots at -70°C. The remaining cells were washed in cold PBS and,

if on coverslips, fixed in acetone at 4°C for 10 mins for immunocytochemistry. In other cases,

the cells were removed from the flasks or wells by mechanical scraping, centrifuged at 300g,

and the cell pellet used for extraction of protein or mRNA.

3.3.4.2 Culture at Different Cell Density

Cells from established cultures grown in serum-supplemented conditions were harvested by

trypsinisation and after centrifugation the resulting cell pellet was resuspended in 1 ml of

serum-free DMEM and a viable cell count performed. Cells were then seeded into the wells of

6-well plates at concentrations of 5×10^5 and 1×10^4, respectively, and allowed to establish in

serum-supplemented conditions for 24 hrs, after which the media was changed for serum-free

DMEM, and the cultures incubated for 48 hrs. Cultures were terminated by removal of the

conditioned media, which was centrifuged and stored at -70°C for future analysis. The cells

were washed in cold PBS and removed from the wells by mechanical scraping, as described

above.

3.3.4.3 Antibody Blocking Studies

For antibody inhibition assays, prior to plating the cells into wells they were incubated with excess concentrations (25 µg/ml) of the appropriate blocking antibody in a small volume (500 µl) of DMEM at 4°C for 30 mins with rotation. The cells were then plated initially in serum-supplemented, and after 24 hrs, serum-free DMEM containing excess blocking antibody. Two sets of antibody inhibition assays were performed:

- effect of anti-α6 integrin and anti-β4 integrin on cell invasion: in these experiments, following prior incubation with antibodies, further blocking antibodies were added to the medium in the assay.

- effect of anti-E-Cadherin on invasion and on MMP expression: in the invasion assays the blocking antibody was added to the medium as described above. To examine the effect of blocking E-Cadherin on MMP expression, the cells were plated onto 1 µm pore filters supported by a frame which was then inserted into the well of a 12-well plate. The antibody was added to the medium in both the upper and lower chambers, allowing access to both the basal and upper aspect of the cell monolayers.

3.3.5 Immunocytochemistry on Cell Lines

3.3.5.1 Single-Labelling Immunofluorescence

Cells for immunocytochemical analysis under the different culture conditions outlined in section 3.3.5 were plated on 13 mm (thickness 0) glass coverslips coated with 5 µg/ml poly-d-lysine. At the termination of the experiment the cells on the coverslips were fixed in acetone at 4°C for 10 mins. In the case of plectin/HD1 antibody, a range of different fixation procedures was evaluated. The cells were then washed in two changes of PBS and analysed for their expression of cell adhesion molecules and cytoskeletal proteins using an indirect

immunofluorescence technique. The coverslips were incubated in 20% Normal Rabbit Serum

(NRS) for 30 minutes to block non-specific binding. This was tipped off the coverslips and the

primary antibodies, diluted in 20% NRS/PBS, applied for 2 hours at room temperature. Initial

studies determined the optimal concentration of antibodies, and the final dilutions used are

outlined in Table 3.2. Following thorough washing in PBS, the coverslips were incubated with

FITC labelled rabbit anti-mouse IgG, diluted 1:50 in PBS, for 1 hour at room temperature in the

dark to prevent photobleaching. After washing, the coverslips were air dried and mounted onto

glass slides in antifade mountant (Citifluor). The presence and pattern of staining observed with

each antibody was recorded using confocal laser scanning microscopy.

Each series of culture experiments and immunocytochemical staining was performed in

duplicate at least three times. Controls for non-specific binding i.e. cells incubated with NRS in

place of primary antibody, and acetone-fixed cryosections of normal breast tissue, were

included as negative and positive controls, respectively, in each run.

3.3.5.2 Dual-Staining for Integrins and Actin Cytoskeleton

Cells grown on coverslips were washed in cold PBS then permeabilised and fixed by sequential

incubation in ice-cold 2-[N-Morpholino]ethanesulfonic acid (MES) buffer for 1 min, washing

in PBS and addition of 3.7% formaldehyde in PBS for 15 mins at room temperature. Other

experiments were carried out after fixing cells in acetone at 4°C for 10 mins in place of

formaldehyde. The cells were then washed in PBS and incubated in 20% NRS in PBS for 30

mins to block non-specific binding. Indirect immunofluorescence for integrin subunits was

carried out as described above, and following the final wash stage, the cells were then

incubated in TRITC-phalloidin for 30 mins at room temperature. All procedures involving the

use of phalloidin were performed in a fume hood, wearing face mask and two pairs of gloves.

A range of dilutions of TRITC-phalloidin was evaluated, from 1:100 to 1:10 (final

concentration of 0.067 µg/ml to 6.7 µg/ml). After thorough washing in PBS, with collection of

all liquid waste in a labelled container for appropriate disposal, the coverslips were mounted

onto glass slides using Citifluor mountant, and viewed under the confocal microscope.

3.3.5.3 Cytoskeletal Solubilisation Procedure

For solubilisation, cells grown on coverslips were washed in PBS and then treated with a High

Salt Buffer (HSB; appendix 1) for 15 mins at room temperature, according to the method

described by Gomez et al (1992). After washing in PBS the cells were then fixed and stained as

described in sections 3.3.6.1 and 3.3.6.2.

Table 3.2: Dilutions of Primary Antibodies used for Immunofluorescence

ANTIBODY	DILUTION
All integrin antibodies	1: 200
E-Cadherin	1: 50
Vinculin	1: 20
Vimentin	1: 200
EMA	1: 500
CK14	1: 5
CK18	neat
ER	1: 100
EGFR	1: 100
Plectin/HD1	neat; 1:10, 1:50, 1:100

3.3.6 Western Blotting

Protein was extracted from cell lines grown under different conditions and Western blot analysis carried out for $\beta 4$ integrin according to the method described in section 2.3.1.

3.3.7 Zymography

Conditioned medium (CM) was harvested from cell lines cultured under different conditions and subjected to zymographic analysis according to the method described in section 2.3.4. CM from HT 1080 cells was used as a positive control and/or purified pro-MMP-2 and pro-MMP-9. To assess the level of MMP-2-activating potential, 1.0 µg of purified recombinant pro-MMP-2 was added to cultures under serum-free conditions and incubated for 24 hrs. After this time the CM was harvested and subjected to substrate gel zymography.

3.3.8 Transmission Electron Microscopy

For examination of cells by electron microscopy, cells were cultured on 1 µm pore polyethylene membranes coated with 5 µg/ml poly-d-lysine. After the culture period, cells were washed in PBS and the cells on their membrane fixed in 4% buffered glutaraldehyde pH 7.4 at 4°C for 24 hrs. Cells were processed, embedded in resin and ultrathin sections cut by the members of Electron Microscopy in the Department of Pathology, Leicester Royal Infirmary. Briefly, membranes were cut into small strips and the specimens were post-fixed in 1% osmium tetroxide for 1 hr at room temperature, washed in Sorenson's buffer and dehydrated through graded alcohols. Samples were infiltrated with 1:1 resin and acetone mixture for 30 mins at 37°C and then neat resin for 1 hr before being transferred to fresh resin, orientated in a BEEM capsule and allowed to polymerise overnight at 60°C. Semithin sections were cut and stained with 1% toluidine blue in 1% borax to check histology and orientation. When appropriate areas

were located, ultrathin sections were cut to a thickness of between 90 - 120 nm, picked up onto

200 mesh copper grids and stained with a saturated solution of uranyl acetate and Reynolds

lead citrate and examined using a Jeol 100CX transmission electron microscope at an

accelerating voltage of either 80 or 100 Kv.

3.4 Results

3.4.1 Invasive Behaviour of Breast Cancer Cell Lines

3.4.1.1 Optimisation of Invasion Assay

Using normal fibroblasts (F518) and fibrosarcoma (HT 1080) cells as negative and positive

controls respectively, a number of parameters were evaluated to optimise the invasion assay to

discriminate between invasive and non-invasive cells.

(i)   *chemotactic gradient:* invasion assays were performed over 48 hrs using barriers coated

with the lowest concentration of Matrigel (1 µg/filter) with varying ratios of fibroblast CM:

serum free DMEM placed in the lower well of the chamber. In the absence of CM, there was no

movement of cells - either F518 or HT 1080 - across the membrane (fig.3.2). With addition of

CM to the lower chamber there was a dramatic effect on invasion by HT 1080 cells, rising from

no significant invasion to a mean invasive index of 72% with 0.5:1 CM : DMEM. There was no

further increase in invasion with increasing amounts of CM. Normal fibroblasts also invaded

through the Matrigel barrier in the presence of CM, with a mean invasion index of 3%.

(ii)   *concentration of Matrigel barrier:* to discriminate between high and low invasive cells

adequately, a range of concentrations of Matrigel was applied to the membranes and invasion

indices compared between F518 and HT 1080 cells (assays carried out over 48 hrs in the

presence of 1:3 CM : DMEM). Figure 3.3 demonstrates that the extent of invasion by the cells

is dependent on the concentration of the Matrigel barrier. At the lowest concentration of

Matrigel (1 µg/filter), HT 1080 cells gave an invasion index of 72%, but the F518 cells also

exhibited invasive activity with a mean invasion index of 3%. When the Matrigel barrier was

increased to 5 µg/filter, the F518 cells no longer exhibited invasive activity over 48 hrs, but a

high invasion index (mean of 61%) was still obtained for HT 1080 cells. The invasion by HT

1080 cells decreased progressively with increasing Matrigel concentration - 42% at 10 µg/filter,

170
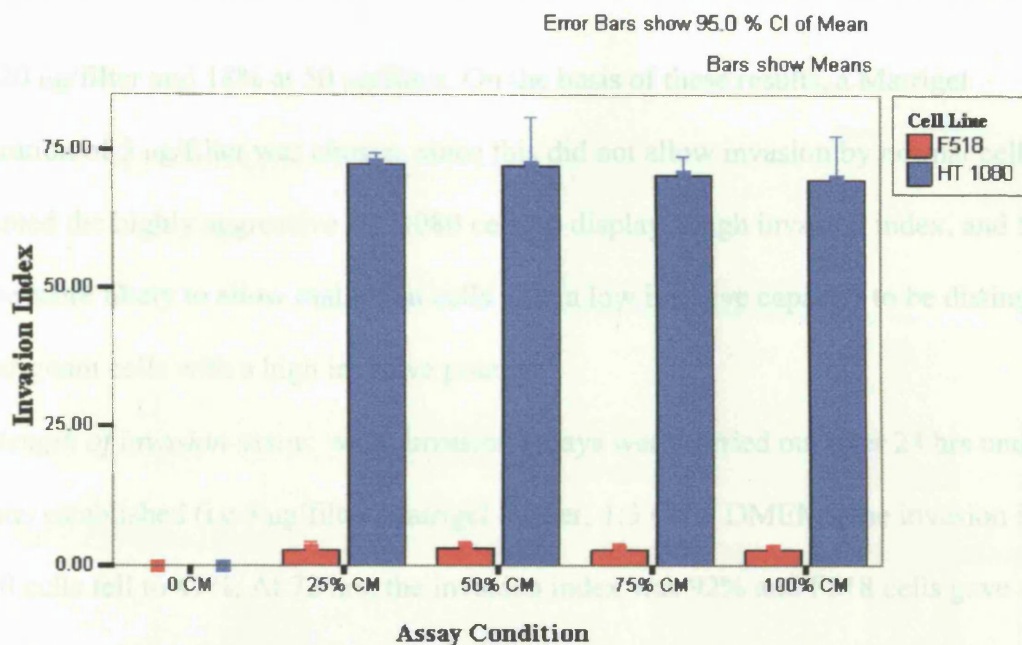
Fig. 3.2: Effect of Fibroblast Conditioned Medium (CM) on Invasion.
x axis indicates proportion of CM to DMEM added to lower chamber of invasion assay
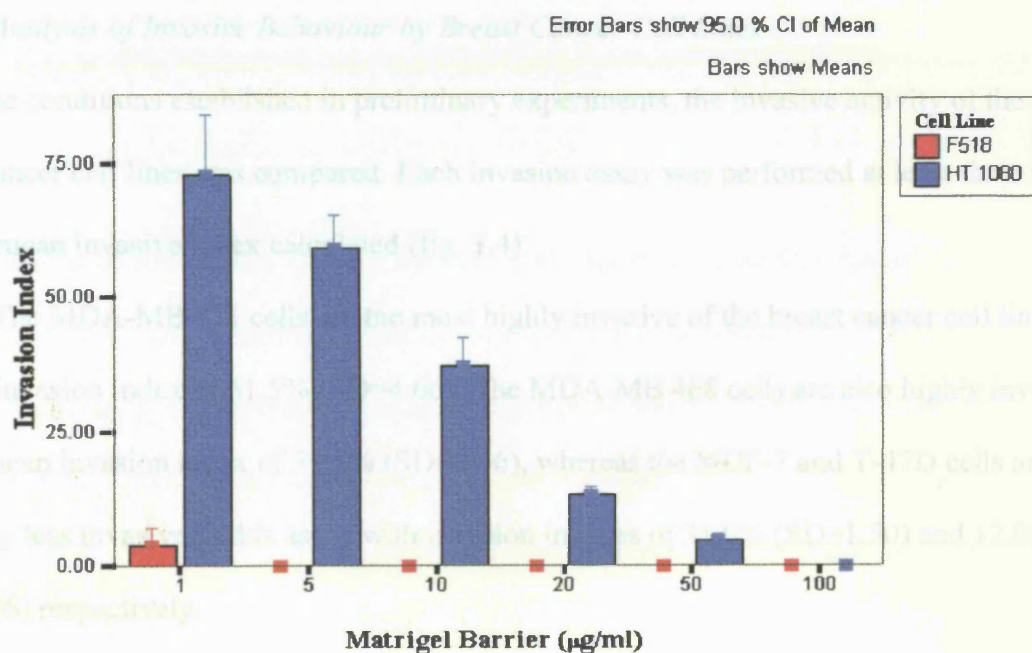
Fig. 3.3: Effect of Matrigel Barrier on Invasion.
x axis indicates concentration of Matrigel in µg/ml

31% at 20 μg/filter and 18% at 50 μg/filter. On the basis of these results, a Matrigel

concentration of 5 μg/filter was chosen, since this did not allow invasion by normal cells but

still enabled the highly aggressive HT 1080 cells to display a high invasion index, and thus

would be more likely to allow malignant cells with a low invasive capacity to be distinguished

from malignant cells with a high invasive potential.

(iii)   *length of invasion assay:* when invasion assays were carried out over 24 hrs under the

conditions established (i.e.5 μg/filter Matrigel barrier; 1:3 CM : DMEM), the invasion index for

HT 1080 cells fell to 49%. At 72 hrs, the invasion index was 92% and F518 cells gave an

invasion index of 3%. Since the requirement was for an assay in which non-malignant cells do

not invade, but where cells of low invasive potential can be distinguished from cells of high

invasive potential, 48 hrs was chosen as the optimum length for the assay.

3.4.1.2 Analysis of Invasive Behaviour by Breast Cancer Cell Lines

Using the conditions established in preliminary experiments, the invasive activity of the four

breast cancer cell lines was compared. Each invasion assay was performed at least three times

and the mean invasive index calculated (fig. 3.4).

The MDA-MB 231 cells are the most highly invasive of the breast cancer cell lines with

a mean invasion index of 61.5% (SD=4.66). The MDA-MB 468 cells are also highly invasive

with a mean invasion index of 51.8% (SD=2.46), whereas the MCF-7 and T-47D cells are

markedly less invasive in this assay with invasion indexes of 31.0% (SD=1.50) and 12.0%

(SD=1.96) respectively.
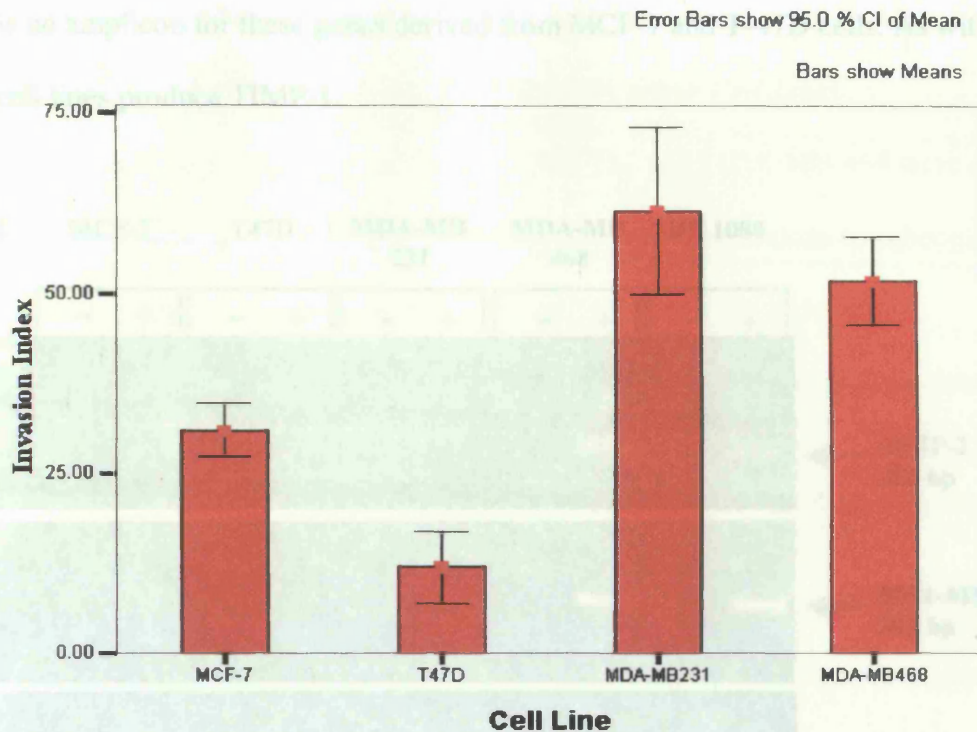
Fig. 3.4: Invasive Behaviour of Breast Cancer Cell Lines.
The bars represent the mean invasion index (MII) of at least three separate assays.

3.4.2 Pattern of MMP and TIMP Expression by Breast Cancer Cell Lines

Each of the cell lines was grown under control conditions (serum-free DMEM plus L-
Glutamine) and after 48 hrs harvested by trypsinisation and the mRNA extracted, reverse
transcribed and analysed for expression of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2
by polymerase chain reaction (PCR). cDNA extracted from HT 1080 cells was used as a
positive control for all genes analysed. Figure 3.5 shows the agarose gel electrophoresis of the
final PCR product (35 cycles of amplification) for each cell line. This demonstrates that only
MDA-MB 468 cells express MMP-2, whereas all express the inhibitor TIMP-2. The MDA-MB

173

231 and MDA-MB 468 cells express both MMP-9 and the activator of MMP-2, MT1-MMP,

but there is no amplicon for these genes derived from MCF-7 and T-47D cells. As with TIMP-

2, all the cell lines produce TIMP-1.
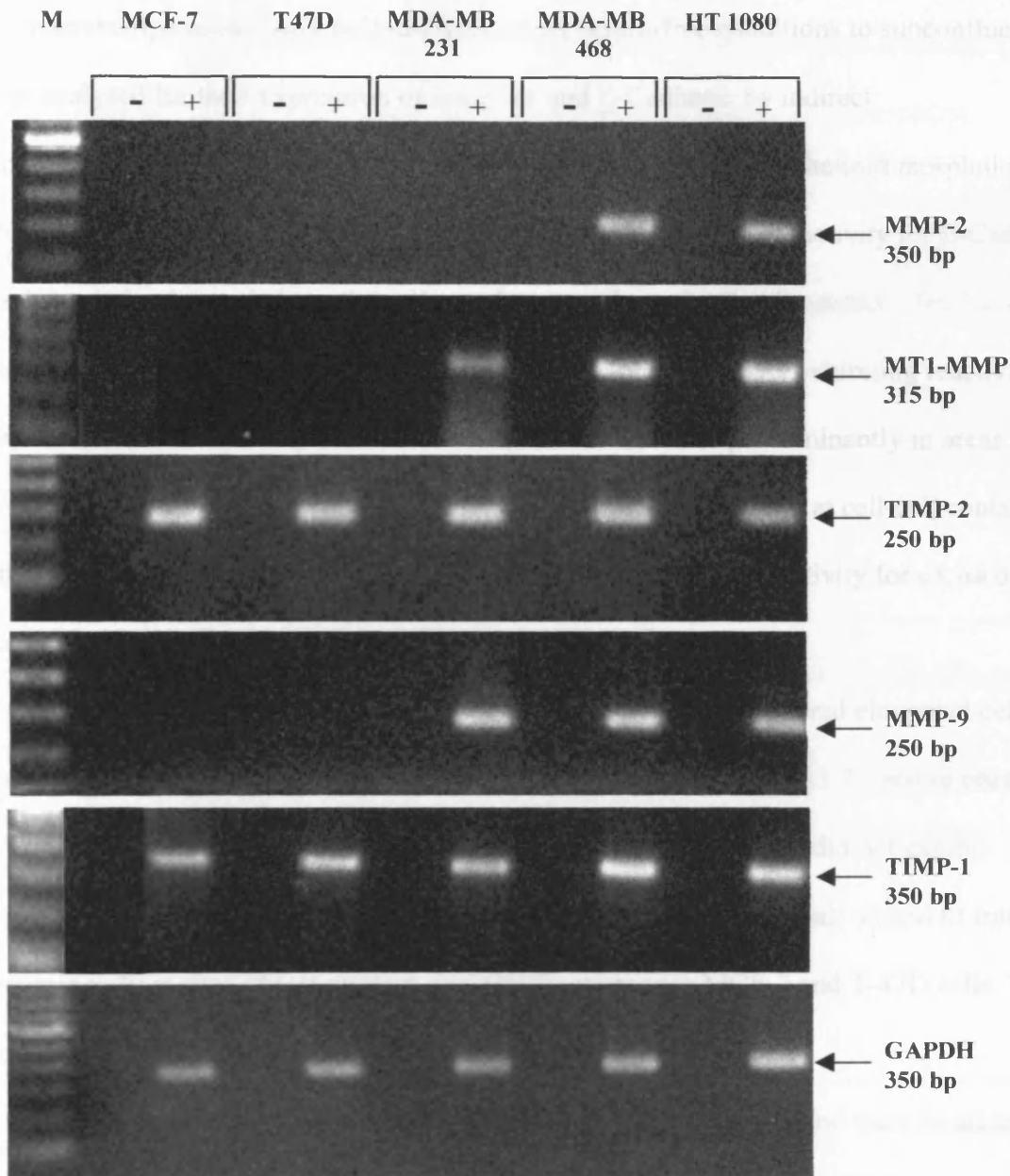
Fig. 3.5: Agarose Gel Electrophoresis of PCR Products for MMPs and TIMPs in Breast Cancer Cell Lines
HT 1080 fibrosarcoma cell line is positive control for each gene. M=Molecular weight markers; - Minus RT; +
Positive RT

174

3.4.3 Characterisation of Breast Cancer Cell Lines

3.4.3.1 Expression of Cell Adhesion Molecules by Breast Cancer Cell Lines

The breast cancer cell lines, MCF-7, T-47D, MDA-MB 231 and MDA-MB 468 were each

grown on coverslips coated with poly-d-lysine under serum-free conditions to subconfluence

and then analysed for their expression of integrins and E-Cadherin by indirect

immunofluorescence. Both the MCF-7 cells and T-47D cells had an epithelioid morphology

and grew as cohesive groups of cells. Both cell lines exhibited strong reactivity for E-Cadherin

with staining being located along the cell membrane at sites of cell-cell contact. They also

showed similar patterns of integrin expression (Fig.3.6), both cell lines exhibiting reactivity for

β1 integrin which was diffusely distributed along cell membranes predominantly in areas of

cell-cell contact. Staining for α2 and α3 integrins was similarly observed at cell-cell contacts,

though α2 reactivity was at a lower level. There was no evidence of reactivity for α5, α6 or β4

integrins.

MDA-MB 468 cells had a distinct morphology; although occasional elongated cells

were observed, the majority of the cells had a 'rounded' appearance (Fig.3.7), and in contrast to

MCF-7 and T-47D cells, they did not grow as cohesive sheets. The cells did not exhibit

membrane reactivity for E-Cadherin, but showed membrane staining for α2, α3 and β1 integrins,

most prominently at sites of cell contact, in a similar manner to MCF-7 and T-47D cells. There

was no evidence of reactivity for α5, α6 or β4 integrins.

The MDA-MB 231 cells displayed a characteristic morphology, and were small and

elongated, with just occasional cells exhibiting a more 'spread' phenotype. They grew as single

cells and did not form cohesive groups. This cell line demonstrated a different pattern of

staining for cell adhesion molecules compared to the other cell lines. They lacked reactivity for

E-Cadherin but there was strong punctate reactivity for β1 integrin at sites of cell-substrate

175

contact (Fig.3.8). The staining pattern for $\alpha 2$ integrin and $\alpha 3$ integrin was similar in intensity

and distribution to that of $\beta 1$ integrin. There was no reactivity for $\alpha 5$ integrin, however, cells did

show reactivity for $\alpha 6$ and $\beta 4$ integrin subunits. These integrin subunits were not strongly

expressed and had a more diffuse distribution than $\alpha 2$, $\alpha 3$ and $\beta 1$ integrins, but were again

observed in a focal, punctate distribution, particularly at the elongated cell extensions.

3.4.3.2 Expression of Other Markers by Breast Cancer Cell Lines

Each of the breast cancer cell lines were examined for their expression of a range of markers

which are characteristic of either luminal epithelial or myoepithelial cells in normal breast in-

vivo. The results are summarised in Table 3.3.

Table 3.3: Markers Expressed by Breast Cancer Cell Lines

Antibody	MCF-7	T47D	MDA-MB 468	MDA-MB 231
ER	+	+	-	-
EMA	+	+	+/-	-
CK14	-	-	-	+/-
CK18	+	+	+	+/-
Vimentin	-	-	+/-	+
EGFR	-	-	+	+

+ : positive   - : negative   +/- : weak positivity

MCF-7 and T47D cells show a similar pattern of reactivity, both being oestrogen receptor

positive, EMA positive and expressing the luminal type cytokeratins CK18. In contrast, both

MDA-MD 468 and MDA-MB 231 are oestrogen receptor negative, MDA-MB 468 are weakly

positive for EMA, whilst MDA-MB 231 are negative, and both express CK18. Both MDA-MB

Fig. 3.6: Morphology and Expression of Cell Adhesion Molecules in MCF-7 Cells
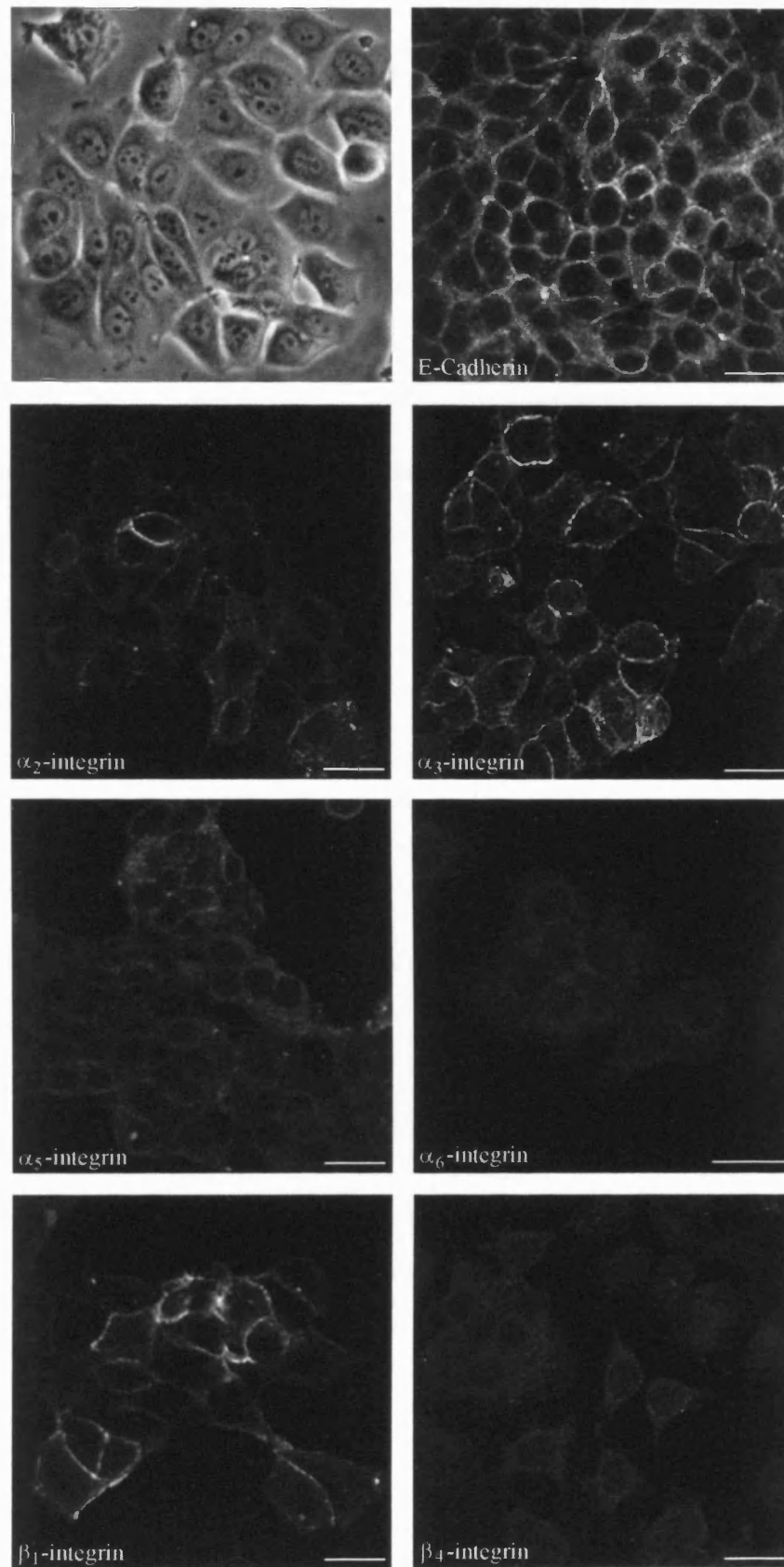
Fig 3.6 : Morphology and Expression of Cell Adhesion Molecules in MCF-7 Cells

(scale bars = 25 μm)

Fig. 3.7: Morphology and Expression of Cell Adhesion Molecules in MDA-MB 468 Cells
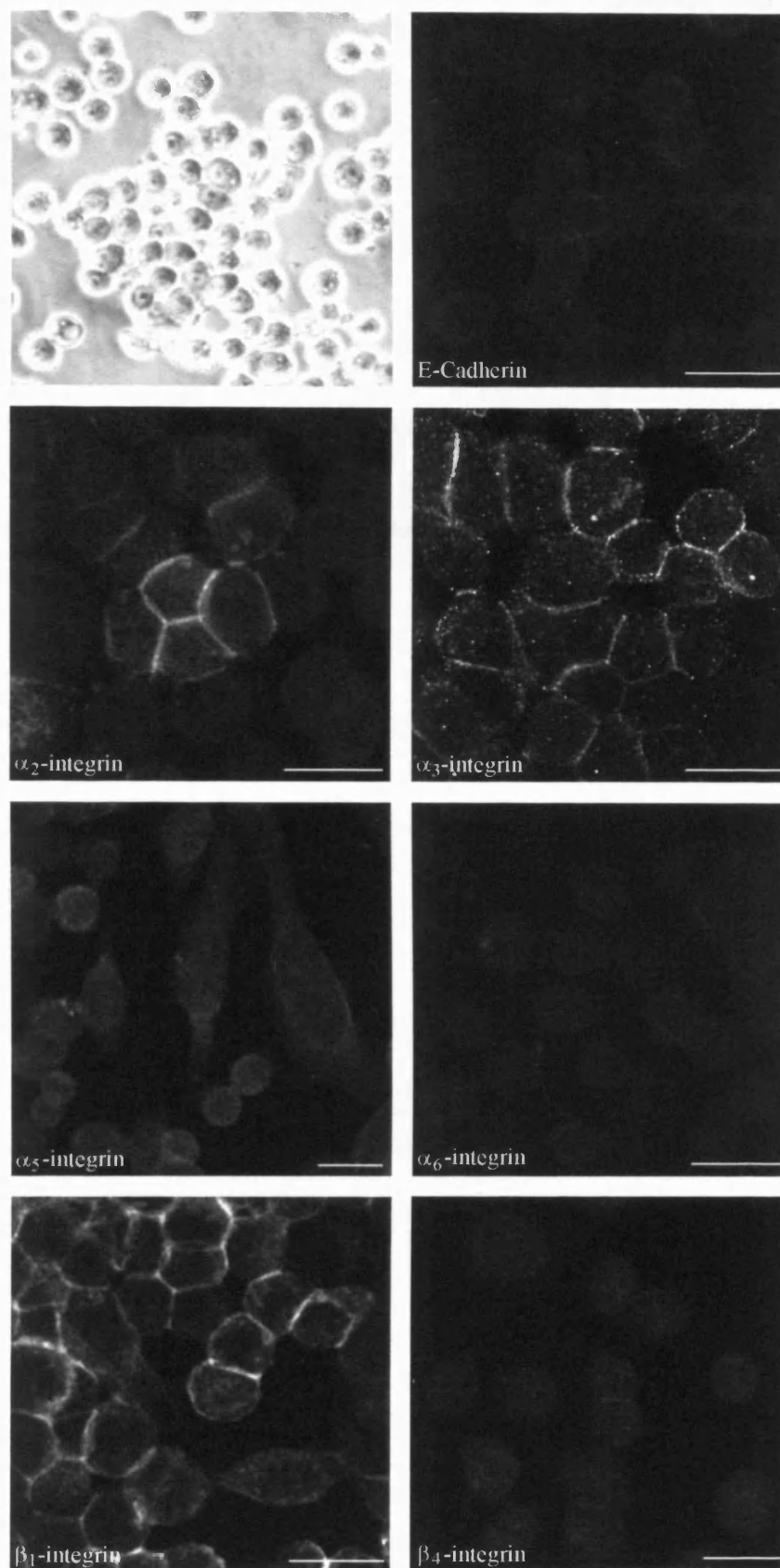
Fig 3.7 : Morphology and Expression of Cell Adhesion Molecules in MDA-MB 468 Cells

(scale bars = 25μm)

Fig. 3.8: Morphology and Expression of Cell Adhesion Molecules in MDA-MB 231 Cells
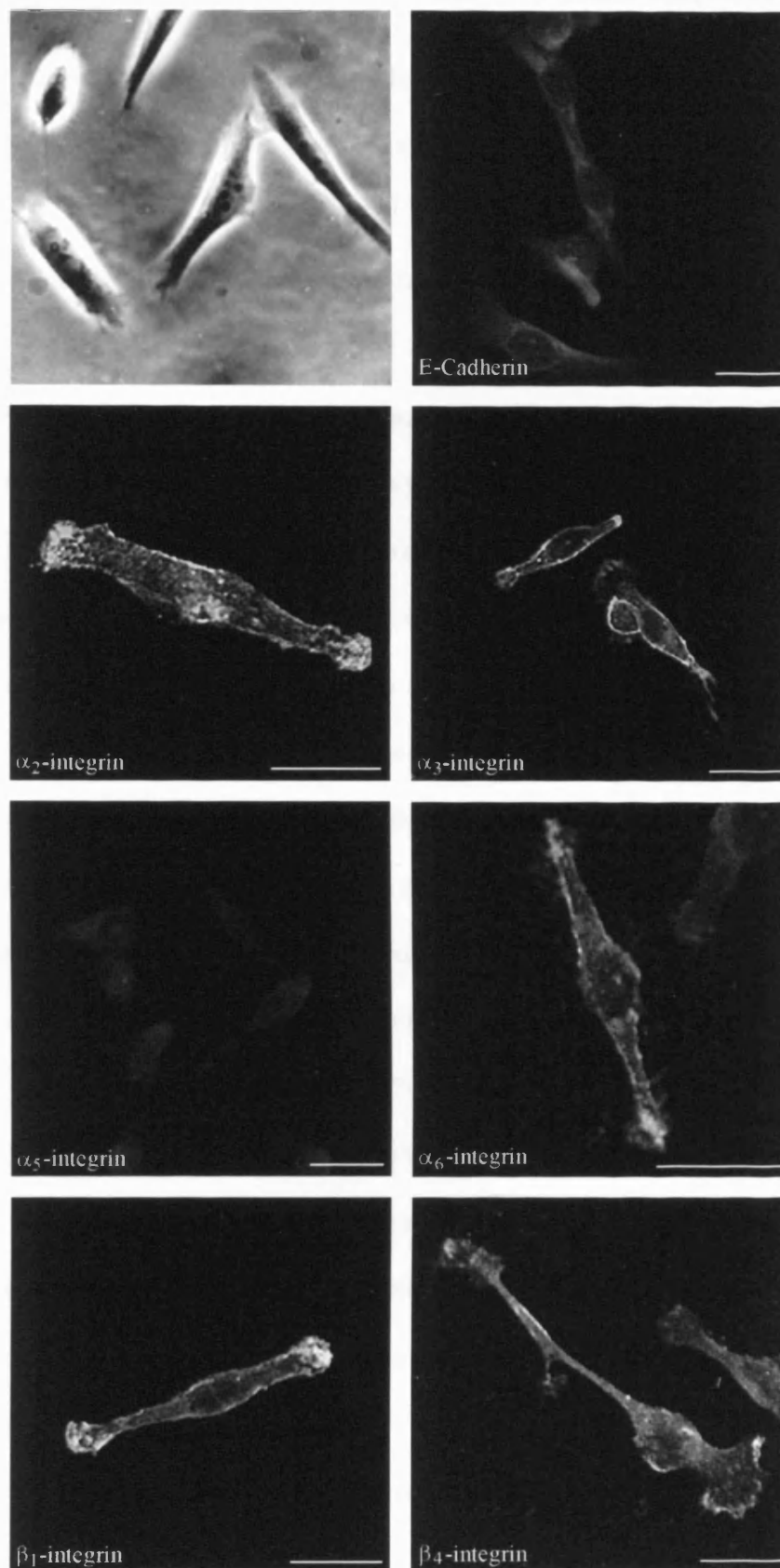
Fig 3.8 : Morphology and Expression of Cell Adhesion Molecules in
MDA-MB 231 Cells

(scale bars = 25μm)

468 and MDA-MB 231 express vimentin and show high level reactivity for EGFR, whilst

MDA-MB 21 cells also show weak positivity in some cells for CK14.

3.4.4 Relationship between E-Cadherin Expression, Invasive Behaviour and Expression of MMPs and TIMPs

From the baseline analysis of the breast cancer cell lines, it is evident that the two cell lines that

lack E-Cadherin - MDA-MB 231 and MDA-MB 468 - synthesise MMP-9, whilst the cell lines

that express E-Cadherin - MCF-7 and T-47D - do not produce this MMP. Since an association

between the infiltrating lobular phenotype - carcinomas which lack E-Cadherin - and

expression of MMP-9 was observed earlier in this study (Chapter 2), the relationship between

E-Cadherin and MMP-9 expression was investigated more directly using the breast cancer cell

lines. For functional E-Cadherin-mediated adhesion, cells must be maintained in contact with

one another to allow interaction of adjacent E-Cadherin molecules. Thus whilst MCF-7 and T-

47D cells strongly express E-Cadherin, when they are cultured at low cell density, with less

than 15% confluence, cell surface E-Cadherin is seen only at rare points of cell-cell contact

(fig. 3.9). In contrast, even at full confluence, MDA-MB 231 and MDA-MB 468 cells do not

exhibit cell surface E-Cadherin (fig. 3.9). This characteristic was exploited to examine the

influence of functional E-Cadherin-mediated adhesion on MMP-9 expression, whereby

expression of MMP-9 was compared in cells grown at low density versus the same cells at high

density (i.e. 85% confluence), and also at high density in the presence of blocking anti-E-

Cadherin antibodies. Expression was analysed at mRNA level, with zymography to assess the

level of enzyme activity. The effect of the different culture conditions on TIMP-1 mRNA

expression was also assessed.

Fig. 3.9: Expression of E-Cadherin by Cells Grown at Low and High Density
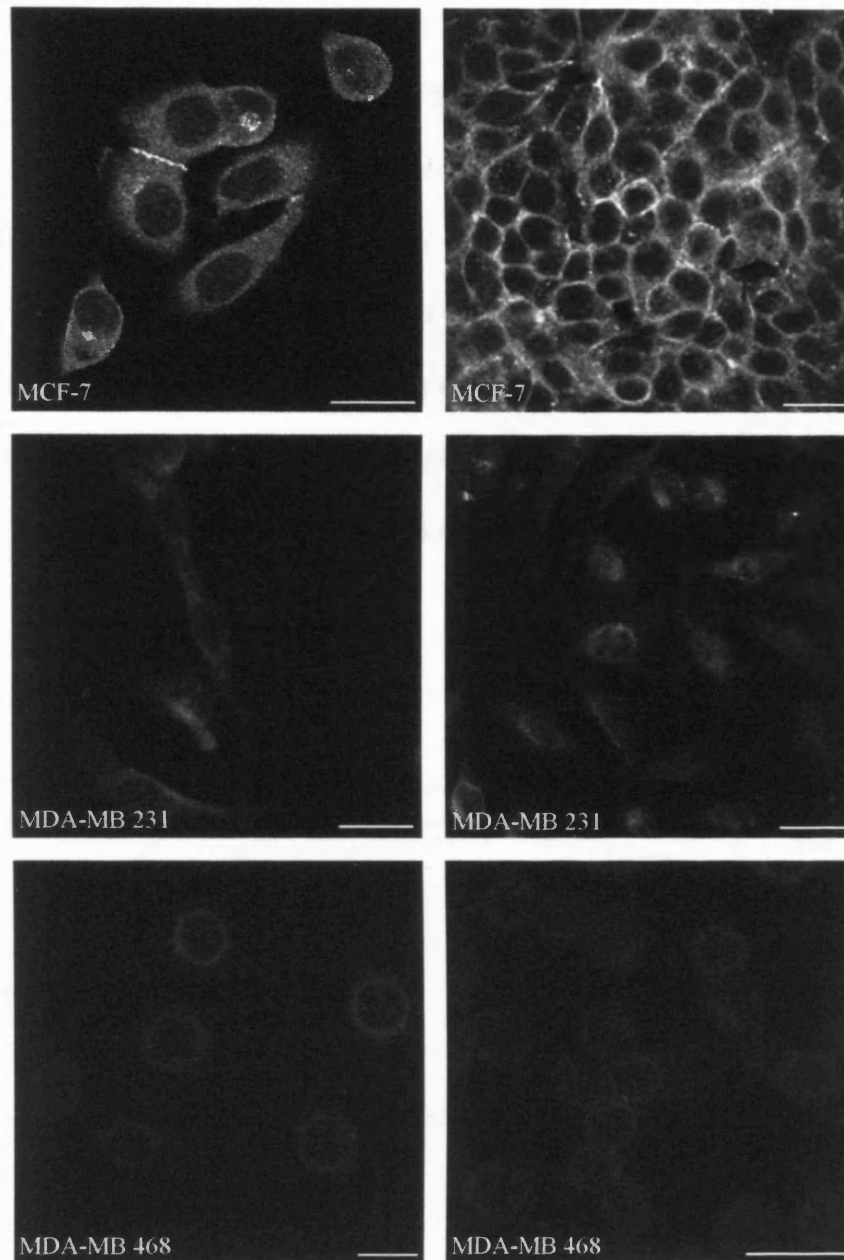
Fig 3.9 : Expression of E-Cadherin by Cells Grown at Low and High Density

(scale bars = 25μm)

3.4.4.1 Analysis of Level of Gene Expression by RT-PCR and ELOSA Quantitation

- Optimisation of Methodology

To establish the linear phase of the PCR amplification for GAPDH and for each of the genes under study, RT-PCR was carried out on mRNA derived from HT 1080 cells. Aliquots of the PCR were removed at 20, 25, 30, 35 and 40 cycles, and each analysed by ELOSA. The optical density reading (OD) was plotted against cycle number for each gene, and the linear range established. Figure 3.10 demonstrates that the PCR for all genes analysed is in the linear phase of the reaction up to 30 cycles but reaches the plateau phase beyond 30 cycles. All future ELOSA analysis was therefore carried out at 30 cycles of amplification.
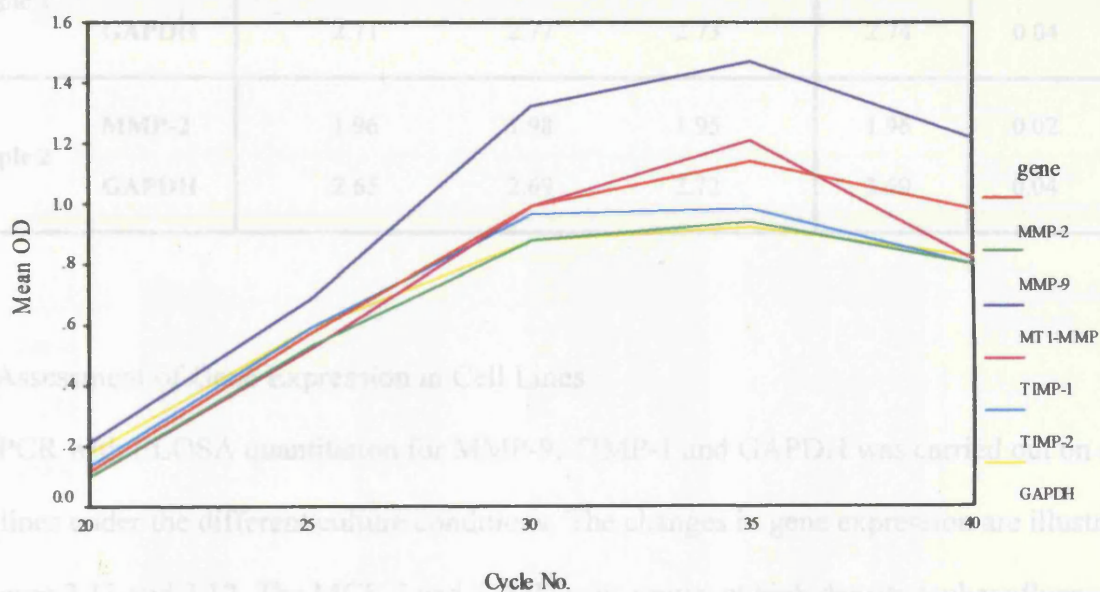
Fig. 3.10: Absorbance Reading for HT 1080 PCR Products in Relation to Cycle Number.

- Assessment of Reproducibility

To establish the level of reproducibility of RT-PCR-ELOSA, both within and between samples, mRNA was extracted from two separate cultures of HT 1080 cells. cDNA was generated from each sample and PCR with ELOSA quantitation was performed in triplicate on each sample for

182

MDA-MB 231 and MDA-MB 468 cells both expressed MMP-9 when grown at either low or high density. There was no significant change in the level of expression when these cells were cultured in the presence of anti- E-Cadherin antibodies.
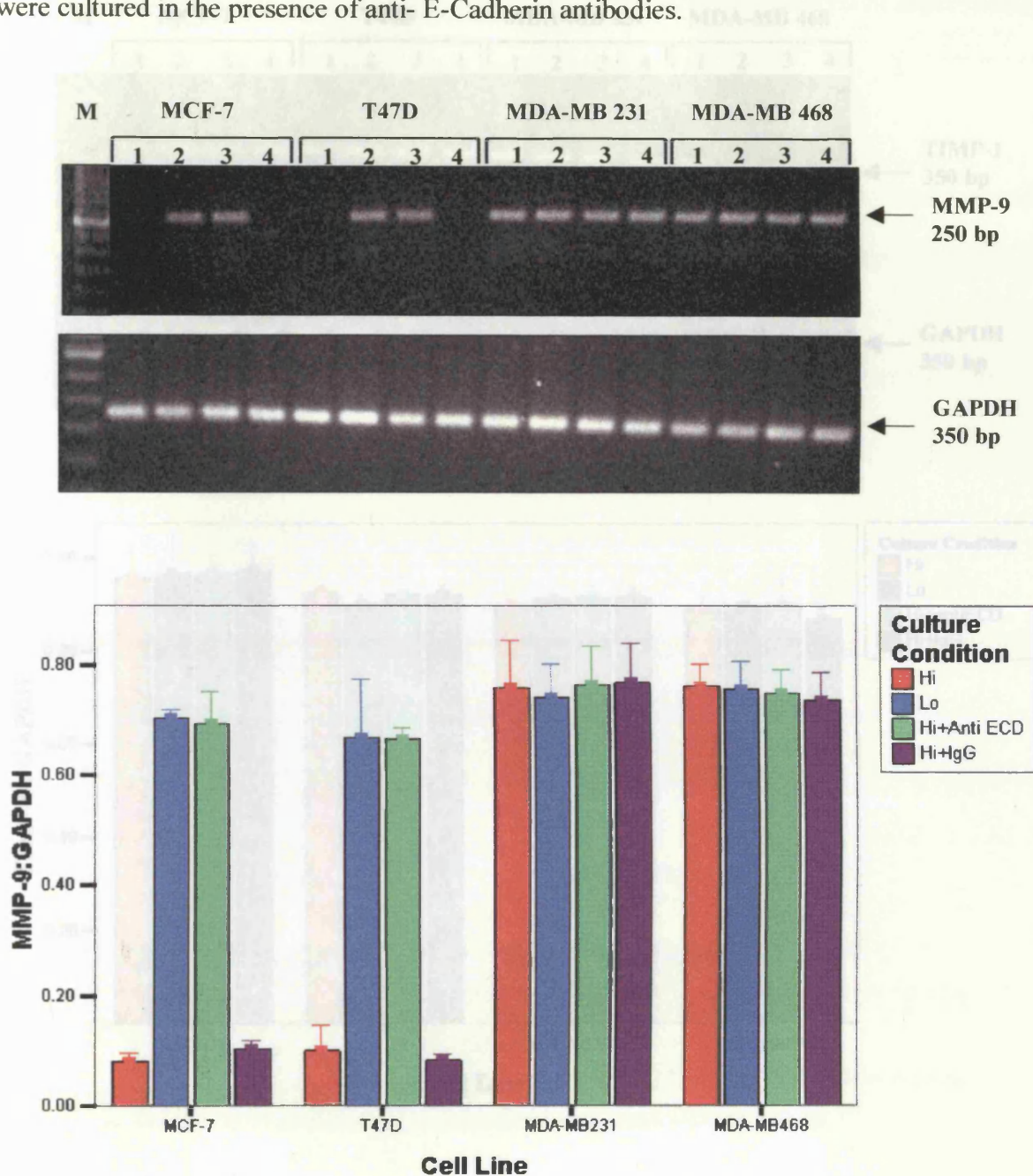
Fig. 3.11: Relationship between Expression of E-Cadherin and MMP-9 Gene Expression.
Top gel illustrates the PCR products after 30 cycles of amplification for MMP-9 and lower gel illustrates bands for GAPDH on the same samples at 30 cycles. All minus RT reactions were negative on gel electrophoresis. The bars on the graph represent mean MMP-9 : GAPDH OD (not corrected for minus RT /background readings) and error bars indicate 95% confidence limits. M=Molecular weight markers. Lanes: 1=Low density; 2=High density; 3=Anti-E-Cadherin antibodies; 4=Mouse IgG
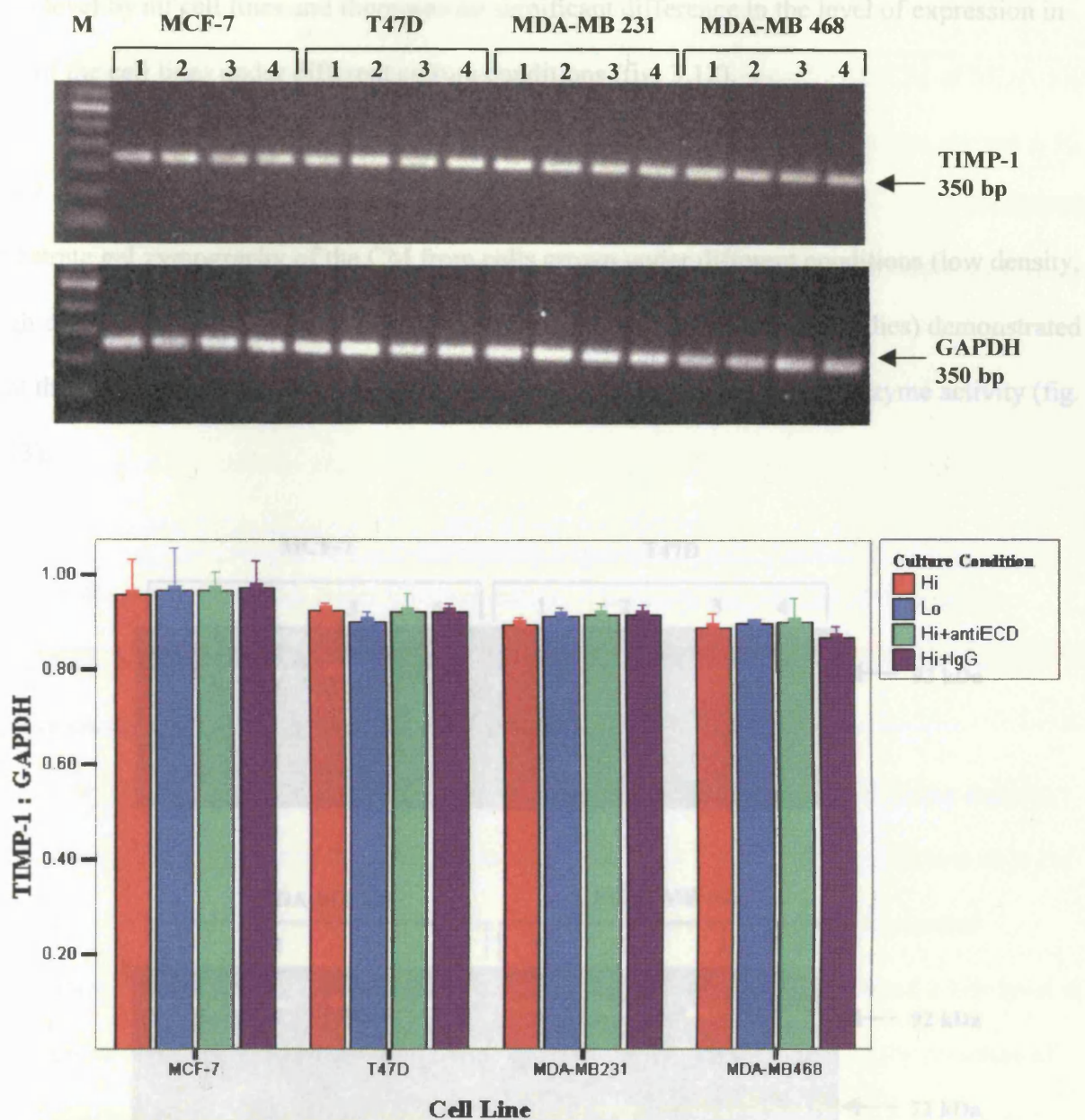
Fig. 3.12: Relationship between Expression of E-Cadherin and TIMP-1 Gene Expression.
Top gel illustrates the PCR products after 30 cycles of amplification for TIMP-1 and lower gel illustrates bands for GAPDH on the same samples at 30 cycles. All minus RT reactions were negative on gel electrophoresis. The bars on the graph represent mean TIMP-1 : GAPDH OD (not corrected for minus RT/background readings) and error bars indicate 95% confidence limits. M=Molecular weight markers. Lanes: 1=Low density; 2=High density; 3=Anti-E-Cadherin antibodies; 4=Mouse IgG

185

In contrast to the changes in level of MMP-9 expression, TIMP-1 was expressed at a high level by all cell lines and there was no significant difference in the level of expression in any of the cell lines under different culture conditions (fig. 3.12).

3.4.4.2 Analysis of Enzyme Activity

Substrate gel zymography of the CM from cells grown under different conditions (low density, high density, in the presence of anti-E-Cadherin antibodies or control antibodies) demonstrated that the changes in levels of gene expression were reflected at the level of enzyme activity (fig. 3.13).
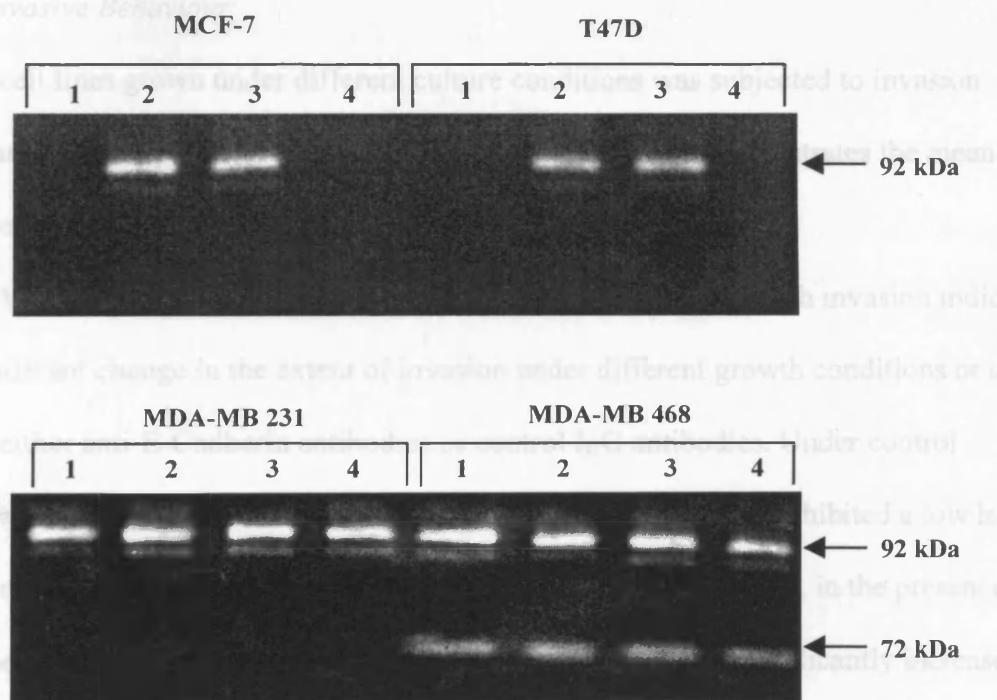
Fig. 3.13: Substrate Gel Zymography on Conditioned Medium from Breast Cancer Cell Lines
MCF-7 and T47D cells show MMP-9 activity only when grown at low density or in the presence of anti-E-Cadherin antibodies. MDA-MB 231 and MDA-MB 468 cells display MMP-9 activity under all culture conditions. Lane 1=High density; 2=Low density; 3= Anti-E-Cadherin antibodies; 4=Mouse IgG

No lytic band was observed in high density cultures of MCF-7 and T47D cells, with strong bands of lysis being seen when these cells were grown in the presence of anti-E-Cadherin antibodies or at low density. Strong lytic bands were observed from CM of MDA-MB 231 and MDA-MB 468 cultures under all growth conditions. The major band was present at 92 kDa indicating latent MMP-9, with a faint band present at ~ 90 kDa, indicating the intermediate form of MMP-9. No bands were seen at 82 kDa, and only MDA-MB 468 cells were seen to show MMP-2 activity, which was of the latent form.

3.4.4.3 Altered E-Cadherin-Mediated Adhesion and MMP-9 Expression is Associated with Enhanced Invasive Behaviour

Each of the cell lines grown under different culture conditions was subjected to invasion assays. All assays were performed at least three times and figure 3.14 illustrates the mean invasive index (MII) for each cell line under different culture conditions.

MDA-MB 231 and MDA-MB 468 cells displayed consistently high invasion indices, with no significant change in the extent of invasion under different growth conditions or in the presence of either anti-E-Cadherin antibodies or control IgG antibodies. Under control conditions, with cells grown to subconfluence, MCF-7 and T-47D cells exhibited a low level of invasion, consistent with results of earlier assays (section 3.4.2). However, in the presence of anti-E-Cadherin antibodies, the invasion index of both cell lines was significantly increased from a mean invasive index (MII) of 32.1% to 57.1% for MCF-7 cells ($F(3,8)=192.5$; $p<0.01$), and from 14.0% to 38.2% for T47D ($F(3,8)=370.6$; $p<0.01$). No change in invasion was seen in the presence of mouse IgG. The extent of invasion observed when these cells were grown at low density was also significantly increased compared to control cells ($p<0.01$ for both MCF-7 and T47D), and was similar to the MII exhibited in the presence of anti-E-Cadherin antibodies.

187
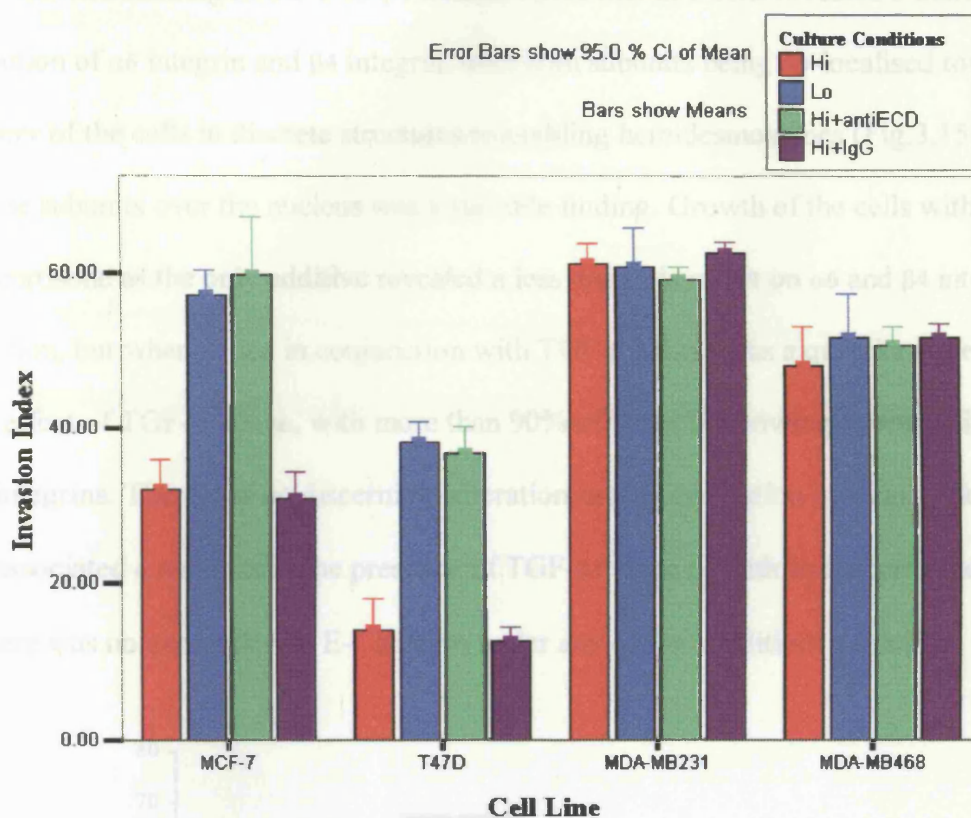
Fig. 3.14: Invasive Activity of Breast Cancer Cell Lines in Relation to Cell Density and Presence of anti-E-Cadherin Antibodies. The bars represent the mean of at least three separate assays.

3.4.5 Modulation of α6β4 Integrin Expression

MCF-7, T-47D and MDA-MB 468 cells did not exhibit any change in morphology, or any significant alteration of E-Cadherin or integrin receptor profile in response to TGF-β1 alone or in combination with hydrocortisone. However, when MDA-MB 231 cells were grown in the presence of TGF-β1, they exhibited a marked morphological change, adopting a larger, flattened, more 'spread' appearance (Fig.3.15 & 3.16).

Immunostaining of the TGF-β1 treated MDA-MB 231 cells revealed a marked change in distribution of α6 integrin and β4 integrin, with both subunits being co-localised towards the periphery of the cells in discrete structures resembling hemidesmosomes (Fig.3.15). Staining for these subunits over the nucleus was a variable finding. Growth of the cells with hydrocortisone as the only additive revealed a less dramatic effect on α6 and β4 integrin expression, but when added in conjunction with TGF-β1, there was a quantitative enhancement of the effect of TGF-β1 alone, with more than 90% of the cells showing strong staining for these integrins. There was no discernible alteration in the distribution of staining for β1 integrin or its associated α subunits in the presence of TGF-β1 alone or with hydrocortisone (Fig.3.15), and there was no expression of E-Cadherin under any of the conditions examined.
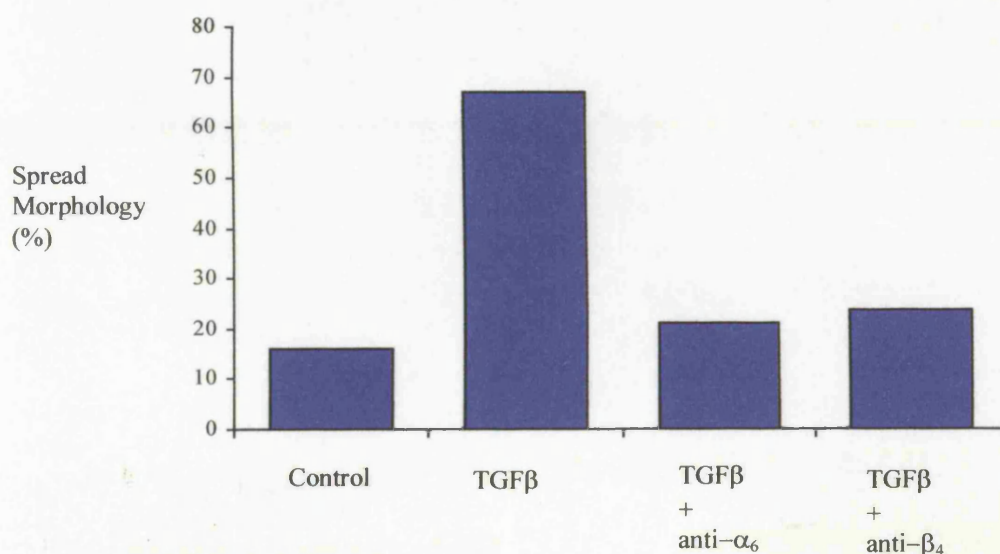
Fig 3.16: Effect of TGF-β1 on Morphology of MDA-MB 231 Cells.
Under control conditions 16% of cells exhibit a spread phenotype. The number of cells showing this phenotype is increased to 67% in the presence of TGF-β1 and this is largely reversed in the presence of anti–α6 and anti–β4 integrin antibodies.

Fig.3.15: Morphology and Expression of Cell Adhesion Molecules in TGF-β1-treated MDA-MB 231 Cells

MDA-MB 231 cells grown in the presence of TGF-β1 (with or without hydrocortisone) demonstrate a marked change in phenotype, adopting a flattened, more epithelioid appearance. The cells do not express E-Cadherin, but retain strong expression of α_2, α_3, and β_1 integrins, which have a punctate disribution at the cell periphery, resembling focal contact formation. There is up-regulation of α_6 and β_4 integrins, which form discrete structures at the cell-substrate interface.
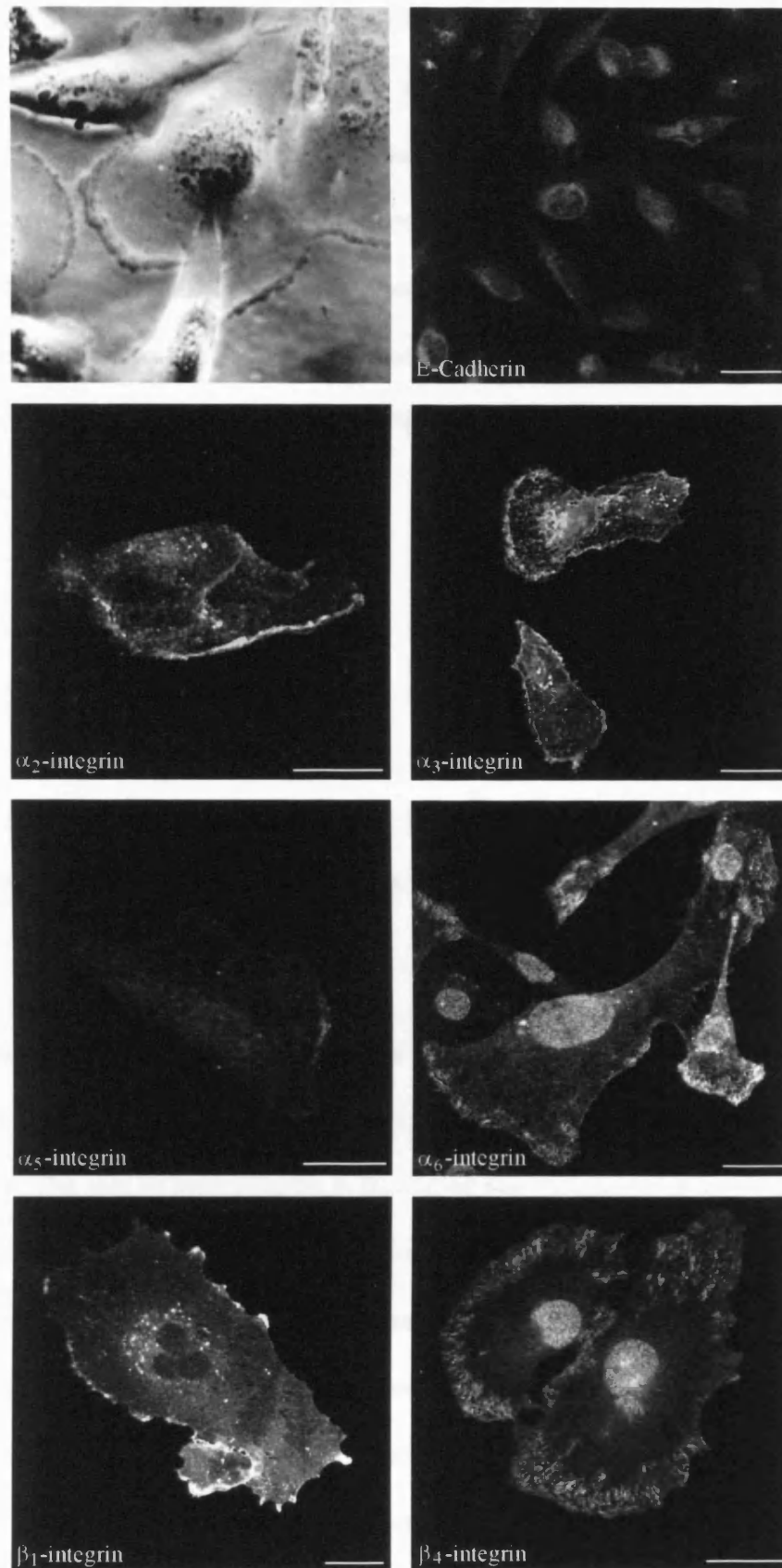
Fig 3.15 : Morphology and Expression of Cell Adhesion Molecules in TGF-β₁-treated MDA-MB 231 Cells

(scale bars = 25μm)

3.4.6 Changes in Level of β4 Integrin Protein in TGF-β1-Treated MDA-MB 231 Cells

To assess whether TGF-β1 treatment of MDA-MB 231 cells leads to increased levels of β4

protein rather than simple redistribution of protein, Western blotting was carried out on equal

protein concentrations of cells grown under control conditions and on cells grown in the

presence of TGF-β1. A major band at 200 kDa was detected. Two further bands at 180 kDa and

140 kDa are also present consistent with proteolytic cleavage of β4 integrin. Figure 3.17

demonstrates stronger bands for β4 integrin in the TGF-β1 treated cells compared to controls.
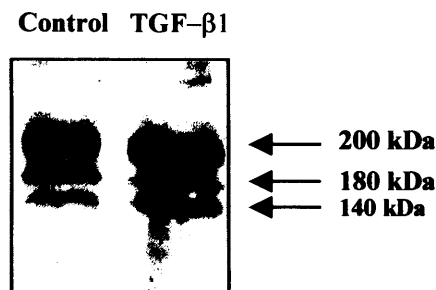
Fig. 3.17: Western Blot Analysis of β4 Integrin Protein in Control and TGF-β1-Treated MDA-MB 231 Cells

3.4.7 Relationship Between α6β4 Integrin Expression and Invasive Behaviour

Under control conditions, MDA-MB 231 is the most highly invasive breast cancer cell line

examined in this study. The role of α6β4 integrin in mediating this invasion was investigated

using a blocking-antibody approach. Figure 3.18a demonstrates that the mean invasive index of

MDA-MB 231 cells is significantly reduced in the presence of either anti-α6-integrin antibodies

or anti-β4-integrin antibodies (p<0.01), but not in the presence of mouse IgG control antibodies.

In contrast the invasive behaviour of MCF-7 cells, which do not express α6β4 integrin, showed

no significant change in the presence of both integrin antibodies and control antibodies.

To assess whether the enhanced expression of $\alpha 6\beta 4$ integrin in TGF-β1-treated MDA-MB 231 cells influences the invasive behaviour of these cells, invasion assays were carried out on cells under control conditions compared to those grown in the presence of TGF-β1, and the effect of integrin blocking antibodies on invasion was also investigated. The same series of invasion assays was performed on MCF-7 cells as a control, since these cells showed no change in integrin expression in response to TGF-β1. Figure 3.18b illustrates the results of the assays which were each performed three times.

As shown previously (section 3.1.2), under control conditions, the MDA-MB 231 cells exhibit higher levels of invasion than MCF-7 cells in this assay (mean invasion index [MII] 62% and 31% respectively). Culture in the presence of TGF-β1 resulted in a significant reduction in invasion by MDA-MB 231 cells (MII 37%; Chi-square=12.4; df=4; p<0.01), and this effect was substantially reversed by preincubation and culture of TGF-β1-treated cells with either anti-β4 integrin antibody or anti-α6 integrin antibody (MII of 60.3% and 57.3% respectively). Preincubation with mouse IgG did not alter the invasion index of the cells. There was a small but significant increase in invasion by TGF-β1-treated MCF-7 cells (MII 36%; F(4,10)=6.5; p<0.008), however, preincubation of the cells with blocking or control antibodies had no significant effect on invasive behaviour over control conditions.

a)
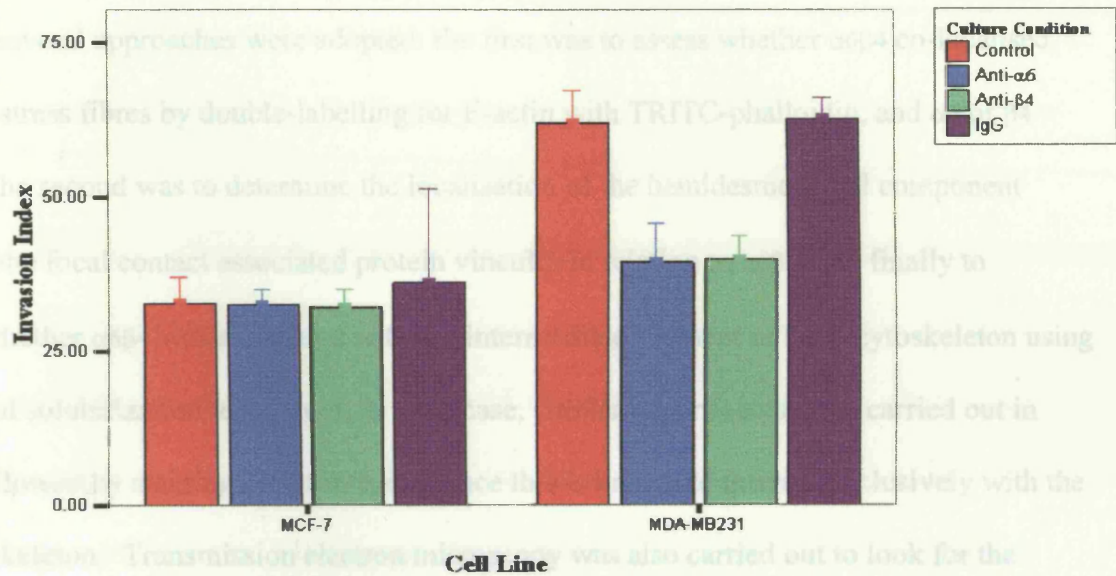
b)
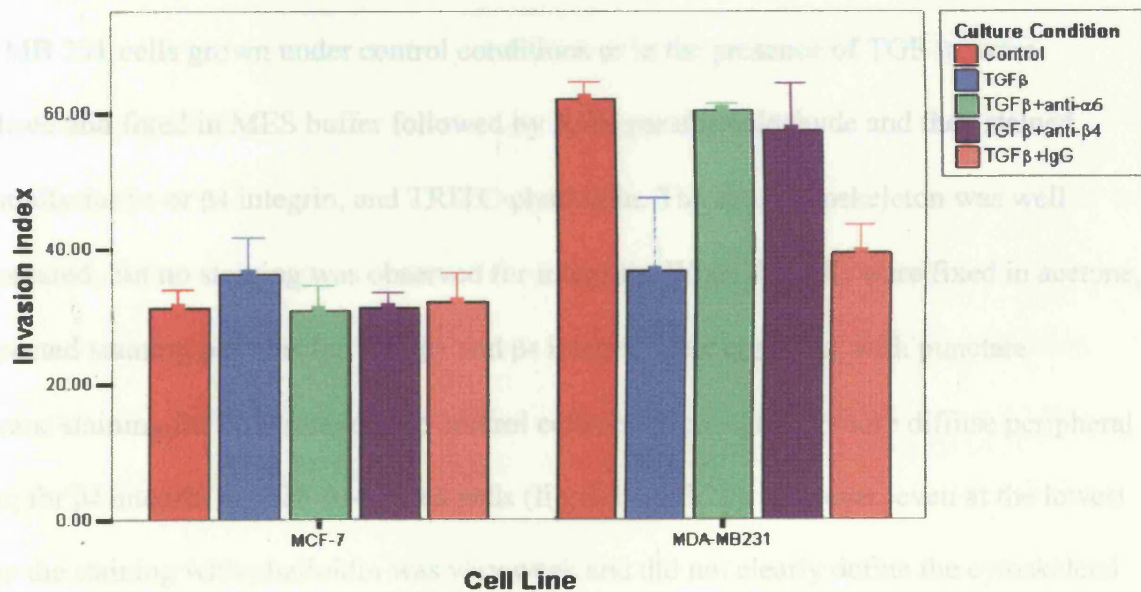
Fig 3.18a & b: The Role of α6β4 Integrin in Tumour Cell Invasion.
(a) in cells grown under control conditions, (b) in cells treated with TGF-β1
Bars indicate the mean of three separate assays; error bars indicate 95% confidence limits.

3.4.8 Cytoskeletal Interactions of α6β4 Integrin in MDA-MB 231 Cells

In order to determine the nature of the cytoskeletal interactions of α6β4 integrin in MDA-MB

231 cells, several approaches were adopted: the first was to assess whether α6β4 co-localised

with actin stress fibres by double-labelling for F-actin with TRITC-phalloidin, and α6 or β4

integrin. The second was to determine the localisation of the hemidesmosomal component

HD1, and the focal contact associated protein vinculin in relation to α6β4, and finally to

evaluate whether α6β4 was associated with the intermediate filament or actin cytoskeleton using

cytoskeletal solubilization techniques. In each case, similar experiments were carried out in

parallel followed by staining for β1 integrin, since this is known to interact exclusively with the

actin cytoskeleton. Transmission electron microscopy was also carried out to look for the

presence of hemidesmosomal structures.

- Co-localisation of β1 and β4 integrins with F-actin

MDA-MB 231 cells grown under control conditions or in the presence of TGF-β1 were

solubilised and fixed in MES buffer followed by 3.7% paraformaldehyde and then stained

sequentially for β1 or β4 integrin, and TRITC-phalloidin. The actin cytoskeleton was well

demonstrated, but no staining was observed for integrins. When the cells were fixed in acetone,

the expected staining patterns for both β1 and β4 integrin were observed, with punctate

membrane staining for both integrins in control cells, but a change to a more diffuse peripheral

staining for β4 integrin in TGF-β1-treated cells (fig. 3.19 & 3.20). However, even at the lowest

dilution the staining with phalloidin was very weak and did not clearly define the cytoskeletal

structure. It was not possible using this technique to discern the co-localisation of stress fibres

with either of the integrin subunits with any degree of certainty. Other approaches were

therefore adopted.

194

- Localisation of HD1 and vinculin

To localise the HD1 protein in MDA-MB 231 cells, indirect immunofluorescence was carried out using a range of fixatives and antibody dilutions, however, none of the conditions employed yielded staining with this antibody. Further studies were carried out on primary normal breast tissue, both frozen and formalin fixed, but despite use of different fixation procedures, including microwave antigen retrieval, no staining was observed.

Strong, discrete staining for vinculin was achieved on acetone-fixed cells. In control MDA-MB 231 cells, punctate staining was identified at the ends of cells, most prominently in lamellipodia, where staining for both $\beta1$ and $\beta4$ integrin was concentrated (fig.3.19 & 3.20). In TGF-$\beta1$-treated cells, punctate staining was identified at the periphery of cells, in a pattern closely reflecting that of $\beta1$ integrin, and suggesting localisation to focal contacts (fig. 3.19 & 3.20).

- Cytoskeletal solubilisation experiments

To investigate the association of $\beta1$ and $\beta4$ integrin with different cytoskeletal systems, prior to fixation and staining, cells were subjected to high salt buffer (HSB) solubilisation washes, which dissolve the actin cytoskeleton, but leave the intermediate filament (IF) cytoskeleton intact (Carter et al, 1990; Gomez et al, 1992). Following treatment with HSB, control MDA-MB 231 cells revealed no convincing staining for either $\beta1$ or $\beta4$ integrin (fig.3.19), and there was little evidence of staining with TRITC-phalloidin, with just occasional thread-like fibres being identified, indicating marked disruption of the actin system. Treatment of TGF-$\beta1$-treated cells with HSB demonstrated loss of staining for $\beta1$ integrin, but staining for $\beta4$ integrin, in discrete peripheral structures was largely maintained. TRITC-phalloidin indicated disruption of the actin cytoskeleton whilst vimentin staining showed an intact IF system (fig. 3.20).

195

Fig. 3.19: Cytoskeletal Associations of β1 and β4 Integrins in Control MDA-MB 231 Cells

When MDA-MB 231 cells grown under control conditions are subjected to High Salt Buffer (HSB) washes to extraxt the actin cytoskeleton, the focal reactivity for β_1 and β_4 integrins is lost (upper four images). Phalloidin demonstrates an intact actin cytoskeletal structure under control conditions and confirms solubilisation following HSB washes (lower right). The intermediate filament system remains intact, as demonstrated by staining for vimentin.
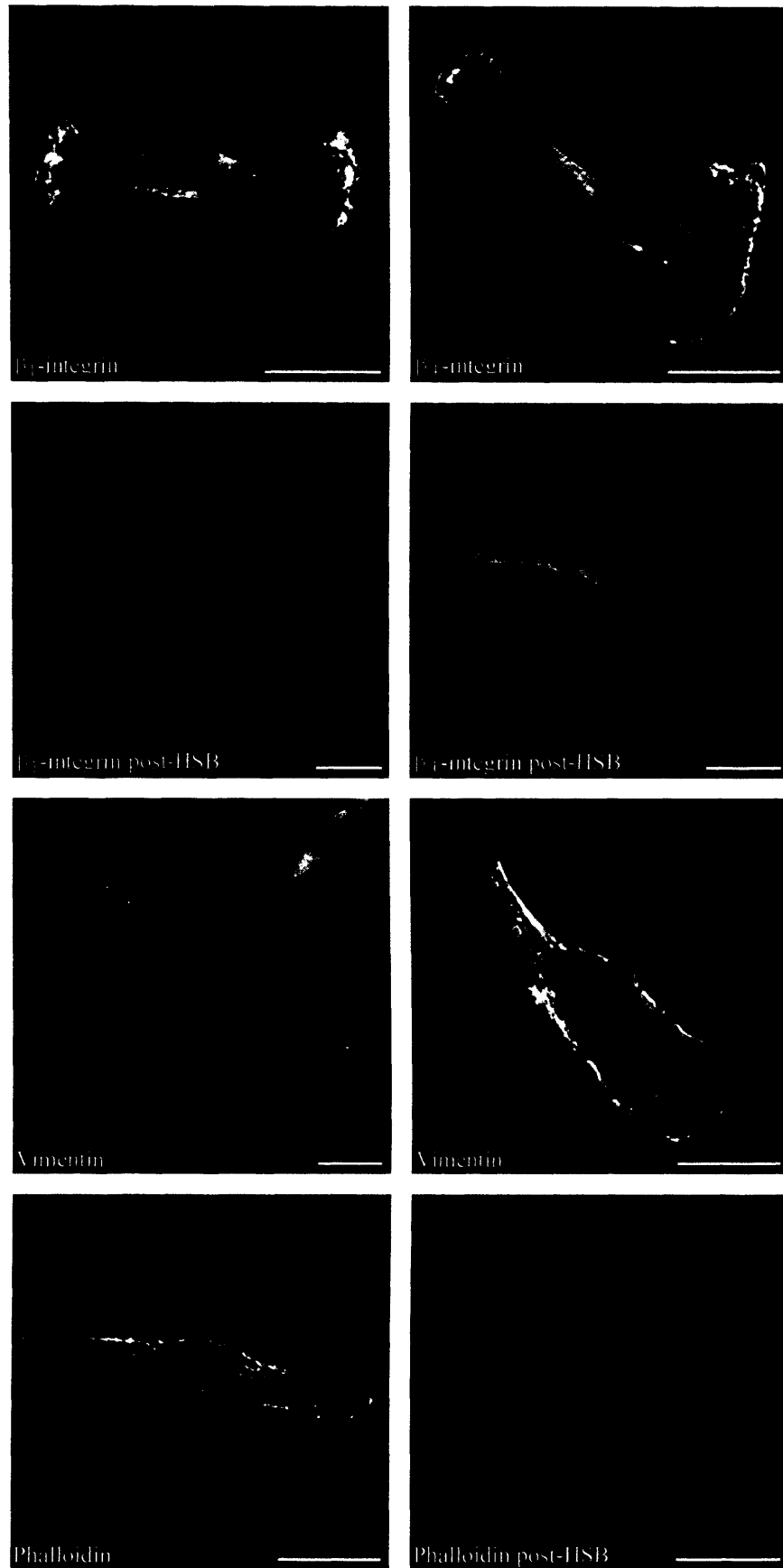
Fig 3.19 : Cytoskeletal Associations of β_1 and β_4 Integrins in Control
MDA-MB 231 Cells

(scale bars = 25μm)

Fig. 3.20: Cytoskeletal Associations of β1 and β4 Integrins in TGF-β1-Treated MDA-MB 231 Cells

MDA-MB 231 cells grown in the presence of TGF-β1 show prominent staining for both β_1 and β_4 integrin, however, β_1 integrin is lost following HSB extraction of the actin cytoskeleton, in contrast to β_4 integrin which is largely retained. Punctate staining for vinculin is observed in a pattern mirroring β_1 integrin, suggesting focal contact formation. Phalloidin staining before and after HSB treatment confirms solubilisation of the actin cytoskeleton; vimentin demonstrates an intact IF system.
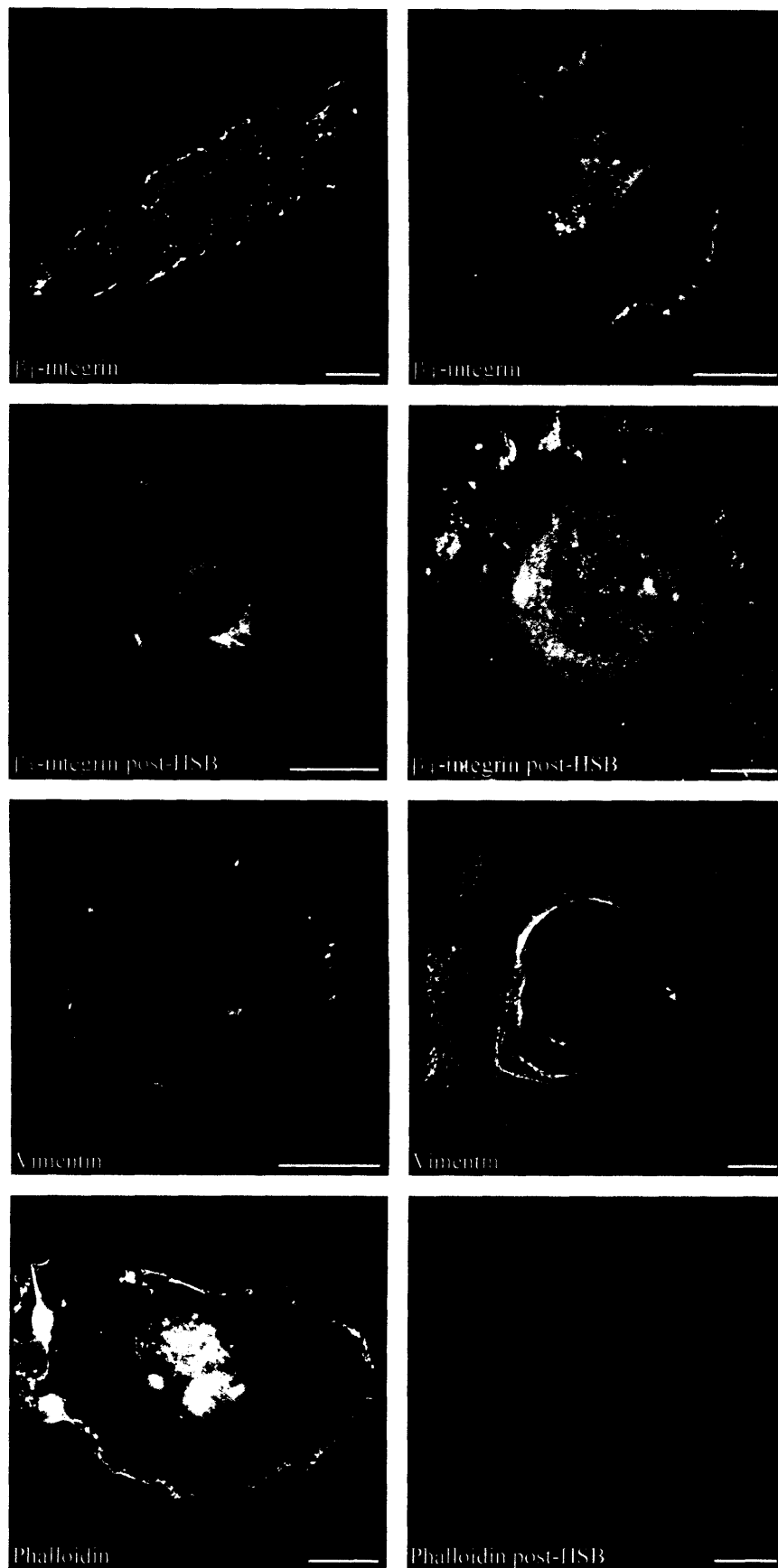
Fig 3.20 : Cytoskeletal Associations of β_1 and β_4 Integrins in TGF-β_1-treated MDA-MB 231 Cells

(scale bars = 25μm)

• Transmission electron microscopy

MDA-MB 231 cells were plated onto poly-d-lysine coated polyethylene membranes and grown under control conditions or in the presence of TGF-β1. These membranes were then processed for transmission electron microscopy and sections were cut maintaining orientation to allow examination of the cell-substrate interface. The aim was to identify whether or not MDA-MB 231 cells form hemidesmosomes (HD). Isolated myoepithelial cells cultured in a similar manner were also examined, since these cells are known to form HD in-vivo.

Multiple sections of each sample were examined: in control cells, no HD were identified, though the cells were rich in focal contacts. The TGF-β1 treated cells had a different appearance in that they formed regularly spaced adhesive structures, approximately 150nm in size, into which filamentous fibres appear to insert (fig. 3.21a). These structures do not have all the features of a classical HD in-vivo (fig. 3.21 b), but similar structures were also observed in myoepithelial cells cultured on poly-d-lysine (fig. 3.21c).
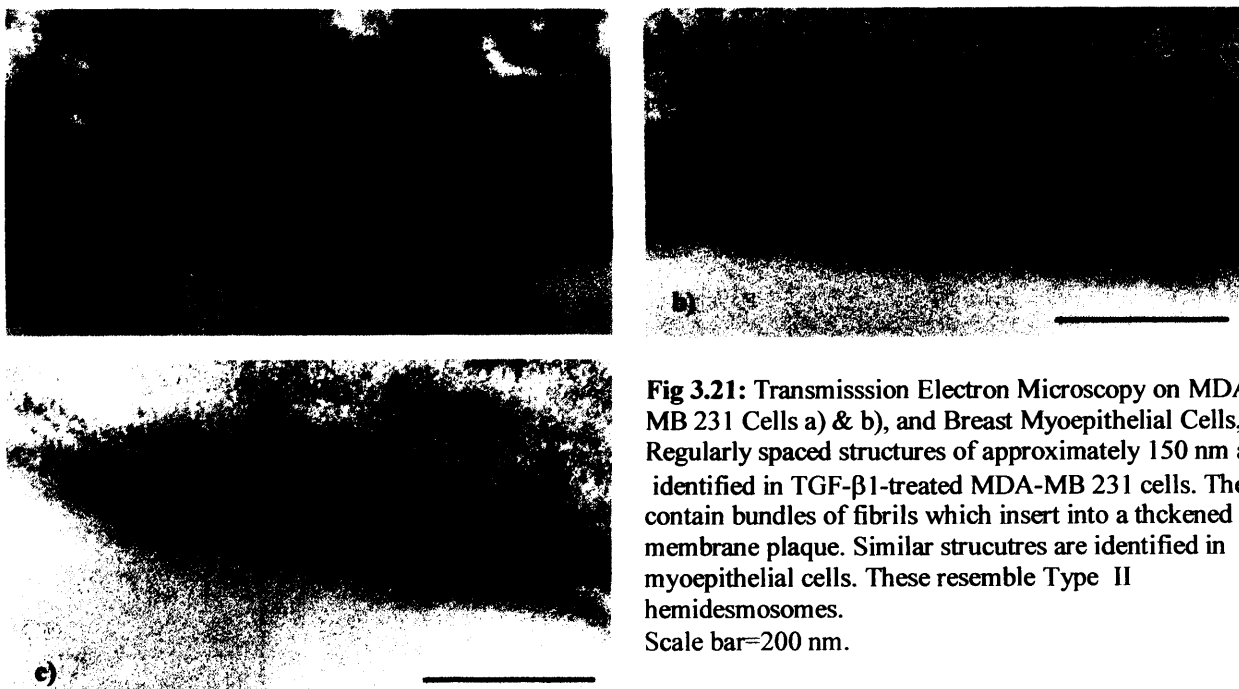
Fig 3.21: Transmisssion Electron Microscopy on MDA-MB 231 Cells a) & b), and Breast Myoepithelial Cells, c). Regularly spaced structures of approximately 150 nm are identified in TGF-β1-treated MDA-MB 231 cells. These contain bundles of fibrils which insert into a thckened membrane plaque. Similar strucutres are identified in myoepithelial cells. These resemble Type II hemidesmosomes.
Scale bar=200 nm.

3.4.9 Relationship Between Altered Invasion in TGF-β1-Treated Cells and Expression of MMPs, MT1-MMP and TIMPs

Treatment of MDA-MB 231 cells with TGF-β1 resulted in a significant reduction in invasive behaviour, whereas TGF-β1 enhanced the invasive behaviour of MCF-7 cells. To investigate whether this effect on invasion was mediated through changes in expression of MMPs and TIMPs, RT-PCR-ELOSA was performed on mRNA extracted from control and TGF-β1-treated cells. The results demonstrate that TGF-β1 leads to a significant increase in gene expression for MMP-9, MT1-MMP, TIMP-1 and TIMP-2 in MDA-MB 231 cells (fig.3.22) but no significant change in expression for any of these genes in MCF-7 cells (Table 3.5). Incubation of TGF-β1-treated MDA-MB 31 cells with anti-α6 or anti-β4 integrin did not lead to any significant change in MMP or TIMP expression (data not shown).

Table 3.5: Effect of TGF-β1 on Level of MMP and TIMP Gene Expression in MDA-MB 231 and MCF-7 Cells++

	MMP-2	MT1-MMP	TIMP-2	MMP-9	TIMP-1
MDA-MB 231 CONTROL	N/E	0.711	0.768	0.617	0.819
MDA-MB 231 + TGFβ	N/E	0.950*	1.111*	0.834*	1.114**
MCF-7 CONTROL	N/E	N/E	0.891	N/E	0.858
MCF-7 + TGFβ	N/E	N/E	0.941	N/E	0.857

++Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background/–RT readings.
*=$p<0.05$; **=$p<0.001$
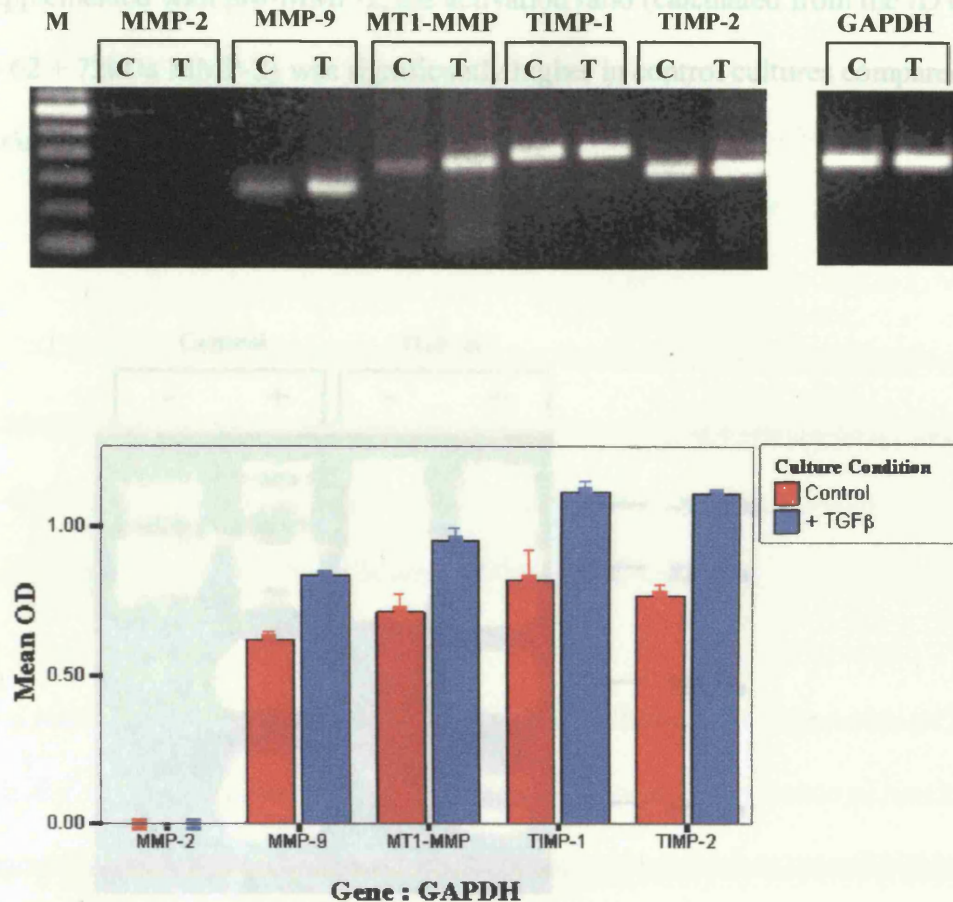
Fig. 3.22: Effect of TGF-β1 on MMP and TIMP Gene Expression in MDA-MB 231 Cells
The gel illustrates the PCR product following 30 cycles of amplification. The graph bars represent the mean gene : GAPDH OD ratio and error bars indicate 95% confidence intervals. M=Molecular weight markers, C=Control cultures, T=+TGF-β1

To assess whether changes in gene transcription translate to a change in enzyme activity, substrate gel zymography was carried out on CM from control and TGF-β1-treated MDA-MB 231 cells. Since these cells do not produce MMP-2, the 'function' of MT1-MMP was analysed indirectly by addition of purified recombinant pro-MMP-2 to cultures, and the degree of activation of this pro-MMP-2 was assessed on zymography (fig. 3.23). The results indicate increased levels of both active and latent MMP-9 in TGF-β1-treated cultures. In those

3.5 Summary of Results

- The ER positive, vimentin negative MCF-7 and T47D cells display relatively low level invasion, retain expression of E-Cadherin and show low level expression of integrin receptors, with no expression of $\alpha6\beta4$ integrin. The cells do not express MMP-2, MT1-MMP or MMP-9 under control conditions. In contrast, the ER negative, vimentin positive MDA-MB 231 and MDA-MB 468 cells are highly invasive, lack functional E-Cadherin and MDA-MB 231 cells exhibit staining for $\alpha6\beta4$ integrin. Both cell lines produce MT1-MMP and MMP-9, and MDA-MB 468 cells also produce MMP-2.

- There is a relationship between E-Cadherin-mediated adhesion and expression of MMP-9. When MCF-7 and T47D cells are grown at low density or in the presence of blocking E-Cadherin antibodies, there is induction of MMP-9 expression both at the mRNA level and in enzyme activity. Blocking of E-Cadherin with induction of MMP-9 is associated with a significant increase in invasion ($p=<0.01$ for both MCF-7 and T47D cells)

- Treatment of MDA-MB 231 cells with TGF-β1 results in up-regulation and redistribution of $\alpha6\beta4$ integrin. In control MDA-MB 231 cells, staining for $\alpha6\beta4$ integrin is lost when the actin cytoskeleton is solubilised, however, the majority of $\alpha6\beta4$ is retained in TGF-β1-treated cells, suggesting a change in localisation from the actin system to the IF system. This is associated with a significant decrease in invasion ($p= <0.01$) which is $\alpha6\beta4$-dependent, and an increase in MMP and TIMP expression in an $\alpha6\beta4$-independent manner. Although increased levels of each MMP mRNA is evident, zymography reveals a reduced ability of TGF-β1-treated cells to activate exogenous pro-MMP-2.

3.6 Discussion

3.6.1 Discussion of Techniques

3.6.1.1 Invasion Assay

Different invasion assays offer different advantages and each have their own limitations.

Assays using reconstituted basement membrane (BM) substance, such as those employed in

this study, are technically relatively simple and have been shown to be highly reproducible

(Hendrix et al, 1989). One potential source of variation is in batch-to-batch variation in

substrate preparation. In an attempt to reduce this, the same batch of Matrigel was used for all

assays in this study, and all assays were carried out in duplicate at least three times.

To quantify the extent of invasion, the proportion of invading cells was measured by

counting under direct visualisation, since this has been shown to be the most accurate method

(Hendrix et al, 1989). One of the limitations of the cell counting methods used in many studies

is that the number of invading cells is expressed as a proportion of the original number of cells

seeded. This does not take account of rates of cell proliferation during the time of the assay,

and could therefore yield spurious results. To address this, in the present study the invasion

index was calculated on the percentage of cells in the lower well in relation to cell counts in the

upper and lower wells at the termination of the assay.

An important question to address is how representative this two-chamber EHS-barrier

invasion assay is of the in-vivo process of cell invasion. Unlike the 'natural' BM barriers such

as human amnion, EHS matrix lacks a complex cross-linked structure (Noel et al, 1991), and in

keeping with this for a given cell line, fewer cells invade human amnion compared to a

reconstituted matrix (Hendrix et al, 1989). A number of groups have found the EHS-barrier

assay to be highly discriminating between malignant and non-malignant cells, and have shown

the extent of invasion in these assays to correlate with in-vivo invasion and metastatic potential

(Terranova et al, 1986; Kramer et al, 1986; Hendrix et al, 1989; Saiki et al, 1994). In contrast one study found no relationship between ability to invade EHS matrix and the malignant phenotype, with normal fibroblasts and keratinocytes also penetrating the matrix barrier (Noel et al, 1991). There are potentially important methodological differences in the assay carried out by this latter group: they layered cells onto a three-dimensional gel of EHS rather than a thin layer of matrix, and assessed invasion as penetration into the gel rather than the ability to penetrate a gel and traverse the pores of a barrier, as employed by Hendrix et al (1989) and others, and as used in my study. It is arguable whether penetration of a gel alone is a reflection of invasive capacity.

As used in the current study, the EHS-barrier invasion assay was found to discriminate between malignant and non-malignant cells at protein concentrations of 5 µg and above, and whilst it is accepted that such a crude assay may not reflect the complex process of invasion, it does allow comparison of in-vitro invasive activity under defined and reproducible conditions.

3.6.1.2 RT-PCR-ELOSA

The method for quantitation of amplified cDNA chosen in this study was an ELOSA technique, based on detection of a specifically hybridised probe to the PCR product. Two issues needed to be addressed, 1) whether the technique offered acceptable reproducibility within and between samples, and 2) confirmation that measurements were taken over the exponential phase of the PCR. The reproducibility assay carried out in this study demonstrates that RT-PCR with ELOSA quantitation can achieve good levels of reproducibility, with no significant differences in the OD readings for a given gene in separate samples. Errors in RT-PCR may be introduced at a number of stages. In this study, mRNA was extracted from cells using oligo d(T) labelled paramagnetic beads, and priming for RT was thus achieved by interaction of oligo d(T) and the

poly-A tail of mRNA species. This technique was chosen because (i) it selects mRNA rather than total RNA and this enhances efficiency of detection of low copy transcripts, (ii) extraction on the magnetic beads allows multiple washing steps which yields clean mRNA preparations, minimising contamination with proteins and genomic DNA, and (iii) since priming for RT was achieved from the oligo d(T) arm of the bead without addition of extrinsic primers, this allowed greater standardisation of the RT reaction.

Exponential amplification during PCR has the ability to magnify small errors. Variations can be minimised by use of mastermix preparations, careful pipetting and use of more dilute reagents where possible to permit pipetting of larger volumes.

The linear phase of the reaction for each gene analysed was determined in preliminary experiments and all future measurements taken over this part of the reaction. The quantitation achieved in this study was essentially a relative measurement and not absolute, with the 'amount' of target gene present being compared to the 'amount' of a housekeeping gene - in this case GAPDH - to account for any differences in cellularity between the samples. Since cell lines and not tissues were used in this study, it is possible to control cellularity in each specimen, and the consistency of readings obtained for GAPDH expression illustrates that the samples were indeed comparable. Use of competitor fragments, either DNA or RNA, can allow absolute quantitation of PCR product, however, for the purposes of this study, an indication of the relative change in gene expression was required without the need for absolute quantitation. It was felt that the current approach with RT-PCR-ELOSA achieved this aim, with an acceptable level of reproducibility.

3.6.1.3 Cytoskeletal Solubilisation Experiments

It was essential in this study to elucidate the nature of interaction of α6β4 with the cytoskeleton

in cells grown under control conditions and in the presence of TGF-β1. Initial attempts were

made at co-localising α6β4 with components of the cytoskeleton, including F-actin, using

TRITC-phalloidin. These experiments were not successful because the conditions necessary to

demonstrate the actin cytoskeleton precluded use of the integrin antibodies, therefore other

methods had to be explored.

Previous workers have described conditions for the controlled solubilisation and

isolation of different cytoskeletal fractions (Capco et al, 1982; Fey et al, 1984; Owaribe et al,

1991; Gomez et al, 1992), and indeed such an approach was adopted by Rabinovitz and

Mercurio to demonstrate the association of α6β4 with the actin cytoskeleton in a colon cancer

cell line (Rabinovitz & Mercurio, 1997). Soluble proteins and phospholipids are removed by

Triton X-100, whilst a high salt buffer removes the salt-labile cytoskeleton leaving the skeletal

framework intact. The skeletal framework consists of the intermediate filament system,

including vimentin and the cytokeratins, desmosomal proteins and the nuclear-matrix proteins

(Fey et al, 1984). Evidence that the cellular structure remains intact after such treatment can be

demonstrated using antibodies to either vimentin or nuclear-matrix proteins (Fey et al, 1984).

The method described by Gomez et al (1992) was adopted because of its relative simplicity.

The use of HSB washes is quite harsh on the cells and the main problem encountered was

lifting of the cells from the coverslip. With strict adherence to a 15 min incubation and use of

gentle washing steps, an adequate number of cells remained on the coverslips. Subsequent

immunofluorescence revealed an intact vimentin network with almost complete loss of actin

filaments, confirming adequate solubilisation of the actin cytoskeleton whilst retaining an intact

skeletal framework.

3.6.2 Discussion of Results

3.6.2.1 Characteristics of Cell Lines

All of the breast cancer cell lines used in this study were derived from metastatic tumour, and therefore all represent late stage tumour progression. In spite of this, the tumour cells exhibit very different characteristics and behaviour in-vitro. The MCF-7 and T47D cells show a classical luminal epithelial phenotype, in that they express oestrogen receptor (ER), E-Cadherin and CK18, and lack vimentin and the basal cytokeratin CK14. This study has also shown that these cells have low level integrin receptor expression and lack $\alpha6\beta4$ integrin, which in-vivo is expressed purely by the basal or myoepithelial cells in normal breast. The MDA-MB 231 and MDA-MB 468 cells are generally regarded as less well differentiated cell lines: they lack ER, both show high level expression of vimentin and lower level expression of CK18, and 231 cells do not express E-Cadherin, whilst 468 cells exhibit very low levels of E-Cadherin which is not localised to the cell surface in adherens junctions. These findings are in agreement with those of other workers (Bartek et al, 1986; Curschellas et al, 1987; Taylor-Papidimitriou et al, 1993). Whilst these features may be interpreted as loss of epithelial differentiation, this expression profile does exhibit similarities to the myoepithelial phenotype. The similarity is particularly strong for MDA-MB 231 cells, which in common with myoepithelial cells also show high level expression of EGFR, integrin receptors, including $\alpha6\beta4$, and lack EMA. It could be postulated therefore that the MDA-MB 231 cells show evidence of a myoepithelial phenotype.

That breast carcinomas may exhibit a basal or myoepithelial phenotype has been proposed by other groups (Rudland, 1993; Taylor-Papidimitriou et al, 1993; Li et al, 1998). Taylor-Papidimitriou et al (1993) suggested that whereas the majority of breast carcinomas clearly exhibit a luminal phenotype - either ER positive or negative, it is evident that a subgroup of approximately 10-20% of tumours express one or more markers of a basal

phenotype, and that this phenotype is generally associated with a poor prognosis (Catoretti et al, 1988; Domagala et al, 1990a, 1990b; Wetzels et al, 1990). It is suggested that this relationship between basal phenotype and poor prognosis may exist because the basal cell contains the stem cell population of the breast (Taylor-Papidimitriou et al, 1993). It has been demonstrated that cancer cell lines expressing markers of a basal phenotype are more invasive than 'luminal' type cells in in-vitro invasion assays (Thompson et al, 1992), and the present study also reveals the basal type MDA-MB 231 cells to be more highly invasive than the luminal MCF-7 and T47D cells. The MDA-MB 468 cells which also display some basal markers have a similar invasive capacity to MDA-MB 231 cells.

All of the cell lines elaborate high levels of TIMP-1 and TIMP-2, but MDA-MB 231 and MDA-MB 468 are again distinct in being the two breast cell lines to express MT1-MMP, with 468 cells also expressing MMP-2. This may indicate that expression of MMPs is associated with the more poorly differentiated cancer cells, but could also be a reflection of the more 'mesenchymal' characteristics of the basal/myoepithelial cells. Expression of MMPs and MT1-MMP by tumour cells as part of the so-called epithelial-mesenchymal transition has previously been suggested (Pulyaeva et al, 1997; Martorana et al, 1998).

3.6.2.2 Relationship Between E-Cadherin Expression, Expression of MMP-9 and Invasive Behaviour

A relationship was demonstrated between the presence of membrane E-Cadherin and expression of MMP-9 in the breast cancer cell lines used in this study. The E-Cadherin in MCF-7 and T47D cells has been shown to be genetically and functionally normal (Hiraguri et al, 1998) and both cell lines have been shown to express normal levels of α- and β-catenin, at the mRNA and protein level (Pierceall et al, 1995). The absence of E-Cadherin in MDA-MB

231 cells is due to hypermethylation of the promotor region Cpg islands (Hiraguri et al, 1998), and whereas MDA-MB 468 cells have been shown to express E-Cadherin, the protein is present at low levels, and these cells do not express α-catenin, possibly as a result of gene mutation (Pierceall et al, 1995). Linkage of E-Cadherin to the actin cytoskeleton via α- and β-catenin/plakoglobin is essential for adhesive function (Hirano et al, 1992), and the lack of α-catenin in these cells explains the absence of membrane E-Cadherin.

In the cell lines expressing membrane E-Cadherin it was demonstrated that MMP-9 could be modulated by culturing the cells at different densities, such that in the absence of cell-cell contacts higher levels of MMP-9 were produced. Changes in the level of MMP-9 expression at different cell density has been reported (Xie et al, 1994; Anzai et al, 1996) although in these studies the role of E-Cadherin was not investigated. In a recent study on mouse skin cancer cell lines a direct link between E-Cadherin expression and control of MMP-9 production was demonstrated by induction of MMP-9 expression following transfection of E-Cadherin positive cells with E-Cadherin antisense cDNA (Llorens et al, 1998). To address whether MMP-9 expression in MCF-7 and T47D cells is controlled by E-Cadherin, subconfluent cultures were grown in the presence of anti-functional E-Cadherin antibodies (Behrens et al, 1989). This demonstrated that blocking E-Cadherin-mediated interactions resulted in up-regulation of MMP-9 expression. The MDA-MB 231 and MDA-MB 468 cells lacking membrane E-Cadherin produced relatively high levels of MMP-9 under all culture conditions.

Expression of MMP-9 may be regulated by a number of agents including growth factors (Shimuzu et al, 1996), cytokines (Okada et al, 1990; Hujanen et al, 1994) and ECM molecules (Tremble et al, 1994). Fibroblasts plated on a mixture of fibronectin and tenascin up-regulate MMP-9 expression (Tremble et al, 1994) and a laminin peptide fragment has been shown to

induce MMP-9 in monocytes (Corcoran et al, 1995). Analysis of the promotor region of both

the human and mouse MMP-9 genes has identified several control elements including TPA

responsive elements that can bind AP1 transcrption factors, an NFkB binding site and closely

related to this a PEA3/Ets binding site, and in the human gene, but not the mouse, there is a

TGF- β inhibitory element (Huhtala et al, 1991; Sato & Seiki, 1993; Sato et al, 1993; Gum et

al, 1996). Interestingly, Llorens et al (1998) have detected a potential LEF-1-binding site in the

murine MMP-9 promotor. Thus it could be hypothesised that inhibition of E-Cadherin results in

free cytoplasmic β-catenin or plakoglobin which could then bind to the LEF-1/Tcf family of

transcription factors to influence gene expression (Behrens et al, 1996). Whether such a direct

relationship between E-Cadherin and MMP-9 exists in humans remains to be confirmed,

however, in human colon cancer cells transfection of E-Cadherin has been shown to reduce

gelatinase secretion (Edvardsen et al, 1993)

In this study, absence or inhibition of E-Cadherin resulted in a more invasive

phenotype, in keeping with the original reports on E-Cadherin as an invasion-suppressor

protein (Frixen et al, 1991; Vleminckx et al, 1991). In view of the accumulating evidence, it

may be reasonable to suggest that at least part of the anti-invasive effect of E-Cadherin may be

due to the regulation of other pro-invasive pathways, such as release of proteolytic enzymes.

3.6.2.3 Modulation of Expression of Cell Adhesion Molecules in Breast Cancer Cell Lines

A wide range of factors have been shown to influence expression and activation of integrin

receptors (Burridge et al, 1988; Sollberg et al, 1992; Juliano & Haskill, 1993; Dogic et al,

1998; Nebe et al, 1998). I have found that growth of MDA-MB 231 cells in the presence of

TGF-β1, alone or in combination with hydrocortisone leads to up-regulation of α6β4 integrin

(Jones et al, 1997). TGF-β1 has been shown to up-regulate β4 integrin in transformed human

keratinocytes, along with other hemidesmosomal proteins such as the Bullous Pemphigoid

Antigens, BPAg1 and BPAg2 (Sollberg et al, 1992). De-novo expression of β4 integrin was not

observed in any of the other cell lines treated with TGF-β1. In addition to up-regulation of the

protein, a change in the distribution of α6β4 was observed, with more diffuse peripheral staining

in place of the focal punctate pattern of staining seen in control cells. This correlated with a

change in cell morphology to a flattened more epithelioid phenotype. These results suggested a

change in cytoskeletal organisation in TGF-β1 -treated MDA-MB 231 cells, a function which

has previously been described for this growth factor (Garbi et al, 1990; Scardigli et al, 1996).

3.6.2.4 Role of α6β4 Integrin in Invasion

To address whether the change in expression of α6β4 integrin affected tumour cell behaviour,

invasion assays were carried out and the role of α6β4 investigated using blocking antibodies.

The results are complex: TGF-β1 -treated cells are significantly less invasive than control

MDA-MB 231 cells, and this inhibition of invasion is α6β4-dependent, however, control MDA-

MB 231 cells are highly invasive cells and the results of antibody-blocking experiments

indicate that this invasion is substantially dependent on α6β4 integrin. These results suggest that

the function of α6β4 changes under different conditions. It is evident from a growing body of

literature that α6β4 integrin has different functions in different situations. The classically

accepted role for α6β4 integrin is as a component of hemidesmosomes where it mediates stable

anchorage associated with non-motile cells (Carter et al, 1990; Sonnenberg et al, 1990), and in

normal breast α6β4 is highly expressed in myoepithelial cells which are rich in

hemidesmosomes. However, hemidesmosomes are rarely found in tumour cells (Bergstraesser

et al, 1995), and are lost in basal keratinocytes at a wound edge, where they adopt a migratory

function (Kurpakus et al, 1991). Recent work on colon cancer cell lines has demonstrated that

211

α6β4 can promote tumour cell invasion by the localised activation and targeting of

phosphatidylinositol 3-kinase (PI3-K), with subsequent activation of the small GTPase Rac,

which has been shown to modulate organisation of the actin cytoskeleton (Shaw et al, 1997).

The results of the present study are in keeping with other studies that suggest a pro-

invasive role for α6β4 integrin (Dedhar et al, 1993; Rabinovitz & Mercurio, 1997), in that the

invasive behaviour of MDA-MB 231 cells under control conditions is an α6β4-dependent

process. However, the invasive-inhibitory effect of α6β4 in TGF-β1 -treated cells suggests that

the function of the integrin has changed in response to enviromental cues, and one could

hypothesise that this change in function is due to translocation of the integrin from 'motility'

associated structures to 'anchorage' associated structures. This hypothesis was investigated by

analysing the cytoskeletal interactions of α6β4 under different conditions.

3.6.2.5 Cytoskeletal Interactions of α6β4 Integrin

In hemidesmosomes, α6β4 is linked to the IF cytoskeleton via HD1/plectin (Borradori &

Sonnenberg, 1996), whereas in colon cancer cells, in which α6β4 was found to promote cell

migration, the integrin was shown to be linked to the actin cytoskeleton (Rabinovitz &

Mercurio, 1997). To investigate whether the cytoskeletal associations of α6β4 are changed in

MDA-MB 231 cells treated with TGF-β1, attempts were made to co-localise the integrin

subunit with the actin cytoskeleton, and with linker proteins classically associated with actin

(vinculin) and the IF system (HD1/plectin). Unfortunately, these experiments were not

successful; the fixation regime which gave optimal staining for actin did not allow successful

localisation of the integrins and vice versa. No staining for HD1 was achieved under a range of

different fixations and antibody dilutions, and for future work alternative antibodies will be

sought.

Therefore, another approach was adopted to try and establish whether α6β4 linked to the actin cytoskeleton or the IF system. The cytoskeletal solubilisation experiments indicated that under control conditions, α6β4 integrin and β1 integrin were attached to the actin cytoskeleton, and vinculin also co-localised with these integrins. In TGF-β1 -treated cells, however, staining for β1 integrin was lost following solubilisation, but staining for α6β4 was largely maintained, suggesting that in these cells it is attached to IF.

Since the classical localisation of α6β4 integrin is in hemidesmosomes, electron microscopy was performed to establish whether HD were formed in MDA-MB 231 cells, either under control conditions or following exposure to TGF-β1. Normal breast myoepithelial cells were also processed for EM for comparison, since these cells are known to form HD in-vivo. The classical trilaminar structure of HD - a dense cytoplasmic plaque where keratin bundles attach, the sub-basal dense plate, and the layer of anchoring fibrils linking the cell to the BM - was not convincingly identified in any of the cells, but there were differences between the control and TGF-β1 treated MDA-MB 231 cells. The control cells exhibited numerous membrane thickenings typical of focal contacts. Such structures were also observed in TGF-β1 treated cells but these cells also formed larger adhesive structures characterised by a cytoplasmic plaque with a fibrillary appearance and a thickened membrane plate. Similar structures were seen in the myoepithelial cells. The appearances resemble those of putative type-II HD which have been described in a variety of cell types in culture (Hieda et al, 1992; Uematsu et al, 1994; Fontao et al, 1997; Nievers et al, 1998). As with type-I HD, α6β4 is a central component of type-II HD, and has been shown to link to the IF cytoskeleton, with no co-localisation to vinculin (Gomez et al, 1992; Fontao et al, 1997). These characteristics were also demonstrated for the TGF-β1-treated MDA-MB 231 cells in this study, and taken together,

213

these findings support the hypothesis that exposure to TGF-β1 leads to translocation of

α6β4 from actin-associated motility structures to IF-associated type-II HD.

3.6.2.6 What Factors Control Localisation and Function of α6β4 Integrin?

There has been considerable interest in understanding the factors that control formation of

hemidesmosomes (Kurpakus et al, 1991; Langhofer et al, 1993; Hormia et al, 1995; Baker et al,

1996; Nievers et al, 1998; Schaapveld et al, 1998). In view of the recent studies indicating that

α6β4 is not exclusively localised to HD but may also be incorporated in motility associated

structures, the mechanism which determines the localisation of α6β4 and the initiation of HD

formation becomes particularly relevant.

There continues to be discussion in the literature as to whether HD formation is

determined by intracellular events and is a ligand-independent process (Nievers et al, 1998;

Schaapveld et al, 1998), or whether HD formation is initiated by contact with the appropriate

extracellular matrix ligand (Baker et al, 1996). The ligand for α6β4 is laminin-5 (Ln-5), also

known as epiligrin, nicein and kalinin (Carter et al, 1991; Domolge-Hultsch et al, 1992; Gil et

al, 1994; Jones et al, 1994; Ryan et al, 1994). When rat bladder cells or the SCC12 keratinocyte

cell line are plated on a Ln-5-rich matrix, HD are formed and this is inhibited by antibodies

specific for the α chain of Ln-5 (Baker et al, 1996). A role for Ln-5 in HD formation is further

supported by reports of mutations in Ln-5 leading to skin blistering diseases characterised by

lack of, or poorly developed, HD (Gil et al, 1994). This would suggest that when cells capable

of forming HD come into contact with Ln-5 then HD formation will be initiated. However, this

is not necessarily the case, since Giannelli et al (1997) indicated that the non-tumourigenic

HUMEC cells and MCF-10 breast cells adopt a migratory and invasive phenotype when plated

on Ln-5. This was shown to be the result of MMP-2-mediated cleavage of the Ln-5 γ2 chain,

which reveals a cryptic site on Ln-5 that then supports migration via $\alpha3\beta1$ integrin (Giannelli et al, 1998). Thus it appears that proteolytic modification of the ECM influences the function of the matrix. Further support for the role of the matrix in determining the function of $\alpha6\beta4$ comes from Mercurios' group, who found that localisation of $\alpha6\beta4$ integrin to actin-containing motility structures only occurred when the cells were plated on laminin-1 (Ln-1), and not on other matrices (Rabinovitz & Mercurio, 1997). It is difficult to marry these findings with the proposal that HD formation is a matrix-independent process (Nievers et al, 1998).

In the current study, both control and TGF-$\beta1$ -treated cells were plated on poly-d-lysine, however, TGF-$\beta1$ is known to influence ECM protein synthesis, therefore one could postulate that TGF-$\beta1$ could promote translocation of $\alpha6\beta4$ to HD-like structures either (i) via a direct effect on cytoskeletal organisation, (ii) via an effect on matrix protein production, for example, switching synthesis from Ln-1 to Ln-5, or (iii) by influencing MMP production, which would alter the nature of the ECM and determine whether it exerted an anchoring or migratory effect on the cells, or a combination of all these. This study has examined only one of these factors, that is, control of MMP synthesis and activity, but parallel studies on ECM synthesis and cytoskeleton-disruption experiments would be worthwhile to provide a fuller picture of this complex process.

3.6.2.7 Relationship Between Altered Invasion of TGF-β1-Treated Cells, and Expression of MMPs and TIMPs

As described above, when MDA-MB 231 cells were treated with TGF-$\beta1$, there was up-regulation and redistribution of $\alpha6\beta4$ integrin and this was associated with a significant reduction in invasive behaviour. To address whether this was related to changes in expression of MMPs and TIMPs, RT-PCR-ELOSA was performed on cells grown in the presence and

absence of TGF-β1. Since TGF-β1 led to an increase in invasion by MCF-7 cells, analysis of MMP and TIMP levels was similarly carried out on these cells. In contrast to the reduced invasive behaviour, TGF-β1 -treated MDA-MB 231 cells revealed elevated levels of expression of MMP-9, MT1-MMP as well as TIMP-1 and TIMP-2. These results appear contradictory to a reduction in invasion, however, on assessing enzyme activity, it was evident that even though TGF-β1-treated cells showed enhanced expression of MT1-MMP, the ability of the cells to activate pro-MMP-2 was reduced. MMP-9 activity was elevated, but this is most marked for the latent pro-enzyme.

A number of in-vitro models have shown TGF-β1 to enhance synthesis of matrix proteins, to decrease proteinase activity and to increase protease inhibitor synthesis (Roberts et al, 1992; Ronco et al, 1992; Ziyadeh et al, 1994), however, this is not a universal finding, since other studies show TGF-β to up-regulate MMP synthesis (Overall et al, 1989; Marti et al, 1994). The results of this study demonstrate stimulation of gene expression of both proteases and inhibitors but shows the net effect to be one of reduced active proteolysis. A number of possible explanations for the observed results can be made: the first is that whereas there is an increase in both enzymes and inhibitors, the stimulation of TIMPs exceeds that of the enzymes with a net anti-proteolytic effect. The second is that excess TIMP-2 has an inhibitory effect on the MMP-2-activating potential of MT1-MMP. Such a concentration-dependent effect has previously been suggested, with low level TIMP-2 being though to promote binding and activation of MMP-2 by MT1-MMP, but higher concentrations of TIMP-2 inhibiting this binding (Strongin et al, 1995; Butler et al, 1998). The findings of this study emphasise the need to examine multiple components of the system (i.e. both enzymes and inhibitors), and at different levels (i.e. both message and enzyme activity).

The induction of MMPs and TIMPs by TGF-β1 was not reversed when the cells were cultured in the presence of α6β4 blocking antibodies, which suggests that whilst TGF-β1 has a co-ordinate effect on both α6β4 and MMP expression, the effect on enzyme expression is not mediated through the integrin receptor.

No significant alteration in message or enzyme activity was detected in MCF-7 cells treated with TGF-β1, and this illustrates the cell-specific effect of TGF-β. The mechanism by which TGF-β1 enhances the invasive behaviour of MCF-7 cells is unclear, but TGF-β1 can regulate other members of the MMP family (Rougier et al, 1997; Uria et al, 1998) and other proteolytic systems (Lund et al, 1991; Arnoletti et al, 1995) which have not been investigated in the current study.

3.7 Conclusions

- MCF-7 and T47D cells show features characteristic of a luminal epithelial phenotype whilst MDA-MB 231 cells exhibit features more characteristic of a basal/myoepithelial phenotype. MDA-MB 468 cells express markers of both cell lineages.

- There is a direct relationship between loss of E-Cadherin function and induction of MMP-9 expression. Blocking E-Cadherin-mediated adhesion promotes MMP-9 release and enhances tumour cell invasion, suggesting that at least part of the invasive-suppressor effect of E-Cadherin is via a co-ordinate regulation of MMP synthesis.

- TGF-β1 up-regulates α6β4 expression in MDA-MB 231 cells, but does not induce de-novo expression of this integrin in cells which do not normally express it. The high invasive capacity of MDA-MB 231 cells is dependent on α6β4 integrin, however, in the presence of TGF-β1, α6β4 is translocated from actin-associated structures to IF-associated structures and has an anti-invasive effect. This is associated with enhanced synthesis of MMP-9, MT1-MMP, TIMP-1 and TIMP-2, but a net reduction in MMP-2-activating potential. The mechanism by which TGF-β1 influences the changes in function and localisation of α6β4 is unclear but may be a direct effect on the cytoskeleton or via matrix protein synthesis. Further work is required to address this.

- MCF-7 cells showed no change in integrin expression, or expression of MMP and TIMPs, however, they did display a significant increase in invasion. It is possible that TGF-β1 is

affecting other proteolytic enzymes which have not been investigated in this study. The

findings emphasise the cell-type-specific effect of TGF-β1.

Chapter 4

The Effect of Cell Interactions on Tumour Cell Invasion and on

MMP Expression

**AIM: To introduce into the cell culture model system (i) stromal fibroblasts and (ii)
normal breast myoepithelial cells, and to analyse the effect of these cellular interactions
on tumour cell invasion and on MMP/TIMP expression and activity.**

4.1 Introduction

In the human breast the epithelial and stromal compartments are separated by the specialised

basement membrane. With development of invasive breast cancer, tumour cells penetrate the

limiting BM and acquire a different relationship with the host stroma as they move through the

extracellular matrix. The stroma is not a passive barrier in this process but an active biological

compartment which responds to and co-operates with the tumour cell population to provide it

with a local environment favouring tumour progression (Starkey, 1990). The importance of

tumour-host interactions in facilitating tumour progression was demonstrated over a decade ago

when it was reported that pre-induction of a reactive stroma at the site of tumour

transplantation dramatically increased the invasion of the carcinoma (Gabbert, 1985). The

stromal compartment is also important for tumour cell growth, providing growth factors and

blood vessels essential for tumours.

Stromal fibroblasts have been shown to influence the growth and tumourigenicity of

tumour cells. Tumour cell growth has been shown to be promoted or inhibited by co-culture

with fibroblasts or with fibroblast CM (Picard et al, 1986; Miller et al, 1989; Cornil et al, 1991;

Mukaida et al, 1991), and co-culture of weakly metastatic tumour clone cells with normal

fibroblasts substantially enhances lung tumour colonisation in-vivo (Picard et al, 1986; Tanaka

et al, 1988)

The frequent localisation of MMP mRNA to the stromal compartment of a tumour also

suggests that the stromal response to malignant cells is important in modulating synthesis and

221

release of pro-invasive factors. Thus the aim of this part of the study is to use the cell culture

system described in Chapter 3 to analyse directly the effects of tumour cell-stromal cell

interaction on tumour invasion and on expression of MMPs and TIMPs.

It is increasingly evident that the BM is not merely a scaffold providing structural

support for cells. Cellular interactions with the BM play a major role in controlling growth,

organisation and differentiation of epithelial cells (Barcellos-Hoff et al, 1989; Roskelly et al,

1995). However, in the resting human breast, luminal epithelial cells, from which the majority

of breast cancers are thought to arise, do not lie in direct contact with BM but are separated

from it by the myoepithelial cell layer (Gusterson et al, 1982; Tsubura et al, 1988; fig. 4.1).
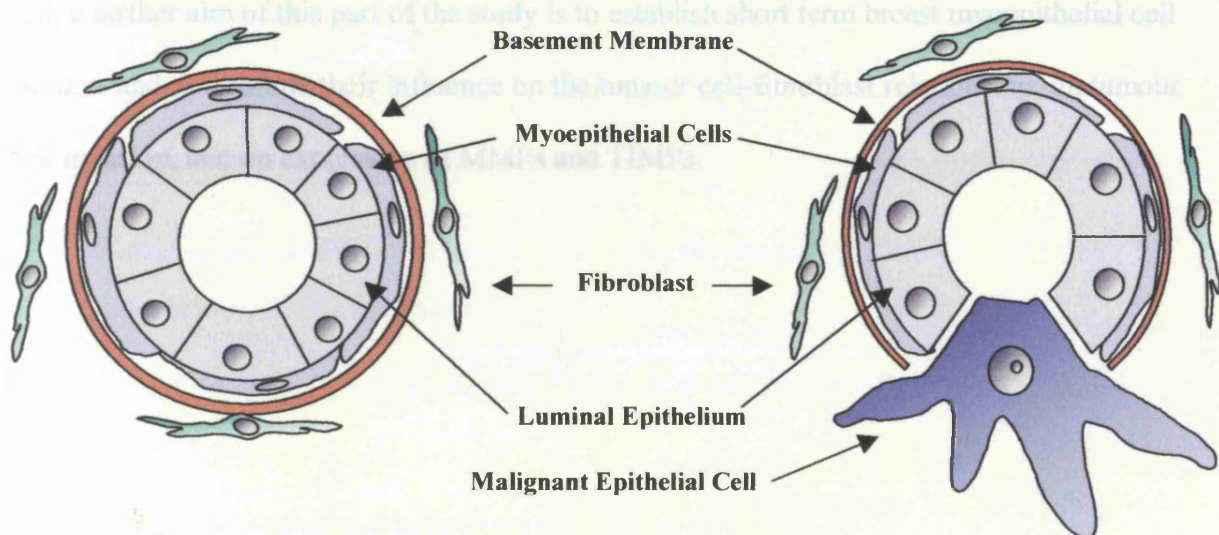
Fig. 4.1: Relationship Between Myoepithelial Cell, Basement Membrane and Luminal Epithelial Cell in Breast Ducts. In normal breast ducts, myoepithelial cells form the interface with the stromal compartment. With development of malignancy, myoepithelial cell layer is lost and epithelial cells have direct relationship with stroma.

Thus the modulatory influences of the BM must be signalled via the myoepithelial cell

population. Myoepithelial cells are well adapted for such a regulatory function. As well as

being intimately connected to both luminal epithelial cells and BM (Koukoulis et al, 1991),

myoepithelial cells express high levels of growth factor receptors such as Epidermal Growth

Factor Receptor (Monaghan et al, 1995) and Fibroblast Growth Factor Receptor (Gomm et al, 1991). Activin, a member of the TGF-β superfamily, is expressed exclusively by myoepithelial cells in the breast, and has been shown to modulate tubule formation in normal breast in-vitro, and to inhibit growth of MCF-7 cells (Liu et al, 1996), providing indirect evidence of a modulatory role for myoepithelial cells. Bani et al (1994) demonstrated that co-culture of a myoepithelial cell line derived from salivary gland pleomorphic adenoma with MCF-7 cells promoted differentiation of the breast cancer cells, with development of apical microvilli, intercellular junctional complexes and occasional pseudoluminar structures. More recently, Shao et al (1998) have shown that myoepithelial cells inhibit proliferation of breast cancer cell lines through secreted paracrine mediators. In order to investigate the role of the myoepithelial cell, a further aim of this part of the study is to establish short term breast myoepithelial cell cultures and to examine their influence on the tumour cell-fibroblast relationship, on tumour cell invasion, and on expression of MMPs and TIMPs.

4.2 Materials

4.2.1 Normal breast tissue

Breast tissue was obtained from cosmetic reduction mammoplasty procedures carried out at the Leicester Royal Infirmary. Tissue was collected in clean plastic bags immediately following completion of the surgical procedure, examined under aseptic conditions and blocks taken for routine histology prior to selection of the tissue for culture. Tissue from women with a known family history of breast cancer or a past history of breast surgery was not used in this study.

4.2.2 Tissue culture reagents

As for section 3.2.2, and in addition, Ham's Nutrient F12 media from Gibco, and human recombinant Epidermal Growth Factor (EGF), hyaluronidase Type IV-S, Collagenase Type IA, penicillin and streptomycin solution, and fungizone all from Sigma. Nylon mesh cell strainers (40 µm) by Falcon from Becton Dickinson.

4.2.3 Materials for Cell Separation

The cell separation method was based on the use of magnetic polystyrene beads from Dynal UK Ltd, as follows:

- Dynabeads M-450 coated with sheep anti-mouse IgG1 (Fc)

- anti-epithelial cell Dynabeads coated with mouse IgG1 monoclonal antibody (BerEP4) specific for two glycopeptide membrane antigens (34 kDa and 39 kDa) expressed on most normal epithelial cells.

- Magnetic Particle Concentrator (MPC) comprising a magnet encased in polyethylene.

4.2.4 Media recipes

Basic media: 90% DMEM, 10% FBS, 100IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone

Enriched media: 1:1 DMEM : Ham's F12, 10% FBS, 5 μg/ml insulin, 5 μg/ml hydrocortisone, 20 ng/ml EGF, 100IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone .

4.2.5 Antibodies

Antibodies as previously described and in addition:

Anti-Smooth Muscle Actin (SMA)

Mouse monoclonal antibody from Dako. This was raised against the N-terminal decapeptide of α-actin and recognises α-actin but not β- or γ-actin. It labels vascular smooth muscle cells as well as myoepithelial cells and some breast stromal cells (Peterson et al, 1980).

Anti-Human Milk Fat Globule Membrane Antigen 1 (HMFG-1)

Mouse monoclonal antibody to HMFG-1, clone 1.10 F3. Directed against an epitope on a mucin-like glycoprotein expressed in milk fat globule membranes. This epitope is well exposed in normal breast epithelial cells.

4.2.6 RT-PCR on Separated Cell Populations

Oligonucleotide primers for PCR were designed with the help of Dr. Howard Pringle, as described in section 3.2.3. The oligonucleotides were synthesised by Oswell or Genosys. Details of the primer sequences are given in Table 4.1.

Table 4.1: Primer Sequences for PCR

GENE	PRIMER SEQUENCE	SIZE	REFERENCE
β 4	F: AGGAGAACCTGAACGAGGTC R: ATCAGCACTGTGTCCACAATG	124	N/A
CK14	F:CCCTACTTCAAGACCATTGAGG R:GTCACGCATCTCGTTCAGAA	319	N/A
CALLA	F:TTGTAAGCAGCCTCAGCC R:TTGTCCACCTTTTCTCGG	460	Gomm et al, 1995
EMA	F:GAGGATCCGCTCCACCTCTCAA R:CCAAGCTTCTGGGCAATGAACT TCTCTGGGTAG	*	Gomm et al, 1995

N/A : Not applicable

* Variable amplicons of 254, 227, 218 & 191 bp generated due to alternative splicing

4.3 Methods

4.3.1 Isolation and Expansion of Normal Breast Cells

Normal breast tissue was obtained from reduction mammoplasty procedures and digested using a modification of the technique described by Stampfer et al (1980). The tissue was dissected free of fat, cut into 1mm cubes and incubated for 12-18 hrs in a digestion mixture of 200 units/ml Collagenase type IA and 125 units/ml Hyaluronidase in DMEM containing 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. Following digestion the fat layer was decanted and the mixed population of organoids and single cells was washed twice in cold Basic Medium. In initial digestions, the suspension was filtered through a 90 µm pore polyester mesh in an attempt to yield organoid-free cultures, however, after several procedures, this step was omitted and the following procedure adopted. After resuspension, a single sedimentation step of 30 mins at 4°C was carried out and the supernatant rich in fibroblasts and blood cells was removed and retained for generation of fibroblast-rich cultures. Slightly different procedures were adopted according to the cell type required: fibroblast rich cultures were maintained in Basic Medium and passaged 2-3 times prior to extraction of contaminating epithelial and myoepithelial cells. For the generation of myoepithelial-rich cultures, organoids were washed in Enriched Medium and transferred to culture flasks where they were maintained in Enriched Medium for approximately 5 days. Contaminating fibroblasts were removed from these cultures by differential trypsinisation. Since epithelial cells were not used for the experiments in this study, methods used to enrich for these cells will not be described.

When the cultures reached subconfluence, cells were harvested by trypsinisation, washed in cold PBS and passed through a 40 µm filter to remove any residual organoids. Cell counts were performed on the resulting suspensions prior to separation of the cell populations.

4.3.2 Separation of Cell Populations

Antibody-coated magnetic Dynabeads were used to give positive selection of myoepithelial and epithelial cells with negative selection of fibroblasts. Initial studies compared different selecting antibodies, as outlined in Table 4.2, and the final method of selection chosen employed beads pre-coated with sheep anti-mouse IgG_1 conjugated with anti-$\beta4$ integrin antibody for myoepithelial cells, and anti-epithelial cell Dynabeads, pre-coated with antibody to BerEP4 for the positive selection of luminal epithelial cells. Further optimisation of the method was achieved by comparing different antibody concentrations required for efficient bead labelling, different bead : target cell numbers, and also by defining the optimal sequence of incubations. The final method of separation used is outlined schematically in figure 4.2, and is described below.

Table 4.2: Range of antibody-labelled beads used for cell separation

EPITHELIAL	MYOEPITHELIAL
EMA-conjugated IgG_1 beads	CALLA-conjugated IgG_1 beads
BerEP4 labelled beads	$\beta4$-integrin-conjugated IgG_1 beads

- *labelling of beads:* Aliquots of the sheep anti-mouse IgG_1 coated beads (1×10^6 beads in 2.5 μl) were washed three times in cold PBS/1% BSA, the supernatant being removed each time using a magnetic particle concentrator (MPC). The washed beads were then incubated with anti-$\beta4$ integrin antibody at a ratio of 2 μg antibody : 10^6 beads for 18 hrs at $4°C$ with rotation. Following incubation the beads were washed three times in cold PBS/1% BSA for 30 mins each wash at $4°C$ with rotation to remove excess unbound antibody. Prior to

incubation with the cell suspensions both anti-epithelial cell beads and β4-integrin labelled beads were washed briefly three times in serum-free DMEM.

- *cell separation steps:* Aliquots of the mixed cell suspension, containing 2 x 10⁶ cells, were incubated sequentially for 30 mins at 4°C with 1 x 10⁶ labelled beads as follows:

  - first round incubation with anti-β4 integrin labelled beads

  - second round incubation with anti-β4 integrin labelled beads

  - incubation with 1 x 10⁶ anti-epithelial cell beads

Following each incubation, specifically bound cells were collected using the MPC and the supernatants transferred to the next incubation step. In the case of fibroblast-rich cultures, the second round incubation step with β4 integrin-labelled beads was omitted, since contaminating myoepithelial cells were in low concentration and efficiently removed by one round of extraction. The final fibroblast-rich supernatant and the bead-bound cell populations were then washed in cold Basic Medium and cell counts on each population carried out before transferring the epithelial cells and fibroblasts to flasks containing Basic Medium, and the myoepithelial cells to flasks containing Enriched Medium. The cell populations were then allowed to further expand over 3-5 days before being harvested for use in experiments.

4.3.3 Characterisation of Separated Cell Populations

In the early experiments, aliquots of cells were plated onto coverslips after each step in the separation procedure. Once the method had been optimised, aliquots of the final selected cell populations only were taken for a series of verification procedures:
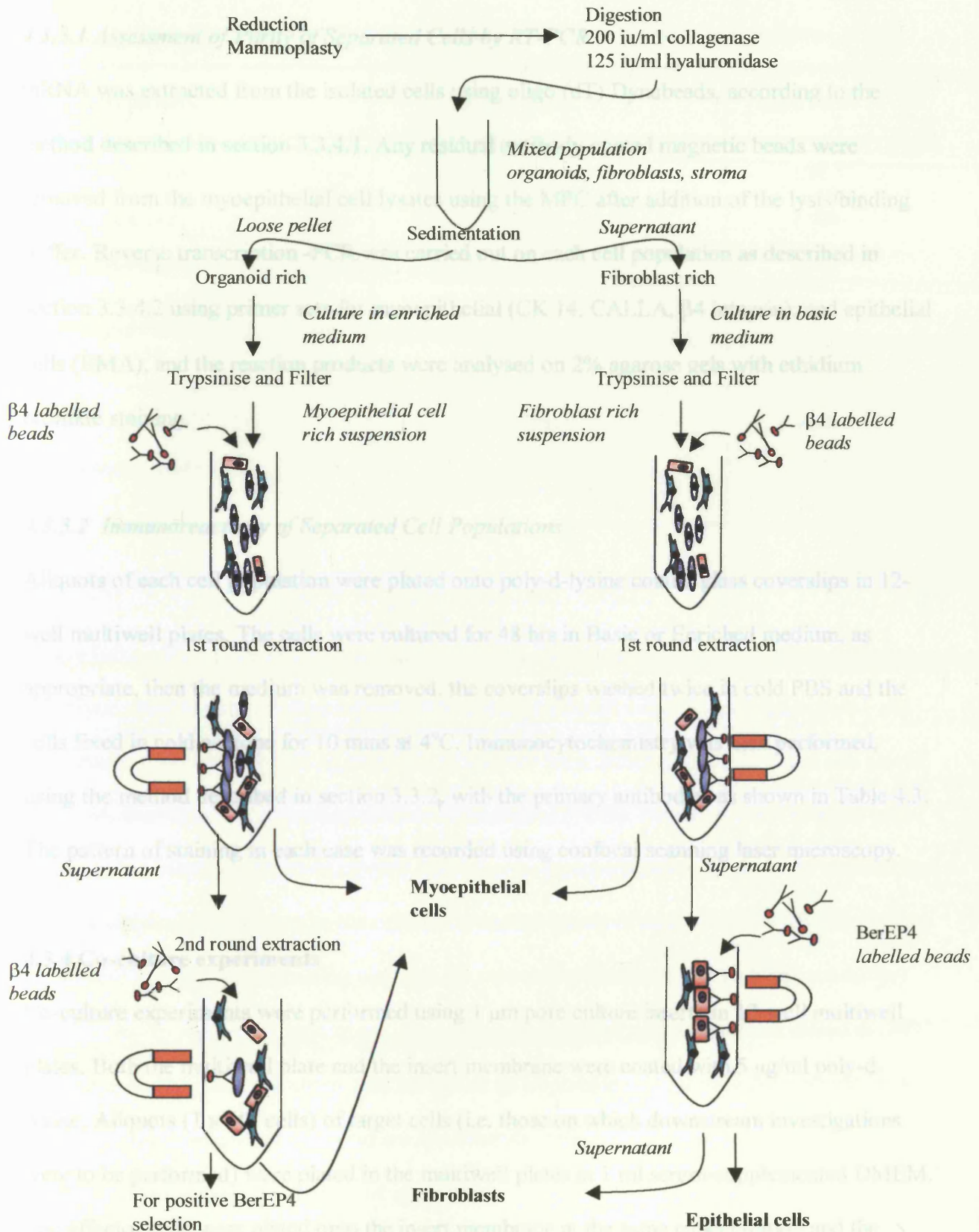
Fig 4.2: Schematic Outline of Cell Separation Procedure

4.3.3.1 Assessment of Purity of Separated Cells by RT-PCR

mRNA was extracted from the isolated cells using oligo (dT) Dynabeads, according to the

method described in section 3.3.4.1. Any residual antibody-coated magnetic beads were

removed from the myoepithelial cell lysates using the MPC after addition of the lysis/binding

buffer. Reverse transcription -PCR was carried out on each cell population as described in

section 3.3.4.2 using primer sets for myoepithelial (CK 14, CALLA, β4 integrin), and epithelial

cells (EMA), and the reaction products were analysed on 2% agarose gels with ethidium

bromide staining.

4.3.3.2 Immunoreactivity of Separated Cell Populations

Aliquots of each cell population were plated onto poly-d-lysine coated glass coverslips in 12-

well multiwell plates. The cells were cultured for 48 hrs in Basic or Enriched medium, as

appropriate, then the medium was removed, the coverslips washed twice in cold PBS and the

cells fixed in cold acetone for 10 mins at 4°C. Immunocytochemistry was then performed,

using the method described in section 3.3.2, with the primary antibodies as shown in Table 4.3.

The pattern of staining in each case was recorded using confocal scanning laser microscopy.

4.3.4 Co-culture experiments

Co-culture experiments were performed using 1 µm pore culture inserts in 12-well multiwell

plates. Both the multiwell plate and the insert membrane were coated with 5 µg/ml poly-d-

lysine. Aliquots (1 x 10^5 cells) of target cells (i.e. those on which downstream investigations

were to be performed) were plated in the multiwell plates in 1 ml serum-supplemented DMEM.

The effector cells were plated onto the insert membrane at the same concentration and the

cultures established in separate multiwell plates in serum-supplemented DMEM for 24 hrs.

231

Table 4.3: Details of Antibodies used to Characterise Bead-Separated Cell Populations

ANTIBODY	TARGET CELL	DILUTION
E-Cadherin	Epithelial & Myoepithelial	1:50
HMFG-1	Epithelial	1:100
β4-integrin	Myoepithelial	1:200
CALLA	Myoepithelial	1:100
CK14	Myoepithelial & subpopulation of Epithelial cells	1:5
CK18	Epithelial	neat
Smooth Muscle Actin	Myoepithelial & subpopulation of stromal cells	1:100

Where three cell populations were being co-cultured, the system illustrated in figure 4.3 was employed, whereby the effector cell population (myoepithelial cells) was seeded onto a 1 μm pore insert, precoated with 50 μg Matrigel, which had been detached from its frame and placed inside another insert so generating a three-compartment system. Parallel control cultures were set up with a Matrigel coated membrane insert but no myoepithelial cells. Once the cultures had been established independently, the cells were washed gently in serum-free DMEM, the inserts introduced into the corresponding well and the co-cultures incubated for 48 hrs in serum-free DMEM. At the end of the experiment, the media was collected, centrifuged and stored at $-70^{\circ}C$ for future analysis, and the target cells harvested by trypsinisation and mRNA extracted for RT-PCR analysis. Two groups of co-culture experiments were performed to investigate:

(i) the effect of tumour cell-fibroblast interactions

(ii) the effect of myoepithelial cells on tumour cells and fibroblasts.

The co-cultures performed are detailed in Table 4.4. Each experiment was performed in duplicate at least twice.
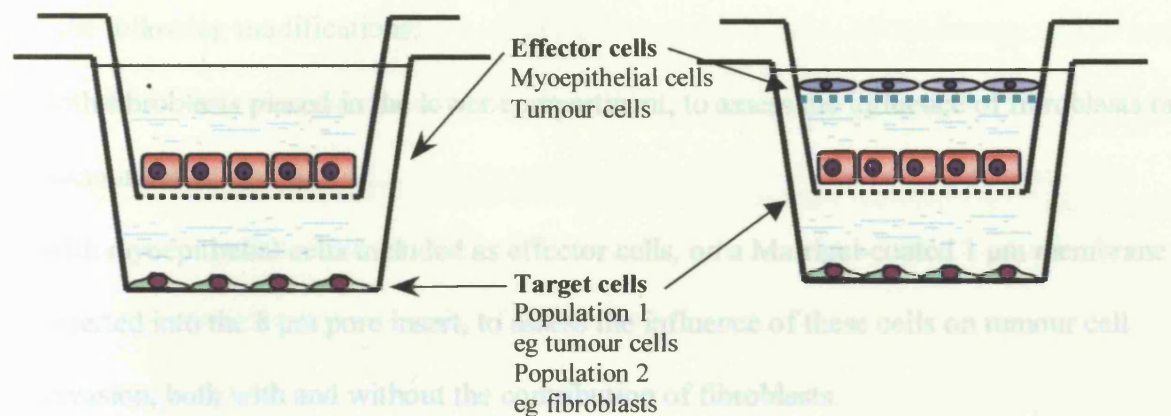
Fig. 4.3: System Employed for Two and Three Population Co-cultures

Table 4.4: Combinations of Co-cultures Performed

TARGET POPULATION	EFFECTOR POPULATION
Fibroblasts F518	Tumour cell line
Primary breast fibroblasts	Tumour cell line
Tumour cell line	Fibroblasts F518
Tumour cell line	Primary breast fibroblasts
Tumour cell line	Myoepithelial cells
F518 & Tumour cell line	Myoepithelial cells
Primary fibroblasts & Tumour cell line	Myoepithelial cells

233

4.3.5 Effect of Fibroblasts and Myoepithelial Cells on Tumour Cell Invasion

Invasion assays were performed to assess the effect of fibroblasts and myoepithelial cells on tumour cell invasion. The assay conditions used were the same as described in section 3.3.1 with the following modifications:

- with fibroblasts placed in the lower compartment, to assess the influence of fibroblasts on tumour cell invasion

- with myoepithelial cells included as effector cells, on a Matrigel-coated 1 μm membrane inserted into the 8 μm pore insert, to assess the influence of these cells on tumour cell invasion, both with and without the contribution of fibroblasts.

Invasion indices were calculated in the same way as for previous invasion assays. Assays were repeated in duplicate at least three times. Statistical analysis was performed using the student t test, or if the samples were found to vary, the Mann Whitney U test.
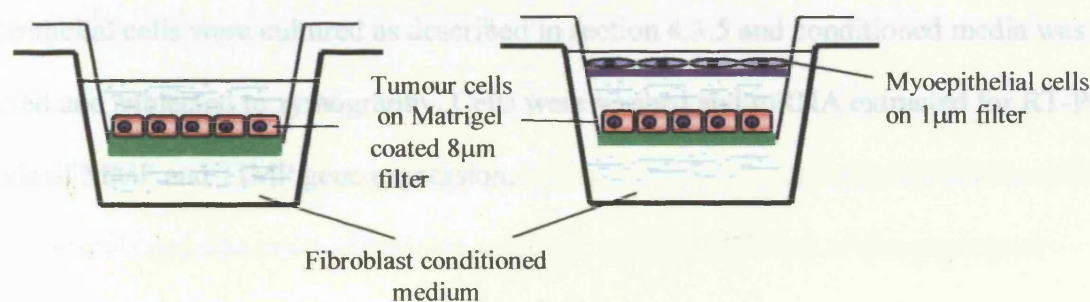
$$\text{Invasion Index} = \frac{N^{\circ} \text{ cells on lower filter}}{N^{\circ} \text{ cells on upper filter + lower filter}} \times 100\%$$

Fig. 4.4: Invasion Assay Modified for use with Co-cultures

4.3.6 Analysis of MMP and TIMP Expression

4.3.6.1 RT-PCR-ELOSA Quantitation of MMP and TIMP Expression

The target cell population from each co-culture experiment was harvested by trypsinisation and mRNA extracted from 1 x 10^5 cells as described in section 3.3.4.1. cDNA was generated and PCR with ELOSA quantitation performed for MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2 and GAPDH, as previously described (section 3.3.3.2 & 3.3.3.4). Statistical analysis was performed on the mean gene: GAPDH ratio taken from four readings using the student t test or if the samples were found to vary, the Mann Whitney U test.

4.3.6.2 Substrate Gel Zymography

CM generated from each of the co-culture experiments was subjected to zymographic analysis as described in section 2.3.4.

4.3.7 Analysis of MMP and TIMP Expression by Myoepithelial Cells

Myoepithelial cells were cultured as described in section 4.3.5 and conditioned media was collected and subjected to zymography. Cells were washed and mRNA extracted for RT-PCR analysis of MMP and TIMP gene expression.

235

4.4 Results

4.4.1 Isolation of Normal Breast Cells

Over 50 reduction mammoplasty specimens were processed for culture during the course of this study. All were obtained from pre-menopausal women aged between 17 and 42 years. Representative histological sections from each case confirmed the absence of significant pathology in these specimens, with just a proportion of cases showing features of mild fibrocystic change.

Initial attempts at digestion and culture of isolated cells separated from organoids were thwarted by problems with infection at an early stage in the process. For this reason, the procedure was simplified, removing as many unnecessary steps as possible: instead of filtering suspensions through mesh to separate organoids from single cells, a sedimentation step was introduced. This yielded organoid rich preparations and a suspension of single cells, rich in fibroblasts. Further digestion of organoids at this stage to yield a single cell preparation resulted in poor cell viability, so subsequently organoids were plated out in different media to promote growth of epithelial, myoepithelial and stromal cells. Culture of organoids in Enriched media favoured the growth of myoepithelial cells over luminal cells, and differential trypsinisation effectively removed most contaminating fibroblasts. Prior to cell separation, organoid rich flasks contained approximately 75% myoepithelial cells, on the basis of morphological characteristics on phase contrast microscopy. The fibroblast-rich cultures, generated from plating out the organoid supernatant, grew rapidly in Basic Media and following 2-3 passages, just prior to separation these flasks contained approximately 90% fibroblasts on the basis of morphological criteria.

4.4.2 Separation of Cell Populations

After approximately 5 days in culture (depending on the degree of confluence) the cells were harvested by trypsinisation and organoids removed, either by sedimentation or by filtering to yield single cell suspensions. Cell counts at this stage ranged between 10-20 x 10^6 cells in myoepithelial-rich cultures and 20 -25 x 10^6 cells in fibroblast-rich cultures.

Initially, CALLA-labelled beads were used to extract myoepithelial cells and HMFG-1-labelled beads to extract epithelial cells. Culture of the resultant isolates, however, demonstrated that the CALLA beads selected both myoepithelial and fibroblastic cells (as determined by morphology on phase contrast microscopy), and the yield of epithelial cells was extremely low, with the cultures containing many unattached beads. Different markers were therefore selected for the isolation procedure, with the myoepithelial-specific β4 integrin and the BerEP4 labelled anti-epithelial beads being chosen for selection of myoepithelial and epithelial cells, respectively. After extraction, bead-labelled cells were plated into multiwell plates to assess visually the nature of cells extracted, and also to determine cell viability following the extraction procedure. From initial extractions, it was evident that BerEP4 labelled beads selected both myoepithelial and epithelial cells, however, the β4-integrin extraction was very efficient, giving high yields of pure myoepithelial cells. It was found that if cells were incubated with β4-integrin beads first, followed by incubation with BerEP4, contamination of the latter extractions with myoepithelial cells was much reduced, and virtually eliminated with the introduction of a second round of β4-integrin extraction. This method was therefore employed for all further separations.

The viability of extracted cells was assessed visually as the approximate percentage of labelled cells not adhering to wells after 12 hrs in culture. In early extraction procedures, viability for both myoepithelial and epithelial cells was low, ranging from 25% -35%. It was

observed that frequently the cells which did not adhere to culture wells were those heavily labelled with beads, therefore the bead to target cell ratio was reduced, and cell viability improved, with approximately 60%-70% of myoepithelial cells adhering and 50%-60% of epithelial cells, and it was noted that many of the selected cells were now labelled by only 2-3 beads. Thus, for positive selection incubations (i.e. for extraction of myoepithelial cells for culture), low bead to target cell ratios were employed (approximately 1:1), whilst for negative selection procedures (i.e. to deplete fibroblast-rich suspensions of residual myoepithelial and epithelial cells) high bead to target cell ratios were employed (approximately 10:1). Magnetic beads were not detached from the selected cells prior to culture, but it was noted that beads were rapidly internalised by the labelled cells (within 8 hours) or shed into the medium, and at the time of introducing myoepithelial cells into co-culture experiments, only occasional cells still contained beads

4.4.3 Characterisation of Separated Cell Populations

Following these extraction procedures, isolated cells were analysed by RT-PCR to assess the purity of the populations and by immunocytochemistry to verify the characteristics of the cells.

4.4.3.1 *Assessment of Purity of Separated Cells by RT-PCR*

RT-PCR on aliquots of cells isolated using β4-integrin labelled beads yielded amplicons for β4-integrin, the basal cytokeratin CK14, and CALLA, with no band generated using primers for EMA. The negatively selected fibroblast population amplified for CALLA only, with no bands obtained following PCR for β4-integrin, CK14 or EMA. RT-PCR for these markers on normal breast tissue showed appropriately sized products with all primer sets (fig. 4.5).
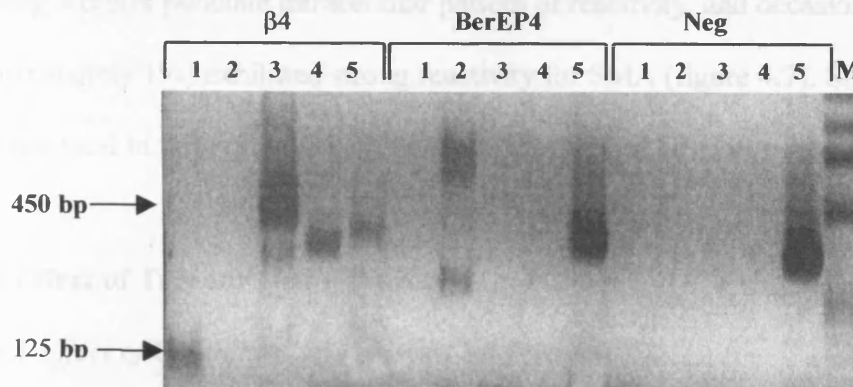
Fig. 4. 5: RT-PCR Analysis of Separated Cell Populations
β4= cells selected on β4-labelled beads; BerEP4=cells selected on BerEP4-labelled beads; Neg= negatively selected cells. Lanes: 1=b4 integrin; 2=EMA; 3= CALLA; 4=CK14; 5=GAPDH

4.4.3.2 Immunoreactivity of Separated Cell Populations

Aliquots of each cell population were seeded onto coverslips in multiwell plates and assessed for their expression of a range of markers typically demonstrated on epithelial, myoepithelial or stromal cells. The β4-integrin selected cells, which grew as sheets of well-demarcated polygonal cells, showed strong polarised expression of β4-integrin. They also exhibited reactivity for CK14 and E-Cadherin, but they did not stain with antibodies to HMFG-1 or CK18. The cells were negative or weakly positive for CALLA and SMA (figure 4.6). In less than 5% of aliquots from total number of cultures, occasional contaminating fibroblasts were identified; these had a typical fibroblastic morphology, did not react with β4-integrin, HMFG-1, CK14 or CK18. When these were identified in a culture, the isolated cells were put through another round of extraction with β4-integrin beads prior to their use in any of the experiments.

The negatively selected cells, yielded after incubation with β4-integrin beads and BerEP4 beads, exhibited a typical fibroblastic morphology. They did not show staining for β4-integrin, E-Cadherin, HMFG-1, CK14 or CK18. A proportion of cells did stain for CALLA,

showing a bright punctate intracellular pattern of reactivity, and occasional cells

(approximately 1%) exhibited strong reactivity for SMA (figure 4.7). Since the epithelial cells

were not used in this study, their characteristics will not be discussed.

4.4.4 Effect of Tumour Cell -Fibroblast Co-culture

4.4.4.1 Effect of Co-culture on Tumour Cell Invasion

Invasion assays were performed for each of the breast cancer cell lines co-cultured with either

primary breast fibroblasts or the dermal fibroblast cell line F518. The results indicate a

significant increase in tumour cell invasion on co-culturing with either set of fibroblasts, and

there was no difference in the effect mediated by breast or dermal fibroblasts (fig. 4.8).
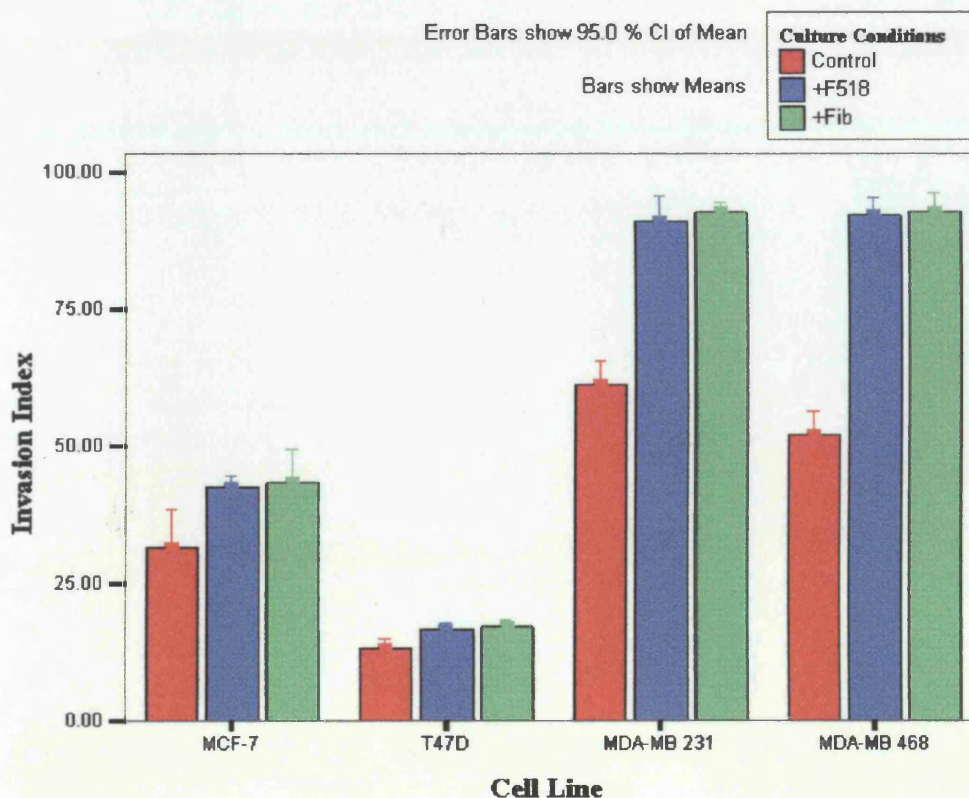
Fig. 4.8: Effect of Co-culture with Fibroblasts on Tumour Cell Invasion.
Bars represent the mean invasion index of three assays and error bars indicate 95% confidence limits of the mean.

240

Fig. 4.6: Characteristics of β4-Integrin Selected Cells

The β_4-integrin-selected cells grow as cohesive well-demarcated sheets. Magnetic beads are internalised by the cells and strong staining for β_4 integrin is observed (upper right). The cells exhibit membrane E-Cadherin, lack HMFG-1, and show strong positivity for the basal CK14 and no CK18 reactivity, in keeping with a myoepithelial phenotype. Very weak, if any, reactivity for the myoepithelial-associated CALLA and SMA is seen following culture for 3-5 days.
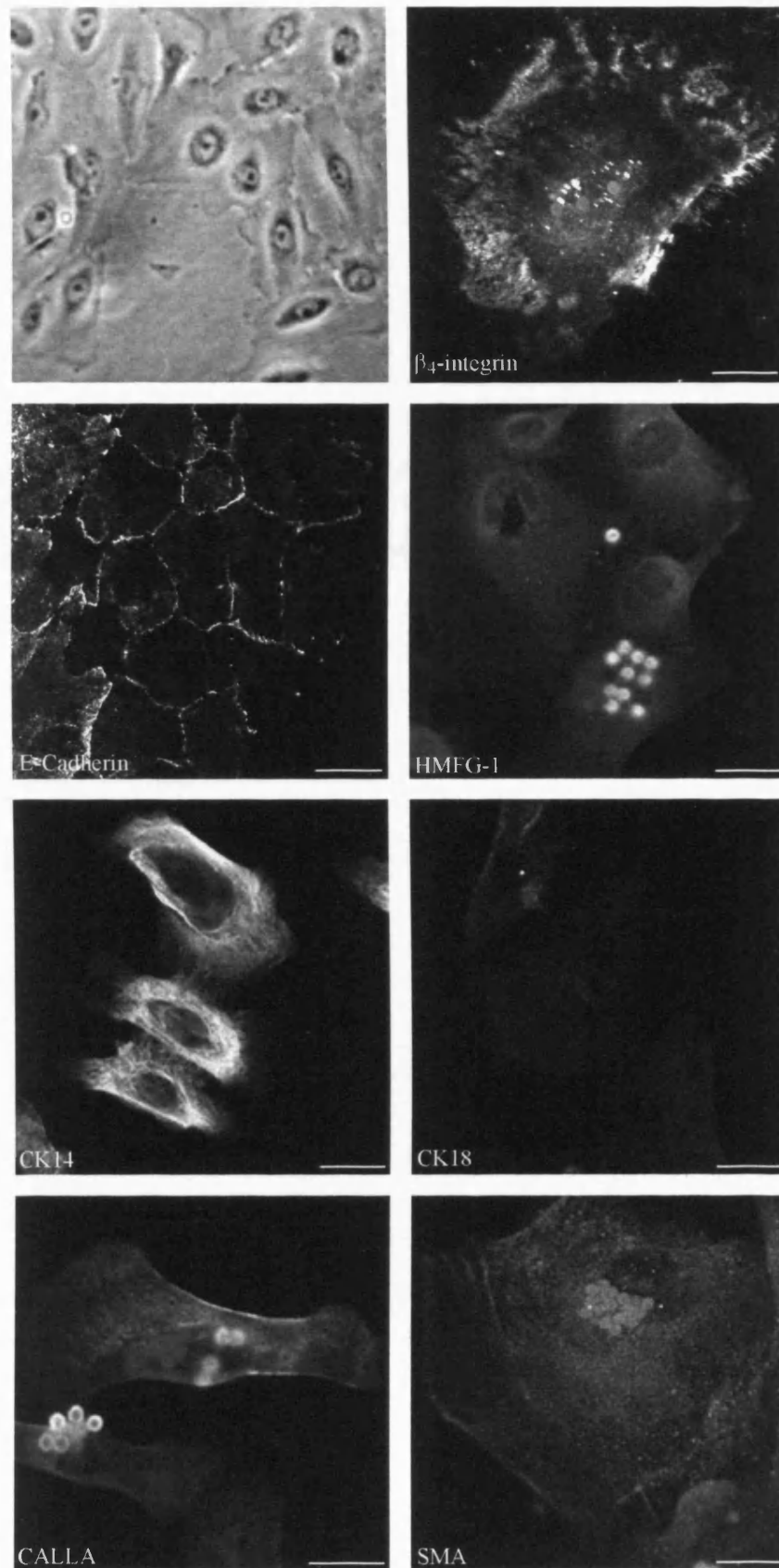
Fig 4.6 : Characteristics of β₄-Integrin Selected Cells

(scale bars = 25 μm)

Fig. 4.7: Characteristics of Negatively Selected Cells

The cells yielded following positive selection with β_4-labelled beads and BerEP4-labelled beads show a typical fibroblastic morphology. In keeping with this, they lack β_4 integrin and E-Cadherin and do not express cytokeratins. A subpopulation of cells exhibit strong punctate cytoplasmic reactivity for CALLA, and whilst the majority of cells lack SMA, occasional cells (~1%) display strong SMA positivity (lower right).
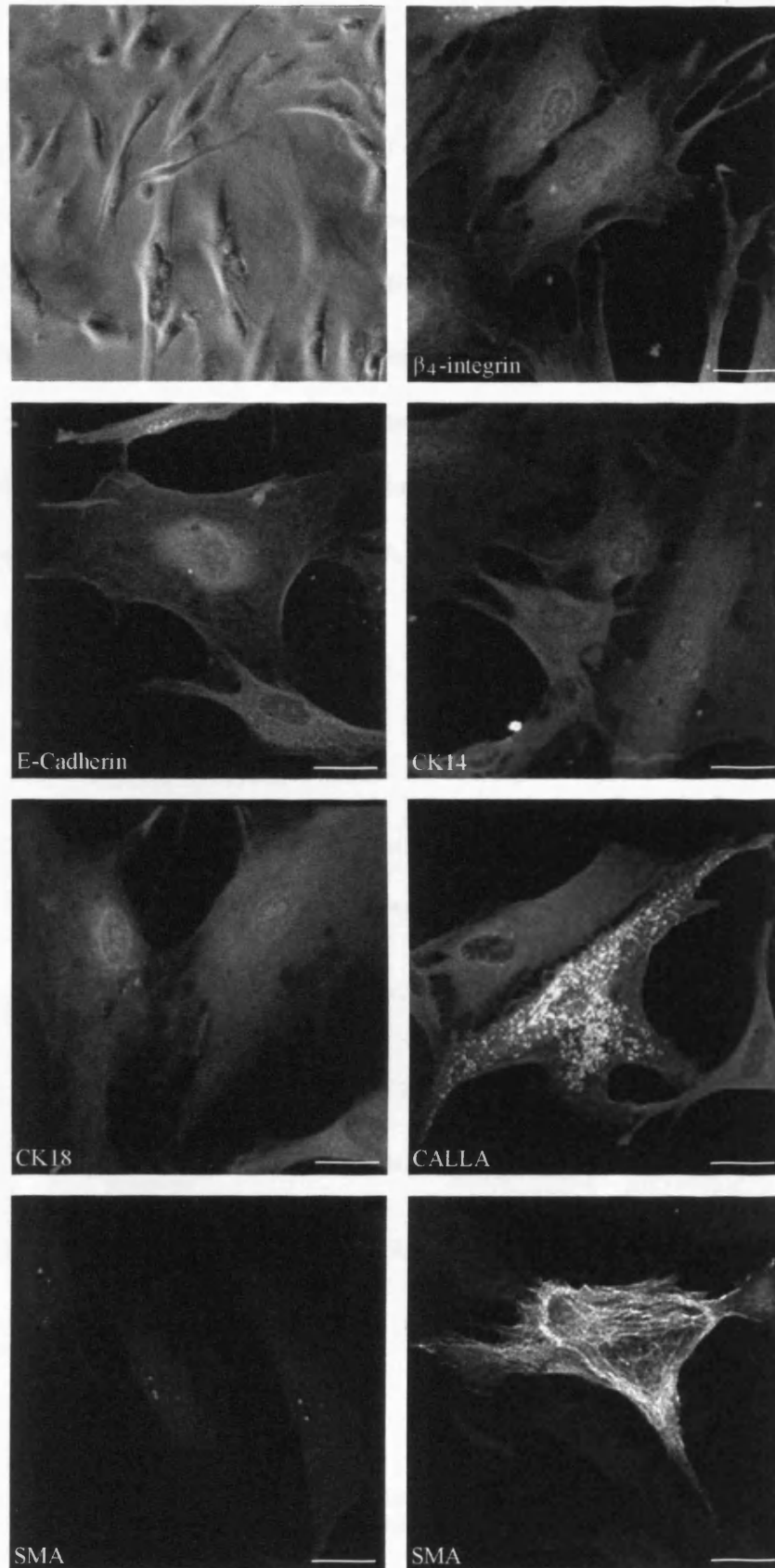
Fig 4.7 : Characteristics of Negatively Selected Cells

(scale bars = 25μm)

When the invasion assays were performed over 48 hours, there was an approximately

1.3 fold increase in invasion exhibited by MCF-7 and T47D cells, increasing from 31.7% for

MCF-7 alone to 42.5% and 43.0% with F518 and primary fibroblasts, respectively, and from

13.1% for T47D cells alone to 16.7% and 17.1% with F518 and primary fibroblasts. MDA-MB

231 cells demonstrated a 1.5 fold increase in invasion and MDA-MB 468 cells a 1.8 fold

increase when assays were carried out over 48 hours, however, because the proportion of these

cells invading after 48 hours was > 90%, assays were repeated over 24 hours (fig. 4.9). This

showed the magnitude of increase in invasion for MDA-MB 231 cells rose from 1.5 fold to 1.8

fold, and for MDA-MB 468 from 1.8 fold to 1.9 fold.
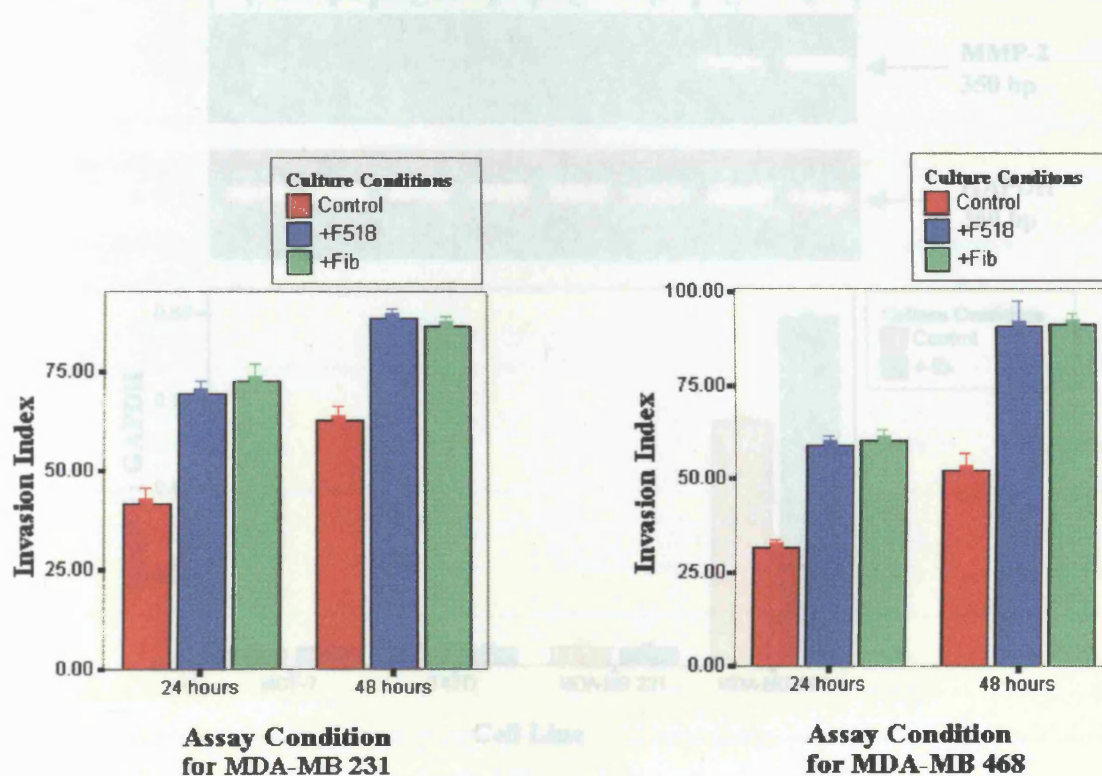
Fig. 4.9: Comparison of Invasion Assays Performed over 24 and 48 hours.
Bars indicate the mean of three assays, error bars indicate 95% confidence intervals.

243

4.4.4.2 Effect of Co-culture on MMP and TIMP Expression

Following co-culture for 48 hours under serum-free conditions, the mRNA was extracted from each population and analysed for levels of MMP and TIMP gene expression.

- Effect of Tumour-Fibroblast Co-culture on Tumour Cell Gene Expression

In the tumour cells there was a significant increase in the expression of MMPs in those tumour cells that already expressed the enzymes under control conditions - there was no de-novo expression of MMPs in MCF-7 or T47D cells (Table 4.5).
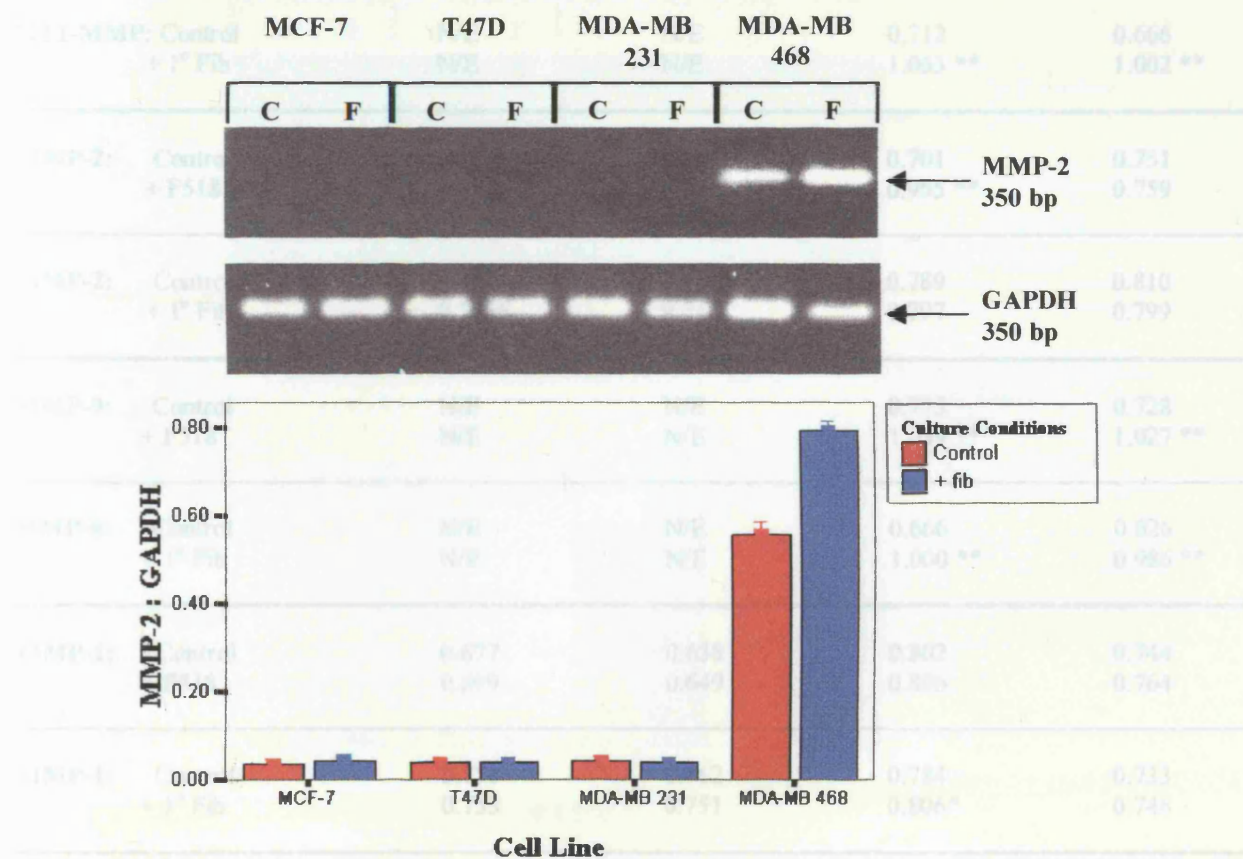
Fig. 4.10: Effect of Tumour Cell-Primary Fibroblast Co-culture on Tumour Cell Expression of MMP-2 Gels show the PCR product following 30 cycles of amplification (C=Control; F=+Fibroblasts). Graph bars represent the mean MMP-2:GAPDH ratio taken from 4 OD readings. Error bars indicate 95% confidence limits of the mean.

244

Table 4.5: Effect of Tumour Cell - Fibroblast Co-culture on Tumour Cell MMP and TIMP Expression ++

		MCF-7	T47D	MDA-MB 231	MDA-MB 468
MMP-2:	Control	N/E	N/E	N/E	0.535
	+ F518	N/E	N/E	N/E	0.746 *
MMP-2:	Control	N/E	N/E	N/E	0.556
	+ 1° Fib	N/E	N/E	N/E	0.794 **
MT1-MMP:	Control	N/E	N/E	0.678	0.650
	+ F518	N/E	N/E	1.020 **	0.967 **
MT1-MMP:	Control	N/E	N/E	0.712	0.666
	+ 1° Fib	N/E	N/E	1.063 **	1.002 **
TIMP-2:	Control	0.769	0.772	0.701	0.751
	+ F518	0.732	0.734	0.955 **	0.759
TIMP-2:	Control	0.708	0.701	0.789	0.810
	+ 1° Fib	0.7556	0.710	0.797	0.799
MMP-9:	Control	N/E	N/E	0.713	0.728
	+ F518	N/E	N/E	1.039 **	1.027 **
MMP-9:	Control	N/E	N/E	0.666	0.626
	+ 1° Fib	N/E	N/E	1.000 **	0.986 **
TIMP-1:	Control	0.677	0.638	0.802	0.744
	+ F518	0.699	0.649	0.806	0.764
TIMP-1:	Control	0.758	0.762	0.784	0.733
	+ 1° Fib	0.753	0.751	0.806*	0.746

++ Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background /minus RT readings.
* = p<0.05; ** = p<0.001; Control = tumour cells alone; F518 = dermal fibroblasts; Fib = normal breast fibroblasts

The level of increase was approximately 1.5 fold for MT1-MMP and MMP-9 ($p<0.001$), and 1.4 fold for MMP-2 ($p<0.05$). There was no significant difference in the magnitude of response to primary fibroblasts or dermal fibroblasts. MDA-MB 231 cells also showed a significant increase in TIMP-1 and TIMP-2 expression in co-culture with primary fibroblasts and in TIMP-2 expression when co-cultured with the F518 dermal fibroblasts. The results are summarised in Table 4.5 and examples are illustrated in fig. 4.10 and fig. 4.11.
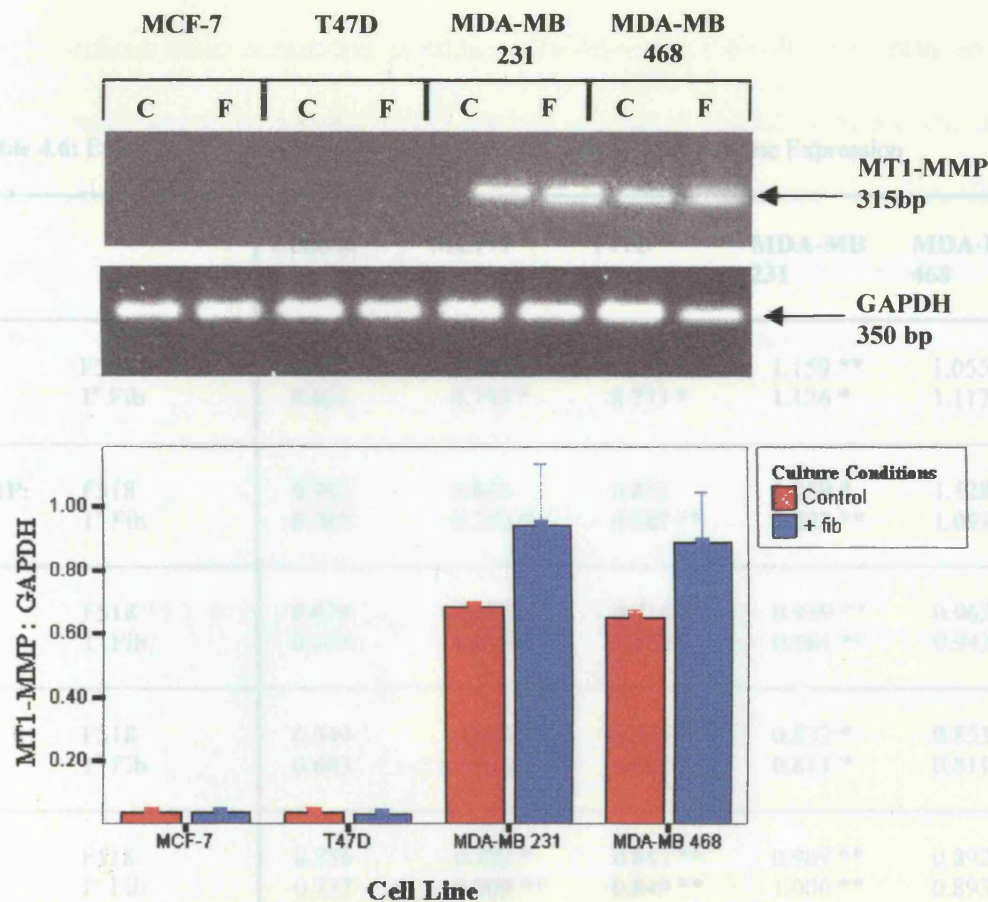
Fig. 4.11: Effect of Tumour Cell – Fibroblast Co-culture on Tumour Cell MT1-MMP Expression. Data represents tumour cells grown with primary breast fibroblasts (C=Control; F=+Fibroblasts). The bars indicate means of four OD readings and the error bars indicate 95% confidence limits.

• Effect of Tumour-Fibroblast Co-culture on Fibroblast Gene Expression

All MMPs were up-regulated in both primary breast and dermal fibroblasts on co-culture with tumour cells though the magnitude of the response varied between the different tumour cells. (Table 4.6). Fibroblasts grown with MCF-7 or T47D cells demonstrated an approximately 1.2 fold increase in expression of MMP-2 and MT1-MMP, with a 1.1 fold increase in MMP-9 expression. In contrast, fibroblasts grown with MDA-MB 231 or MDA-MB 468 cells exhibited a 1.6 - 1.7 fold increase in each of these MMPs.

Table 4.6: Effect of Tumour Cell - Fibroblast Co-culture on Fibroblast Gene Expression

		Control	MCF-7	T47D	MDA-MB 231	MDA-MB 468
MMP-2:	F518	0.677	0.788 **	0.743 *	1.159 **	1.055 **
	1° Fib	0.691	0.798 *	0.773 *	1.126 *	1.117 *
MT1-MMP:	F518	0.782	0.843	0.832	1.259 *	1.128 *
	1° Fib	0.709	0.772 *	0.789 **	1.222 **	1.091 **
TIMP-2:	F518	0.679	0.697	0.714 *	0.939 **	0.962 **
	1° Fib	0.639	0.664	0.665	0.981 **	0.943 **
MMP-9:	F518	0.549	0.665 **	0.654 **	0.832 *	0.853 **
	1° Fib	0.643	0.668	0.687 *	0.811 *	0.811 *
TIMP-1:	F518	0.756	0.792 *	0.842 **	0.989 **	0.892 **
	1° Fib	0.737	0.809 **	0.849 **	1.000 **	0.893 **

++ Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background /minus RT readings.
* = $p<0.05$;   ** = $p<0.001$;   Control = fibroblasts alone;   F518 = dermal fibroblasts;   Fib = normal breast fibroblasts

There was also enhanced expression of TIMPs by fibroblasts in the majority of the co-cultures. A 1.4 fold increase in TIMP-2 was demonstrated by fibroblasts co-cultured with MDA-MB 231 and MDA-MB 468 cells, and 1.2-1.3 fold increase in TIMP-1. A lower level increase was observed in fibroblasts grown with MCF-7 and T47D cells. The results are summarised in Table 4.6 and examples are illustrated in fig. 4.12 and 4.13.

4.4.4.3 Effect of Tumour Cell – Fibroblast Co-culture on Enzyme Activity

Following co-culture under serum-free conditions for 48 hours, the culture medium was removed and equal protein concentrations (1 mg/ml) applied to gelatin substrate gels for zymography. Each set of co-cultures was run on the same gel to minimise variation. The ID for MMP-9, pro-MMP-2 and active MMP-2 is shown in table 4.7a & b, and a representative zymogram is illustrated in fig. 4.14. Zymography demonstrates an increase in the level of MMP-9, pro- and active MMP-2 in all co-cultures compared to fibroblasts grown alone.
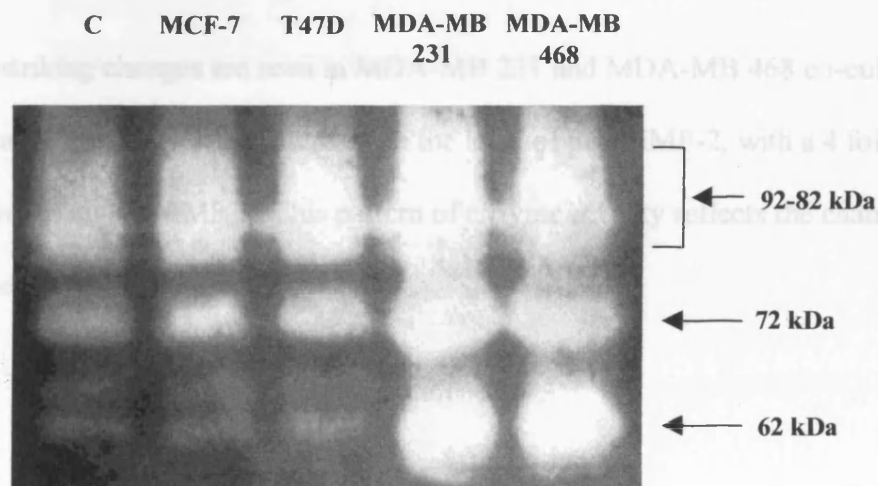
Fig. 4.14: Substrate Gel Zymogram Demonstrating Change in Enzyme Activity on Tumour Cell–Primary Fibroblast Co-culture.
C=Fibroblasts alone; other lanes indicate co-culture of fibroblasts with respective tumour cell line.

Table 4.7: Effect of Tumour Cell Co-culture with Primary Fibroblasts on Enzyme Activity*

	CONTROL	MCF-7	T47D	MDA-MB 231	MDA-MB 468
Fib					
MMP-9	690	870	875	1620	1580
pro-MMP-2	315	430	525	980	950
active MMP-2	145	220	297	1450	1625
F518					
MMP-9	580	720	780	1480	1650
pro-MMP-2	250	390	480	1660	1590
active MMP-2	120	190	210	1300	995

*Values indicate Integrated Density. Fib=Primary Fibroblasts; F518= Dermal Fibroblasts

The most striking changes are seen in MDA-MB 231 and MDA-MB 468 co-cultures where there is an approximately 3 fold increase in the level of pro-MMP-2, with a 4 fold increase in the level of active MMP-2. This pattern of enzyme activity reflects the changes observed in gene expression.
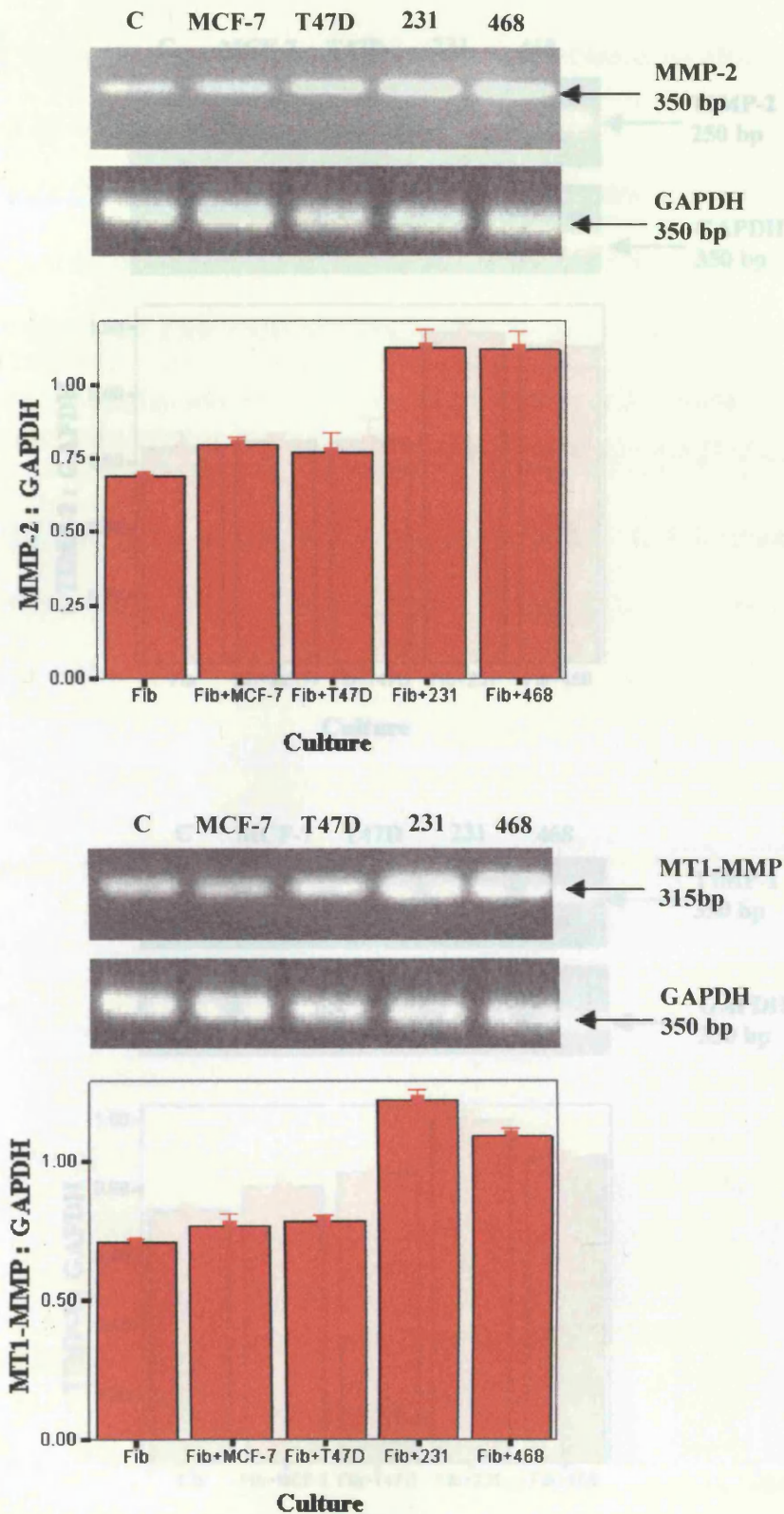
Fig. 4.12: Effect of Tumour Cell-Fibroblast Co-culture on Fibroblast MMP-2 and MT1-MMP Expression. Gels show the PCR product following 30 cycles of amplification. Graph bars represent the mean Enzyme : GAPDH ratio taken from four OD readings. Error bars indicate 95% confidence limits of the means.
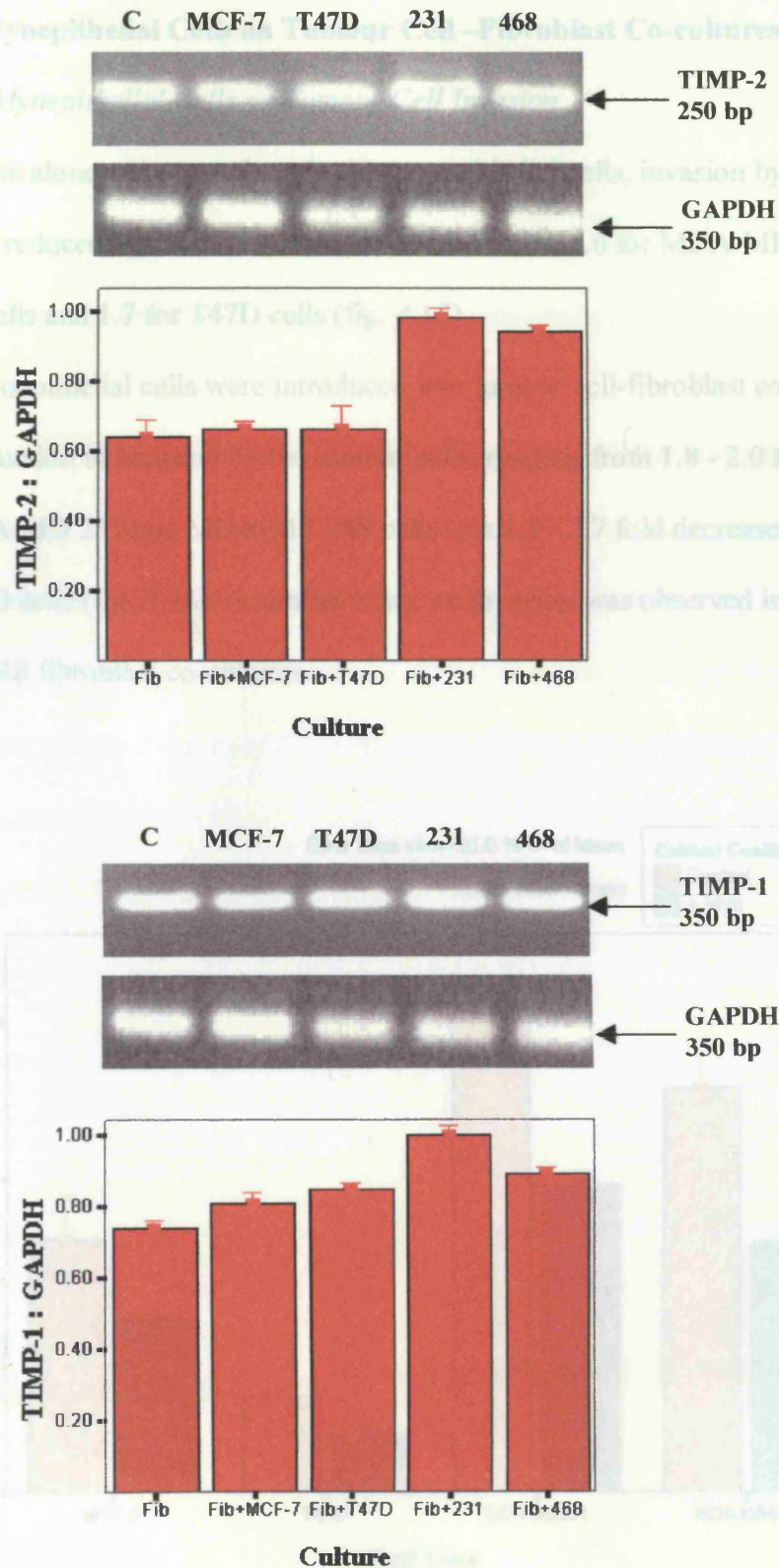
Fig. 4.13: Effect of Tumour Cell-Fibroblast Co-culture on Fibroblast TIMP Expression.
Gels show the PCR product following 30 cycles of amplification. Graph bars represent the mean TIMP : GAPDH ratio taken from four OD readings. Error bars indicate 95% confidence limits of the means.

251

4.4.5 Effect of Myoepithelial Cells on Tumour Cell –Fibroblast Co-cultures

4.4.5.1 Effect of Myoepithelial Cells on Tumour Cell Invasion

When tumour cells alone were co-cultured with myoepithelial cells, invasion by all cell lines was significantly reduced by the order of 1.5 for MCF-7 cells, 1.6 for MDA-MB 231 and MDA-MB 468 cells and 1.7 for T47D cells (fig. 4.15).

When myoepithelial cells were introduced into tumour cell-fibroblast co-cultures there was a similar reduction in invasion by the tumour cells, ranging from 1.8 - 2.0 fold reduction in invasion by MDA-MD 231 and MDA-MB 468 cells to a 1.9 - 2.7 fold decrease in invasion by MCF-7 and T47D cells (fig. 4.16). A similar effect on invasion was observed in both primary fibroblast and F518 fibroblast co-cultures.
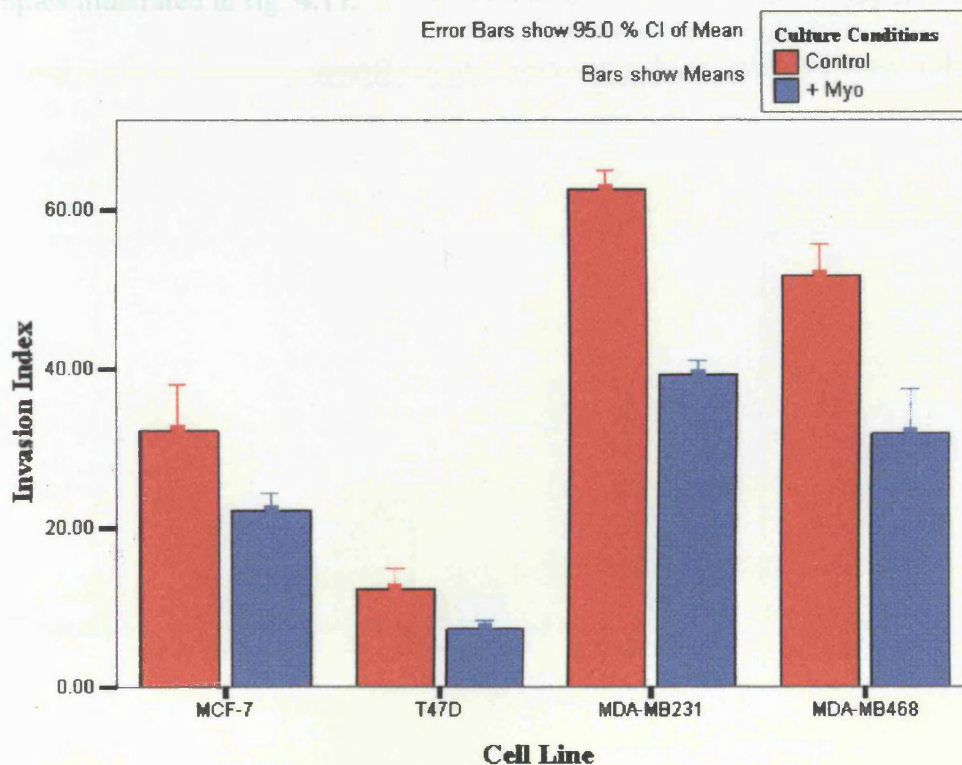
Fig. 4.15: Effect of Myoepithelial cells on Tumour Cell Invasion
Graph bars indicate the mean invasion index of 3 assays

4.4.5.2 Effect of Myoepithelial Cells on MMP and TIMP Expression

- Effect of Myoepithelial Cells on Tumour Cell Gene Expression

When myoepithelial cells were introduced into tumour-fibroblast co-cultures, there was significant down-regulation of MMP gene expression in all tumour cells compared to their expression in control cultures (i.e. with fibroblasts; Table 4.8). MMP-2, MT1-MMP and MMP-9 were down-regulated by a factor of 1.5 - 2.0 in tumour cells, to similar or lower levels than detected in tumour cells grown without fibroblasts (Controls, table 4.5). There were no significant differences observed between the two sets of fibroblasts. No alteration in TIMP-1 or TIMP-2 levels were detected in any of the co-cultures. The results are summarised in Table 4.8 and examples illustrated in fig. 4.17.
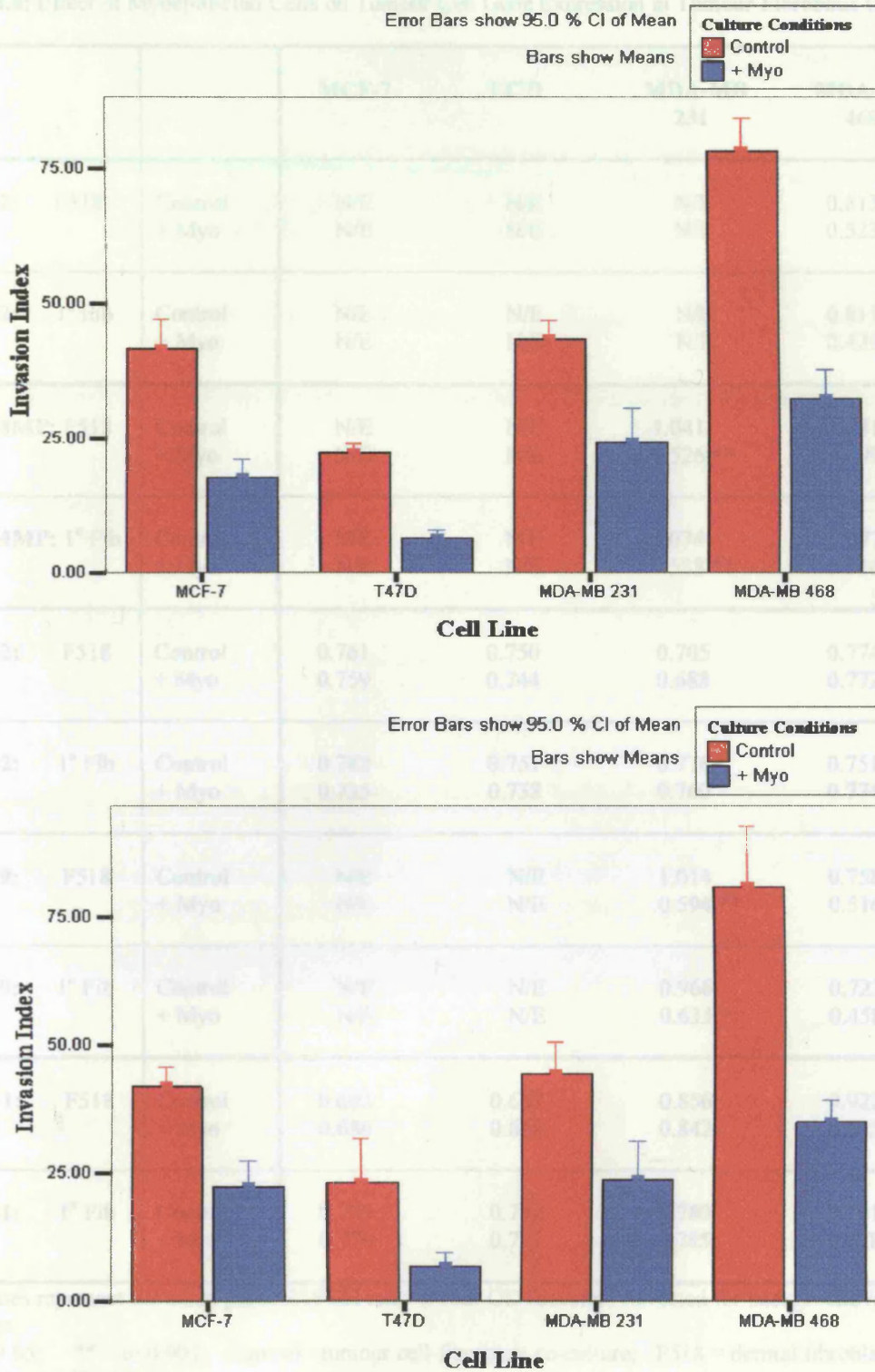
Fig. 4.16: Effect of Myoepithelial Cells on Tumour Cell Invasion in Co-culture with a) F518 dermal fibroblasts and b) primary breast fibroblasts.

Table 4.8: Effect of Myoepithelial Cells on Tumour Cell Gene Expression in Tumour-Fibroblast Co-cultures

		MCF-7	T47D	MDA-MB 231	MDA-MB 468
MMP-2: F518	Control + Myo	N/E N/E	N/E N/E	N/E N/E	0.813 0.523 **
MMP-2: 1° Fib	Control + Myo	N/E N/E	N/E N/E	N/E N/E	0.811 0.439 **
MT1-MMP: F518	Control + Myo	N/E N/E	N/E N/E	1.041 0.526 **	1.031 0.499 **
MT1-MMP: 1° Fib	Control + Myo	N/E N/E	N/E N/E	1.074 0.588 **	1.077 0.516 **
TIMP-2: F518	Control + Myo	0.761 0.759	0.750 0.744	0.705 0.688	0.774 0.772
TIMP-2: 1° Fib	Control + Myo	0.742 0.735	0.751 0.738	0.776 0.760	0.751 0.774
MMP-9: F518	Control + Myo	N/E N/E	N/E N/E	1.014 0.594 **	0.758 0.516 **
MMP-9: 1° Fib	Control + Myo	N/E N/E	N/E N/E	0.966 0.633 *	0.722 0.458 **
TIMP-1: F518	Control + Myo	0.692 0.686	0.667 0.668	0.856 0.842	0.922 0.915
TIMP-1: 1° Fib	Control + Myo	0.795 0.776	0.712 0.701	0.780 0.785	0.781 0.778

++ Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background /minus RT readings.
* = $p<0.05$; ** = $p<0.001$; Control =tumour cell-fibroblast co-culture; F518 = dermal fibroblasts; Fib = normal breast fibroblasts
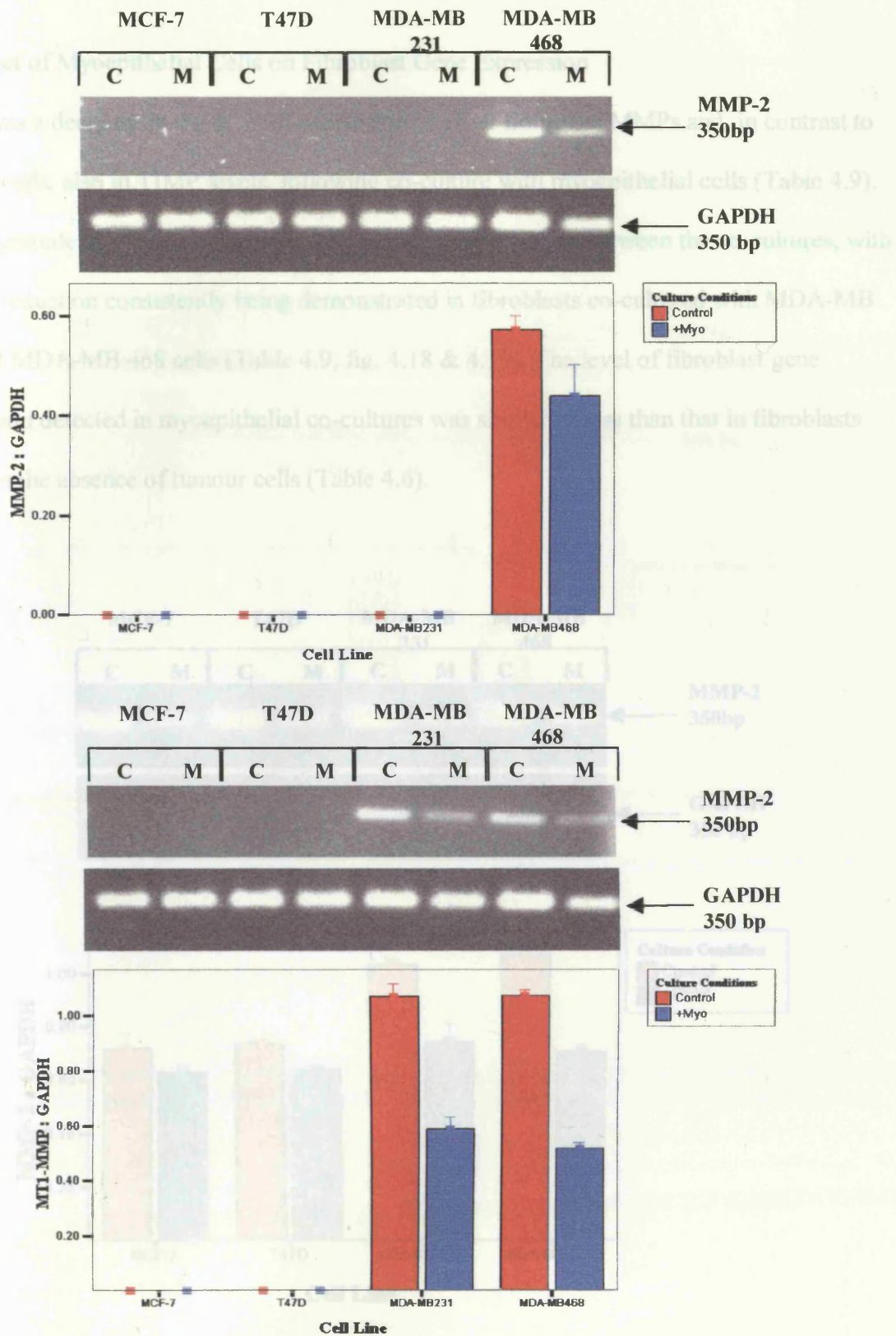
Fig 4.17: Effect of Myoepithelial Cells on Tumour Cell Gene Expression.
Gels illustrate PCR product following 30 cycles of amplification (C=Controls; M=+ Myoepithelial cells) Graph bars represent mean gene: GAPDH ratio taken from 4 OD readings, error bars indicate 95% confidence limits.

- Effect of Myoepithelial Cells on Fibroblast Gene Expression

There was a decrease in the level of expression of all of fibroblast MMPs and, in contrast to tumour cells, also in TIMP levels, following co-culture with myoepithelial cells (Table 4.9). The magnitude of the level of change in gene expression varied between the co-cultures, with greater reduction consistently being demonstrated in fibroblasts co-cultured with MDA-MB 231 and MDA-MB 468 cells (Table 4.9, fig. 4.18 & 4.19). The level of fibroblast gene expression detected in myoepithelial co-cultures was similar or less than that in fibroblasts alone, in the absence of tumour cells (Table 4.6).
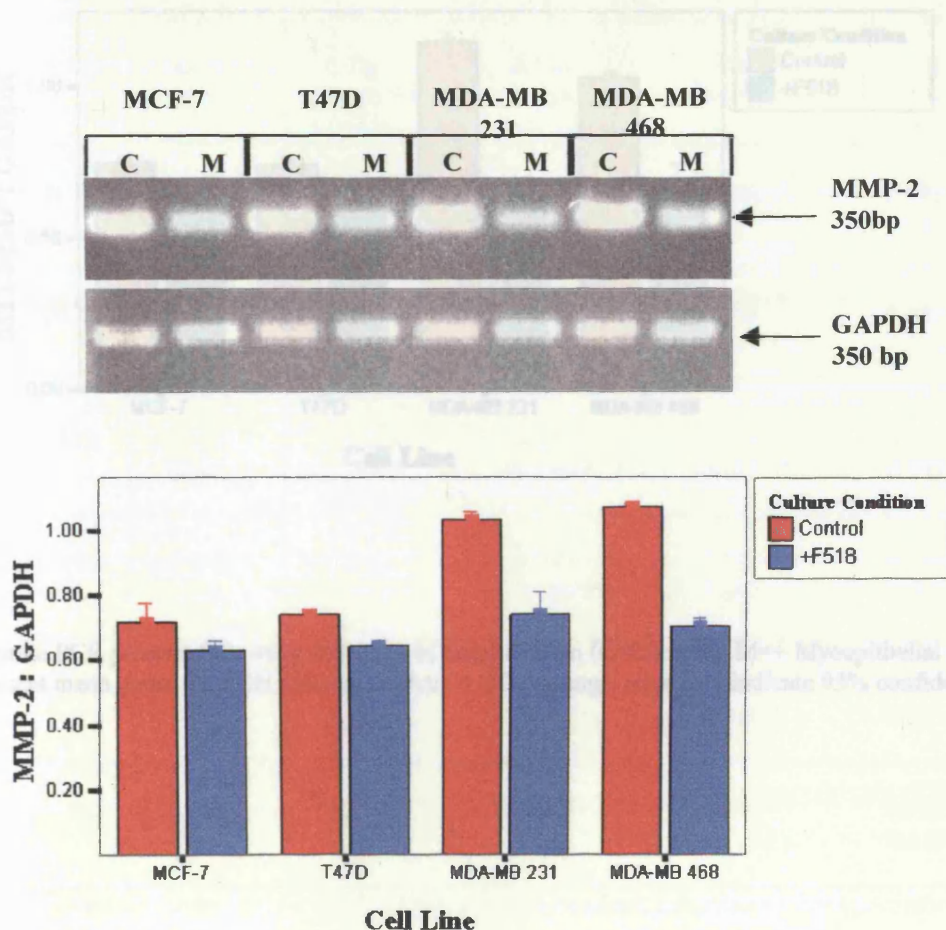
Fig 4.18: Effect of Myoepithelial Cells on Fibroblast Gene Expression.
Gels illustrate PCR product following 30 cycles of amplification (C=Controls; M=+ Myoepithelial cells) Graph bars represent mean gene: GAPDH ratio taken from 4 OD readings, error bars indicate 95% confidence limits.

257
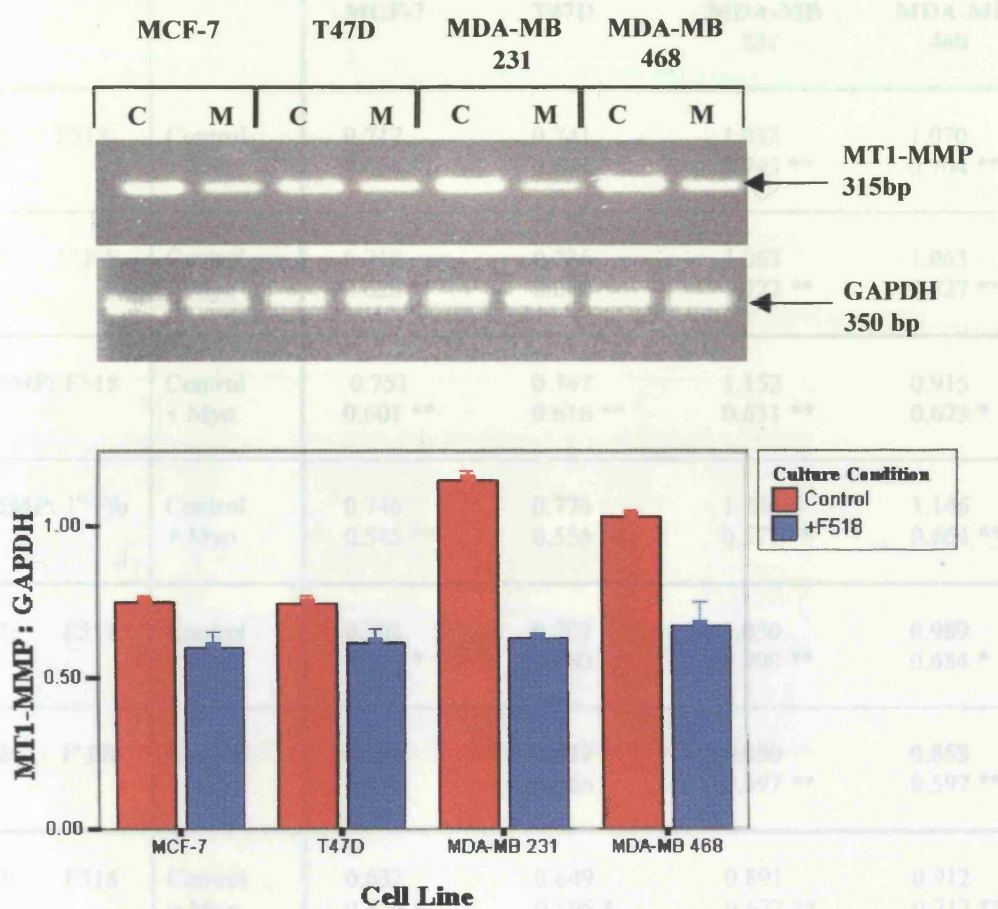
Gels illustrate PCR product following 30 cycles of amplification (C=Controls; M=+ Myoepithelial cells) Graph bars represent mean gene: GAPDH ratio taken from 4 OD readings, error bars indicate 95% confidence limits.

Table 4.9: Effect of Myoepithelial Cells on Fibroblast Gene Expression in Tumour Cell - Fibroblast

Co-Cultures

|			MCF-7	T47D	MDA-MB 231	MDA-MB 468
MMP-2:	F518	Control + Myo	0.717 0.634 *	0.741 0.644 **	1.032 0.743 **	1.070 0.704 **
MMP-2:	1° Fib	Control + Myo	0.716 0.628 **	0.736 0.642 *	1.063 0.727 **	1.063 0.727 **
MT1-MMP:	F518	Control + Myo	0.751 0.601 **	0.747 0.616 **	1.152 0.631 **	0.915 0.673 *
MT1-MMP:	1° Fib	Control + Myo	0.746 0.545 **	0.776 0.556 **	1.146 0.577 **	1.146 0.664 **
TIMP-2:	F518	Control + Myo	0.708 0.656 *	0.703 0.660 **	1.030 0.709 **	0.989 0.684 *
TIMP-2:	1° Fib	Control + Myo	0.655 0.615	0.627 0.586	0.850 0.597 **	0.858 0.597 **
MMP-9:	F518	Control + Myo	0.632 0.574 *	0.649 0.595 *	0.891 0.672 **	0.912 0.712 **
MMP-9:	1° Fib	Control + Myo	0.637 0.565 **	0.703 0.606 **	0.721 0.643 **	0.721 0.643 **
TIMP-1:	F518	Control + Myo	0.785 0.607 *	0.738 0.629 *	1.001 0.643 *	0.979 0.661 *
TIMP-1:	1° Fib	Control + Myo	0.769 0.647	0.746 0.616 *	0.969 0.615 *	0.924 0.615 *

++ Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background /minus RT readings.
* = $p < 0.05$;   ** = $p < 0.001$;   Control =tumour cell-fibroblast co-culture;   F518 = dermal fibroblasts;   Fib = normal breast fibroblasts

4.4.5.3 Effect of Myoepithelial Cells on Enzyme Activity

Conditioned medium was harvested from tumour-fibroblast co-cultures in the presence and

absence of myoepithelial cells and subjected to substrate gel zymography. The results

demonstrate marked down-regulation of MMP-9, pro-MMP-2 and active MMP-2 in cultures

exposed to myoepithelial cells (fig. 4.20).
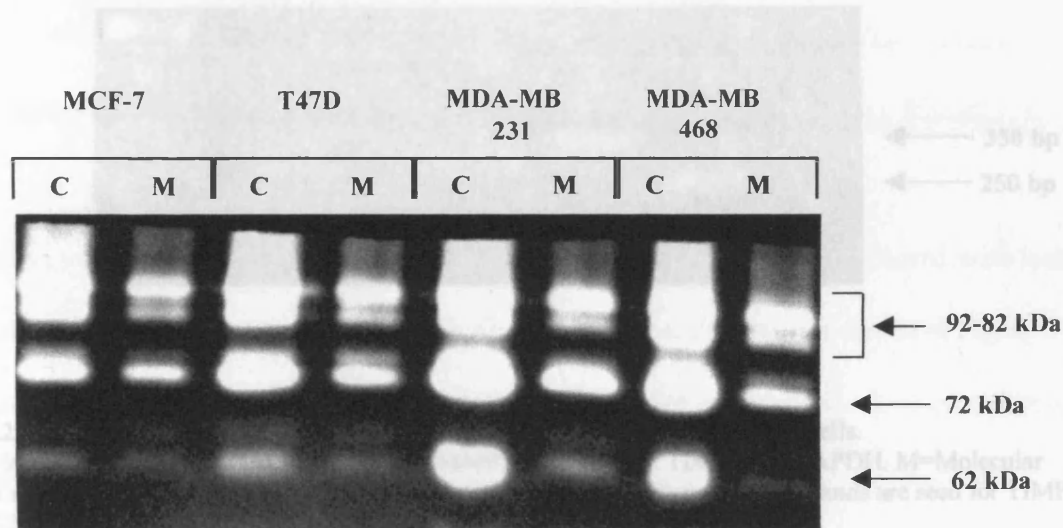
Fig. 4.20: Substrate Gel Zymogram on Conditioned Medium from Control (C=Tumour-Primary Fibroblast) and Myoepithelial (M) Co-cultures.

4.4.6 Expression of MMPs and TIMPs by Myoepithelial Cells

Separated myoepithelial cells were cultured on poly-d-lysine under serum-free conditions for

24 hrs, and the cells then harvested for mRNA extraction and RT-PCR. CM was subjected to

zymography and western blotting, with the cellular protein fraction for MT1-MMP blotting.

The results indicate that myoepithelial cells synthesis each component of the two

gelatinase systems (fig. 4.21), however, the level of TIMP-1 and TIMP-2 amplified is markedly

higher than the corresponding level of MMPs (ELOSA quantitation not performed). This

pattern is partly reflected on zymography, where MMP-2 is present almost entirely in the latent

form, and MMP-9 is present in both active and latent forms in approximately similar amounts

(fig. 4.22).
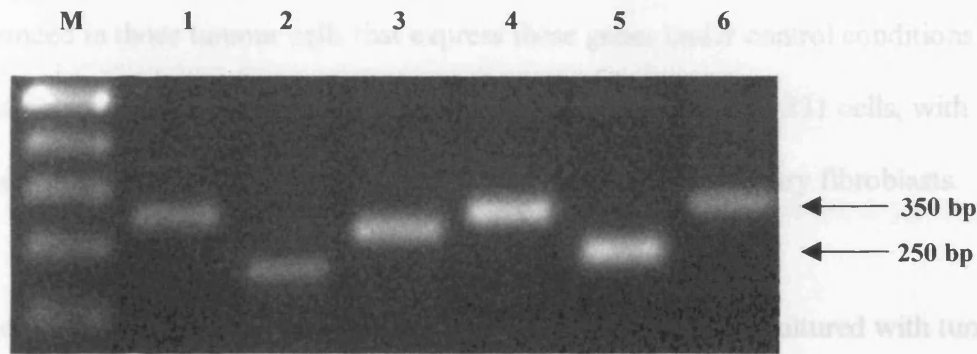
Fig. 4.21: Agarose Gel Electrophoresis for RT-PCR Products from Myoepithelial Cells.
Lanes indicate: 1: MMP-2; 2: MMP-9; 3: MT1-MMP; 4: TIMP-1; 5: TIMP-2; 6: GAPDH. M=Molecular
weight markers.Each reaction ws subjected to 30 cycles of amplification. Stronger bands are seen for TIMP-1 and
TIMP-2 relative to MMP-2 and MMP-9, and also relative to GAPDH.
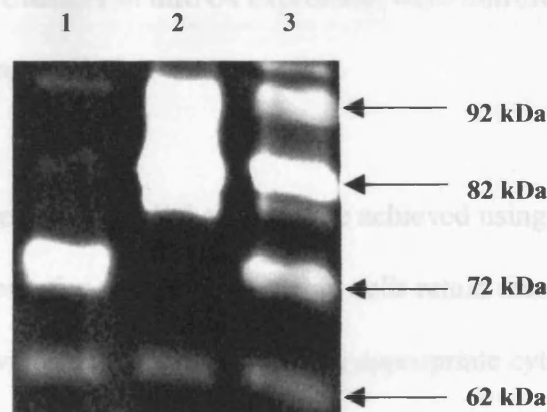
Fig. 4.22: Substrate Gel Zymogram on Conditioned Medium from Myoepithelial Cells.
The gel indicates very low level of active MMP-2, though a strong lytic band is present at 72 kDa indicating latent
MMP-2. Both active and latent MMP-9 is evident in approximately equivelent amounts. Standards include 10 ng
purified pro-MMP-2 (1), which shows some autoactivation, and 50 ng purified pro-MMP-9 (2) which also
indicates autoactivation.

4.5 Summary of Results

• Co-culture of breast cancer cell lines with fibroblasts derived from normal breast or dermal fibroblasts results in enhanced tumour cell invasion. This is reflected by changes in both tumour cell and fibroblast gene expression. MMP-2, MT1-MMP and MMP-9 expression was enhanced in those tumour cells that express these genes under control conditions though changes in TIMP expression were detected only in MDA-MB 231 cells, with increased levels of both TIMP-1 and TIMP-2 in co-culture with primary fibroblasts.

• Increased levels of all MMP mRNA were detected in fibroblasts co-cultured with tumour cells, with the greatest increases being observed in co-cultures with the most highly invasive tumour cells. Fibroblast TIMP levels were also enhanced in most co-cultures except TIMP-2 following co-culture with MCF-7 cells and in primary fibroblasts co-cultured with T47D cells. Changes in mRNA expression were mirrored by increased proteolytic activity on zymography.

• Short-term cultures of pure myoepithelial cells can be achieved using tissue digestion and immunomagnetic bead separation. The myoepithelial cells retain many of the characteristics of their in-vivo counterparts, including appropriate cytokeratin and cell adhesion molecule profile.

• Introduction of myoepithelial cells into tumour cell cultures or tumour cell-fibroblast co-cultures results in a significant reduction in tumour cell invasion. This is associated with down-regulation of MMP expression in tumour cells, with no significant alteration in TIMP levels, and down-regulation of MMP levels in fibroblasts with a variable effect on TIMP

262

levels. The reduced levels of gene expression are reflected by a marked reduction in

proteolytic activity on zymography.

- Myoepithelial cells in culture express each component of the MMP-2/MT1-MMP/TIMP-2

  and MMP-9/TIMP-2 systems, but display low net proteolytic activity on substrate gel

  zymography of myoepithelial conditioned medium.

4.6 Discussion

4.6.1 Discussion of Techniques

Short-term culture of separated breast cell populations from primary tissue is not a trivial task, however, the identification of markers specific for each cell type and the availability of antibody-labelled magnetic beads has greatly facilitated development of these techniques.

Methods for isolation and culture of epithelial clumps or organoids from human breast was first described by Stampfer et al (1980), and most techniques adopt various modifications of this method. More recently, techniques have been developed for the separation of different cell populations in the breast, exploiting their distinct antigenic profile (O'Hare et al, 1991; Clarke et al, 1994; Gomm et al, 1997). The method used in the current study is similar to that described by Monaghan et al (1994) and Gomm et al (1997). The major problem encountered in early attempts was yeast infection, occurring the day following plating out of cells. The method was simplified, reducing filtering steps in an attempt to overcome the problem. It became evident however, that the method of handling of the tissue at Surgical theatres had an impact on subsequent incidence of infection. When tissue was placed directly into pathology specimen buckets, the rate of infection was very high, compared to when tissue was received in plastic bags. The procedure adopted by theatre staff was modified accordingly, and infection became less of a problem. Another problem encountered was difficulty in generating a single cell suspension. Cell viability was very low under conditions required to digest organoids, so it was decided to plate out organoids, expand these into mixed population monolayers which were then successfully harvested and filtered to yield a predominantly single cell suspension.

Since the aim was to extract myoepithelial cells from these cultures, a specialised medium was prepared to promote the growth of myoepithelial cells over other cell types. It has

been shown that Epidermal Growth Factor (EGF) and Insulin promote the growth of these cells

therefore a recipe similar to that used by Peterson & van Deurs (1988) was employed.

One of the central issues in all cell culture work is how representative the culture model

is of the in-vivo situation. Cells may dramatically alter their phenotype when moved from the

3-dimensional tissue situation to a 2-dimensional monolayer. Culture conditions can have a

profound effect on phenotype, in particular cells which have a low level of proliferation in-

vivo, such as in the breast, may undergo unexpected changes when placed in culture conditions

that favour their rapid proliferation. The very rapid rate of proliferation of myoepithelial cells

in culture compared to their relative quiescence in-vivo has previously been observed (O'Hare

et al, 1991). Thus it was important to asses not only the purity of cells isolated but also to

establish their characteristics in comparison to their in-vivo counterpart.

The cell separation procedure identified an early change in phenotype. Whereas

CALLA is specific to myopeithelial cells in tissue, CALLA-labelled beads extracted both

myoepithelial cells and fibroblasts, and immunohistochemistry confirmed that this antigen was

switched on in a proportion of cultured breast fibroblasts. This necessitated changing the

antibody used for myoepithelial cell selection, and $\beta 4$ integrin was chosen because of its high

level of expression in–vivo. A concern was that cells isolated in this manner may be

functionally altered, however, when an appropriate bead-to-cell ratio was established, the

labelling beads were seen to be rapidly internalised and myoepithelial cells re-expressed $\beta 4$

integrin. When isolated myoepithelial cells were examined immunohistochemically for a range

of luminal and myoepithelial markers, weak or absent staining for CALLA and for SMA was

observed. These changes have been reported previously, and appear to relate to the rate of

proliferation of the cells (Petersen & van Deurs, 1988). Thus it has to be concluded that whilst

these isolated cells display many of the features of their tissue counterparts culture-related

changes are also evident. Current efforts are being directed at determining the time sequence of these phenotypic changes and analysing how they may influence the behaviour of myoepithelial cells.

Generation of fibroblast-rich cultures from digested breast tissue is not problematic. In this study fibroblast-rich suspensions were subjected to a round of extraction with β4-labelled beads and BerEP4 beads to remove any residual myoepithelial and luminal epithelial cells, respectively. It is recognised that breast fibroblasts are not a homogeneous population. Markers have been developed that distinguish between interlobular and intralobular fibroblasts (Atherton et al, 1992; Atherton et al, 1994) and there may be further functionally distinct subgroups. In this study, no attempts were made to separate different fibroblast populations, but it was interesting to note the presence of a minor population exhibiting strong expression of SMA, whilst others showed expression of CALLA.

4.6.2 Discussion of Results

4.6.2.1 Effect of Fibroblasts on Tumour Cell Invasion

Fibroblasts have been shown to influence the tumourigenicity of a number of different types of

tumours, having an effect both on tumour growth and on the development of metastases in-vivo

(Camps et al, 1990; Noel et al, 1998; Diamanche-Boitrel, et al, 1994; Gregoire & Lieubeau,

1995; Price, 1996). The absolute dependence of tumour cells on fibroblast-derived factors for

invasion has been demonstrated in this study by the lack of invasion that occurs when fibroblast

CM is not included in the in-vitro invasion assay system (chapter 3). A complex paracrine

effect is implied with the demonstration of enhanced invasion by all breast cancer cell lines

when co-cultured in the presence of either primary fibroblasts derived from normal breast or

normal dermal fibroblasts. This is in keeping with the findings of other studies on breast

(Popowicz et al, 1995) and other tumour cells (Saiki et al, 1994). Some studies indicate that the

effect of tumour-stromal co-culture is dependent on the origin of the fibroblasts, for example

Saiki et al (1994) found that transformed fibroblasts were more potent in inducing melanoma

cell invasion than normal dermal fibroblasts. The effect of tumour-derived fibroblasts was not

examined in this study, but no significant difference was detected between the effects of the

two normal fibroblast sources used. It was evident, however, that the individual tumour cell

lines exhibited varying levels of response to co-culture. The two cell lines with a lower basal

invasive activity (MCF-7 and T47D) displayed an approximately 1.3 fold increase in invasion

on co-culture whereas the two cell lines with higher basal invasion (MDA-MB 231 and MDA-

MB 468) exhibited a 1.6 - 1.7 fold increase in invasion when exposed to fibroblasts. A similar

relationship between metastatic capability of tumour cells and their subsequent invasive

response to fibroblasts has previously been reported both for breast (Popowicz et al, 1995) and

melanoma cell lines (Saiki et al, 1994). These results suggest a complex inter-dependent

relationship between tumour cell and fibroblasts, in which either the highly invasive tumour cells are more effective in priming fibroblasts to release pro-invasive factors, or the tumour cells are more sensitive to the effect of these factors (or a combination of the two).

To address whether the enhanced invasion of tumour cells in co-culture with fibroblasts is at least in part due to changes in MMP and TIMP expression, both cell populations were analysed by RT-PCR and ELOSA quantitation for their level of gene expression. When cultured alone, both primary breast fibroblasts and dermal fibroblasts constitutively expressed MMP-2, MT1-MMP, TIMP-2, and MMP-9 and TIMP-1, which may be a non-specific effect of tissue culture since this is not reflected in the resting state in-vivo. A significant increase in the level of MMP-2 mRNA was detected in both sets of fibroblasts in response to each of the breast tumour cell lines. A similar pattern of response was exhibited for MT1-MMP, though in F518 cells co-cultured with MCF-7 and T47D cells this increase did not reach significance. Interestingly, the level of increase in expression was consistently greater in fibroblasts exposed to MDA-MB 231 and MDA-MB 468 than those exposed to MCF-7 and T47D cells, reflecting the effect of co-culture on tumour invasion. This suggests that factors intrinsic to the malignant cell determine the nature and potency of the stromal response. The level of TIMP-2 expression was also significantly increased on co-culture with MDA-MB 231 and MDA-MB 468, and in F518 by T47D cells, although the magnitude of the increase was less than that observed for MMP-2 and MT1-MMP, with a 0.8 factor increase for TIMP-2 compared to 1.7 fold increase for the proteolytic enzymes.

Expression of MMP-9 was also increased in response to all breast cell lines, and there was a less marked increase in TIMP-2 gene expression which reached significance for fibroblasts co-cultured with MDA-MB 231 and MDA-MB 468 cells, thus demonstrating a similar pattern of response as the MMP-2 enzyme system. The changes in gene expression are

268

mirrored by enhanced net proteolytic activity, together with increased levels of active MMP-2, which would be predicted due to enhanced expression of the activator MT1-MMP.

Tumour-cell stimulation of MMP expression by fibroblasts has been reported in a number of systems. Boyd & Balkwill (1999) demonstrated a 2-8 fold increase in pro-MMP-2 and TIMP-2 protein release from dermal fibroblasts following co-culture with ovarian carcinoma cell lines. Interestingly, they found that the stimulatory response was dependent on tumour cell-stromal cell contact. Tumour cell derived Collagenase Stimulatory factor (TCSF) also known as EMMPRIN (Extracellular MMP Inducer) is a cell surface receptor expressed on the plasma membrane of a number of tumour cells that has been shown to induce fibroblast expression of MMP-1, MMP-2 and MMP-3 (Kataoka et al, 1993; Biswas et al, 1995), however, stimulation of MMPs is also observed in co-culture systems which do not express this molecule (Boyd & Balkwill, 1999) so other mechanisms must be involved. A number of studies have indicated that MMP induction is a β1-integrin-dependent mechanism (Himelstein et al, 1994; Segain et al, 1996; Boyd & Balkwill, 1999). The requirement for cell-cell contact for alteration of MMP expression is not consistent, however, with several reports indicating stimulatory effects in the absence of cell-cell contact (Lengyel et al, 1995; Ito et al, 1995; Ornstein et al, 1999). Polette et al (1997) reported enhanced MT1-MMP gene expression in fibroblasts in response to soluble factors secreted by breast tumour cells. It appears likely that both soluble and cell membrane associated factors are involved in eliciting MMP expression, and the lower magnitude of increase in MMPs noted in this study compared to Boyd & Balkwill (1999) may reflect the 'super-enhancing' effect of cell contact. In keeping with this suggestion, Kataoka et al (1997) found that exposure of cells to fibroblast conditioned media only partially reproduced the enhanced MMP expression observed in contact co-cultures.

One of the major advantages of performing dual-chamber co-cultures is the ability to analyse each cell type in the co-culture separately. This study demonstrated that as well as stimulating fibroblast synthesis of MMPs, tumour cell-stromal co-cultures also affected tumour cell gene expression. Enhanced expression of MMP-2, MT1-MMP and MMP-9 was exhibited in those tumour cells that synthesised these enzymes under control conditions but there was no evidence of de-novo expression of MMPs in tumour cells, and there was no significant change in the level of TIMP-1 and TIMP-2 expression. These results indicate that the tumour cell-stromal cell relationship is reciprocal in nature, as has been suggested by other studies (Nabeshima et al, 1994; Lengyel et al, 1995; Kataoka et al, 1997), but also indicates that the tumour cell and fibroblast response is distinct, in that there was no modulation of TIMP expression in tumour cells in contrast to that seen in fibroblasts.

4.6.2.2 The Effect of Myoepithelial Cells on Tumour Cells and Fibroblasts

This study has demonstrated that co-culture of normal breast myoepithelial cells with tumour cells alone or in culture with fibroblasts leads to a significant reduction in breast cancer cell invasion. Furthermore, this is reflected by reduced tumour cell expression of MMP-2, MT1-MMP and MMP-9, and reduced fibroblast expression of these MMPs and TIMPs, with associated decrease in proteolytic activity.

Little attention has focused on the role of the myoepithelial cell in the breast. It lies at the interface between the BM and the luminal epithelial cell (Rudland & Hughes, 1989) and displays strong expression of $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$ integrins with prominent hemidesmosome formation along its basal aspect (Glukhova et al, 1995). Its contractile nature is reflected by the presence of actin and myosin filaments (Gusterson et al, 1982; Lazard et al, 1993), but the epithelial nature of these cells is confirmed by their expression of intermediate filament

cytokeratins, in particular CK 14 which is characteristically expressed by in the basal cell layer of stratified epithelia (Wetzels et al, 1991; Ronnov-Jessen et al, 1996). Although traditionally the primary function of myoepithelial cells is a contractile role during lactation, it is evident that these cells are well adapted for a regulatory role in the breast. They express a range of growth factors and their receptors (Gomm et al, 1991; Monaghan et al, 1995; Gomm et al, 1997), and many enzymes, including CALLA, a neutral endopeptidase involved in the metabolism of polypeptides and thought to be important in the processing of hormonal and growth factor signals (Gusterson et al, 1986; Kenny et al, 1989). Myoepithelial cells synthesise BM and as demonstrated in this and other studies (Monteagudo et al, 1990) also produce proteolytic enzymes which are likely to be involved in the physiological remodelling of the BM. The mechanism by which myoepithelial cells modulate tumour cell invasion and MMP gene expression is unclear. Since the cell populations were not in contact in the culture system used in this study, paracrine mechanisms must be invoked. Myoepithelial cells produce TIMP-1 and TIMP-2, as shown in this study, and other proteinase inhibitors (Barsky et al, 1997; Kedeshian et al, 1998). Thus inhibition of tumour or fibroblast-derived MMPs and blockade of surface MT1-MMP by released TIMPs could certainly be one mechanism by which myoepithelial cells could reduce invasion. This does not, however, explain the down-regulation of tumour cell and fibroblast gene expression, which implies a more complex regulatory mechanism than simply enzyme inhibition. Different growth factors and cytokines have been shown to influence MMP and TIMP gene expression in a system-specific manner (Festuccia et al,1996; Shimuizu et al, 1996; Khan & Falcone, 1997; Uria et al, 1998). Further work would be required in order to identify which factor or factors is responsible in this system.

The finding that myoepithelial cells exert an anti-invasive effect on the tumour-fibroblast system is of particular relevance given that loss of myoepithelial cells is a consistent

271

finding in invasive breast carcinomas (Ahmed, 1974; Gusterson et al, 1982). Recent studies

suggest that myoepithelial cells are probably derived from a luminal progenitor cell, therefore

their loss in breast carcinomas is most likely due to a combination of overgrowth by a

malignant luminal cell population coupled with failure to renew this cell population. The

implication that loss of myoepithelial cells removes an important regulatory system is of great

interest. This study has highlighted one aspect of control exerted by myoepithelial cells. Recent

reports from Barskys' lab indicate that myoepithelial cells inhibit proliferation of breast cancer

cells (Shao et al, 1998) and inhibit progression of DCIS through an ER-beta-mediated

mechanism (Grossman et al, 1999). Further evidence that myoepithelial cells possess anti-

tumourigenic properties is reported in a recent study indicating that a myoepithelium-derived

serine proteinase inhibitor (MEPI) inhibits breast cancer cell growth and metastasis in a mouse

model (Xiao et al, 1999). Thus evidence is beginning to accumulate to support the importance

of the myoepithelial cell in the control of the breast microenvironment and its potential role as a

tumour suppressor.

4.7 Conclusions

- A reciprocal relationship exists between tumour cells and fibroblasts that promotes invasion. Co-culture, even in the absence of cell contact, leads to enhanced expression of MMPs and increased proteolytic activity. Whilst there is evidence that cell contact may be important in the tumour cell-stromal cell relationship, paracrine pathways must also exist.

- When myoepithelial cells are co-cultured with tumour cells, either alone or in combination with fibroblasts, there is a significant reduction in tumour cell invasion, accompanied by down-regulation of MMP expression. Myoepithelial cells produce high levels of proteinase inhibitors and this in part may explain their anti-invasive effect, however, their effect on gene expression suggests a more complex pathway is involved.

- Myoepithelial cells may play an important role in maintaining the normal breast microenvironment. Techniques for isolating and growing pure populations will enable the function of this cell to be examined in detail.

Chapter 5

Final Discussion and Future Directions

This thesis has examined the expression of two protease systems, the MMP-2/MT1-MMP/TIMP-2 and MMP-9/TIMP-1 systems in primary breast cancers and in a series of breast cancer cell lines, and related pattern of expression to the expression profile of E-Cadherin and integrins and to invasive behaviour. The relationship between cell adhesion molecule expression, MMP synthesis and invasive potential was then explored by manipulating the expression and function of cell adhesion molecules and analysing the effects on MMP expression and invasion. Finally the impact of cellular interactions was addressed by constructing co-culture systems to examine the tumour-stromal cell relationship and to analyse the effect of normal myoepithelial cells on invasion and MMP expression.

The MMP-2/MT1-MMP/TIMP-2 system was found to be widely expressed in breast carcinomas and the presence of tumour cell membrane reactivity for MMP-2 and MT1-MMP correlated with a positive lymph node status, and with the level of MMP-2 activity. Previous in-situ hybridisation studies have clearly demonstrated that MMP-2 is derived from stromal fibroblasts (Bassett et al, 1990; Poulsom et al, 1992; Pollette et al, 1994;Heppner et al, 1996) whilst there is some controversy surrounding the source of MT1-MMP (Okada et al, 1995; Heppner et al, 1996; Gilles et al, 1997). This study has shown that MT1-MMP mRNA is detected in tumour cells in some cases. There remains a discrepancy between the wide distribution of both MMP-2 and MT1-MMP in tumour cells and the lack of MMP-2 and focal nature of MT1-MMP mRNA in tumour cells. The most likely explanations for this is either the level of mRNA expression in tumour cells falls below the level of sensitivity of in-situ hybridisation approaches, or tumour cells internalise MMPs, possibly for degradation. The MMP-9/TIMP-1 system is less widely expressed than MMP-2, and the most striking observation in this study was the association of tumour cell reactivity for MMP-9 with the ILC phenotype.

As for MT1-MMP, MMP-9 mRNA was detected only focally within tumour cells. Again this may reflect a relatively low level of synthesis of this enzyme.

Changes in cell adhesion molecule expression were observed frequently in primary carcinomas. In keeping with other studies, this study showed loss of E-Cadherin in ILC, and an inverse correlation between tumour cell reactivity for MMP-9 and loss of E-Cadherin, both in ILC and IDC was detected. A lower level of expression of integrin receptors was observed in many carcinomas compared to normal breast ducts and acini, the extent of which was inversely correlated with tumour differentiation for $\alpha 2$, $\alpha 3$ and $\beta 1$ integrins. Most tumours did not express $\alpha 6 \beta 4$ integrin, but when present it was associated with more poorly differentiated tumours. These findings are in keeping with those of other workers (Koukoulis et al, 1991; Friedrichs et al, 1995; Tagliabue et al, 1998).

Established breast cancer cell lines were used to examine more directly the relationship between invasion, MMP expression and cell adhesion molecule profile. In-vitro invasion assays demonstrated low level invasion by MCF-7 and T47D cells, with markedly higher levels of invasion by MDA-MB 231 and MDA-MB 468 cells. MCF-7 ad T47D cells were shown not to express MMP-2, MT1-MMP or MMP-9 and retain E-Cadherin, together with membrane expression of $\alpha 2$, $\alpha 3$ and $\beta 1$ integrins. In contrast, MDA-MB 231 and MDA-MB 468 cells both express MT1-MMP and MMP-9, and MDA-MB 468 also express MMP-2. Both cell lines lack membrane E-Cadherin and MDA-MB 231 cells express $\alpha 6 \beta 4$ integrin. It is of interest to note the parallels between the cell lines and primary tissues, with retained E-Cadherin and lack of MMP expression being associated with a less aggressive phenotype and conversely the association of $\alpha 6 \beta 4$ expression and tumour cell MMPs relating to more aggressive behaviour. These parallels go some way towards validating the use of these cell lines as appropriate models of tumour behaviour.

The relationship between E-Cadherin expression and MMP-9 production was examined further by blocking E-Cadherin and by growing cells at low density thereby preventing E-Cadherin homotypic interactions. These experiments indicated that E-Cadherin-mediated interactions inhibit MMP-9 synthesis by tumour cells, so co-ordinating the processes of reduced cell adhesion and enhanced proteolysis, to favour invasion. A LEF-1 transcription motif has been identified in the promotor region of MMP-9 in the mouse (Llorens et al, 1998), though it remains to be established if this is reflected in the human gene. It would be of interest to study the dynamics of the β-catenin complex in this system to help further dissect this signalling pathway.

The MDA-MB 231 cells are the only cell line of those studied that express $\alpha6\beta4$ integrin. My previous work in this laboratory has demonstrated up-regulation and redistribution of this integrin heterodimer in the presence of TGF-$\beta1$. The role of $\alpha6\beta4$ in malignancy appears increasingly complex. This integrin has been reported to promote invasion of some cancer cell lines (Rabinovitz & Mercurio, 1997), and this observation was confirmed in the current study. However, with redistribution of the integrin, the function changes, invasion assays revealing a significant reduction in invasive behaviour via an $\alpha6\beta4$-dependent mechanism. There was also an associated change in MMP expression, with up-regulation of all MMPs and TIMPs examined but with a net reduction in proteolytic activity. This was not blocked by $\alpha6\beta4$ integrin, thus indicating a cascade of related but independent events in response to this growth factor.

In order to address the mechanism by which $\alpha6\beta4$ integrin might exert different functions, cytoskeletal solubilisation experiments were performed to characterise the nature of interaction with the integrin under different conditions. These experiments suggest that the change in function is associated with translocation of $\alpha6\beta4$ from the actin cytoskeleton to the intermediate filament cytoskeletal system. Recently it has been suggested that plectin/HD1 may regulate

cytoskeletal dynamics, since it can crosslink the actin and IF systems (Andra et al, 1998).

Unfortunately, this study was unsuccessful in demonstrating the distribution of HD1, but this is

an area worth pursuing. The distribution of $\alpha 6\beta 4$ in MDA-MB 231 cells raises a number of

interesting questions. Does the localisation of this integrin to the IF system indicate nascent

hemidesmosome formation? Electron microscopy identified structures resembling putative type

II HD, but ideally isolation of these structures and identification of the component proteins

would be required to fully characterise them. Such techniques have been described (Owaribe et

al, 1991). The potential ability of these cells to form HD, which are normally expressed by the

breast myoepithelial cells, raises the suggestion that MDA-MB 231 cells exhibit a more basal or

myoepithelial phenotype. If luminal epithelial and myoepithelial cells arise from a common

progenitor cell, and there is recent evidence to suggest this may be the case (Pechoux et al,

1999), then it would seem plausible that a malignant clone may arise at different points in this

differentiation pathway. It is accepted that the majority of breast cancers display a luminal

epithelial phenotype, however, identification of a group of tumours that are differentiating along

a different path raises the possibility of alternative therapeutic approaches for such tumours. It

would be difficult to establish whether such a distinct subgroup exists but a comprehensive

immunohistochemical study using a range of antibodies to 'myoepithelial' and 'luminal' markers

could be of value.

It is evident both from tissue studies and in-vitro work that tumour-stromal cell

interactions are vital to the progression of malignancy (Diamanche-Boitrel, et al, 1994; Popowicz

et al, 1995). This study has confirmed a co-operative relationship between tumour cells and

fibroblasts. There are limitations in the use of dual-chamber co-culture systems, most obviously

the lack of cell-cell contact, however, the advantage of this system is that it allows both cell

populations to be easily retrieved at the completion of the experiment and analysed separately.

This demonstrated that not only do tumour cells stimulate MMP expression in fibroblasts, but also fibroblasts have a reciprocal action on gene expression in tumour cells.

One of the most exciting findings was the effect of introducing myoepithelial cells into the co-culture system. As with the tumour-fibroblast co-cultures, each cell population was in a separate chamber, thus the ability of myoepithelial cells to reduce tumour cell invasion and to down-regulate MMP gene expression must be mediated through paracrine mechanisms. It is evident from this and other studies (Kedeshian et al, 1998; Xiao et al, 1999) that myoepithelial cells secrete high levels of proteinase inhibitors, and these undoubtedly contribute to the anti-invasive effect of the cells, however, there have been no reports to suggest that high levels of TIMPs can influence MMP gene expression and therefore the action of other secreted factors must be invoked. Identification of this factor or factors, using protein purification would be of particular importance.

The role of the myoepithelial cell in the breast is still poorly understood, but as their unique characteristics are elucidated, the potential for these cells as major regulators of function becomes more apparent. With the ability now to reproducibly isolate pure populations of myoepithelial cells, it will be possible to address the role of these cells. A more physiologically relevant culture system is essential, such as the models developed in Bissells' laboratory (Streuli & Bissell, 1990; Streuli et al, 1991; Weaver et al, 1995). Current work in our laboratory is using such a 3-dimensional model to introduce myoepithelial cells into culture with both normal luminal epithelial cells and tumour cells to evaluate the extent to which these cells can modulate cell behaviour. It may yet be that this 'ignored cell' (Walker, 1988) will prove to have a central role in the control of the breast microenvironment.

Appendices

Appendix 1: Buffers and Solutions

3-aminopropyltriethoxysilane soln.	1% 3-aminopropyltriethoxysilane in dry acetone
Mayer's haematoxylin soln.	0.1% haematoxylin, 5% ammonium or potassium alum, 0.02% sodium iodide, 0.1% acid, 5% chloral hydrate
Eosin soln.	1% aqueous water soluble eosin
TBS (20X)	0.05 M Tris-HCL, 0.15 M NaCl, 0.1% w/v BSA, pH 7.6
PBS (20X)	2.6 M NaCl, 60 mM Na_2HPO_4, 140 mM NaH_2PO_4, pH 7.4
Homogenising buffer	0.05 M Tris-HCl (pH 7.6), 2 M/l urea, 1 g/L NaCl, 1 g/L EDTA (pH 8.0), 1ml/l Brij 35, 0.1mM/l Phenylmethanesulphonyl fluoride (PMSF)
Resolving Buffer	1.5 M Tris-HCl, pH 8.8
Stacking Buffer	0.5 M Tris-HCl, pH 6.8
Reservoir Buffer	0.25 M Tris, 1.92 M glycine, 1% SDS: pH8.3
2 X Reducing Sample Buffer	0.05 M Tris-HCl (pH 6.7), 4% SDS, 20% glycerol, 0.2% bromophenol blue, 1.4M β-mercaptoethanol
Blotting Buffer	0.25 M Tris, 1.92 M glycine, 1% SDS, 20% methanol: pH 8.3

Blocking Buffer	2.5 mM Tris-HCl (pH 7.6), 7.5 mM NaCl, 5% dry defatted milk
Washing Buffer	2.5 mM Tris-HCl (pH 7.6), 7.5 mM NaCl, 0.1% ml Polyoxyethylene sorbiton monolaureate (Tween 20)
Diaminobenzidine	0.1% 3,4,3,4 Tetra-amino Biphenyl Hydrochloride (DAB) in PBS, 3% hydrogen peroxide
Citric Acid Buffer	10 mM citirc acid, pH 6.0
2x Non-Reducing Sample Buffer	0.5 M Tris-HCl pH 6.8, 10% glycerol 10% SDS, 0.2% bromophenol blue
Tank Buffer	0.25 M Tris, 1.92 M glycine, 1% SDS, pH8.3
Incubation Buffer	0.5M Tris, 0.1M CaCl$_2$, 0.05% Brij, pH7.6
Staining Solution	0.05% Coomassie Blue G-250, 30% methanol, 10% acetic acid
Destaining Solution	30% methanol, 10% acetic acid
20 X Standard Saline Citrate (SSC)	3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0
ISH Blocking Solution	TBS containing 0.1% Triton X-100, 3% w/v BSA
ISH Substrate Buffer	0.1 M Tris-HCl 0.05 M MgCl$_2$, 0.1 M NaCl. pH 9.5

Paraformaldehyde	4% paraformaldehyde in 10 X DEPC/PBS, diluted 1:10 in DEPC H_2O to give 0.4% solution
10 X PE	0.5 M Tris-HCl, 1% sodium pyrophosphate, 2% polyvinylpyrrolidine, 2% Ficoll, 50 mM EDTa, pH 7.5
10 X TE	0.1 M Tris-HCl, 0.01 M EDTA, pH 8.0
ISH Blocking Solution	TBS, 0.1% BSA, 0.01% Triton X-100
Buffer 3	0.1 M Tris-HCl, 0.05 M $MgCl2$, 0.1 M NaCl, pH 9.5
Diluent Buffer	30 % 20 X SSC in DEPC H_2O, 0.2 mg salmon sperm DNA
BCIP	0.5% 5-bromo-4-chloro-3-indolylphosphate w/v in dimethyl formamide
NBT	0.4mM nitroblue tetrazolium in 70% dimethyl formamide
10 x AJ PCR buffer	45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)_2SO4$, 4.5 mM $MgCl_2$, 200 μM each dNTPs, 110 μg/ml BSA, 6.7 mM β-mercaptoethanol, 4.4 μM EDTA pH 8.0, 501.8 μl PCR H_2O
Cova buffer 1	0.02% w/v Tween 20, 1 x PBS (filtered)
ELOSA wash buffer 2	11.69% w/v NaCl, 1% w/v $MgSO_4.7H_2O$, 0.5% w/v Tween 20, 1 x PBS (filtered)

ELOSA detection substrate — 1 mg/ml PNPP in DEA, 1 M diethanolamine pH 9.8

Lysis/binding buffer — 100 MM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA pH 8.0, 1% SDS, 5 mM DTT

Washing buffer — 10 mM Tris-HCl pH 8.0, 150 mM LiCl, 1 mM EDTA pH 8.0

Washing buffer with SDS — 10 mM Tris-HCl pH 8.0, 150 mM LiCl, 1 mM EDTA pH 8.0, 0.1% SDS

50 x Tris/Acetate/EDTA buffer (TAE) — 2 M Tris-HCl pH 8.0, 1 M glacial acetic acid, 0.005 M EDTA pH 8.0

5 x Gel Loading buffer — 1 x TAE, 50% glycerol

High Salt Buffer (HSB) — 140 mM NaCl, 1.5 M KCl, 10 mM Tris-HCl pH 7.6, 0.5% Triton X-100

Appendix 2: Western Blotting

10% Resolving Gel: — 2.5 mls Resolving buffer, 3.3 mls 30% Acryamide/Bis-acrylanide, 100 μl 10% w/v SDS, 5 μl TEMED, 50 μl 10% APS, 4.0 mls ddH$_2$O

3.75% Stacking Gel: — 2.5 mls Stacking buffer, 1.3 mls 30% Acryamide/Bis-acrylanide, 100 μl 10% w/v SDS, 10 μl TEMED, 50 μl 10% APS, 6.1 mls ddH$_2$O

Appendix 3: Substrate Gel Zymogram

10% Gelatin Substrate Gel: — 10 mg collagen (Type III, Sigma) in 4.0 mls ddH$_2$O, 2.5 mls Resolving buffer, 3.3 mls 30% Acryamide/Bis-acrylanide, 100 μl 10% w/v SDS, 5 μl TEMED, 50 μl 10% APS

Appendix 4: Zymography on Primary Breast Carcinomas

Case No.	RW No	IHC	Pro-MMP-2*	Active MMP-2*
1	628	high	570	498
2	616	low	3210	926
3	824	high	358	485
4	767	high	220	390
5	844	low	1265	560
6	861	low	1325	522
7	867	low	1572	483
8	845	high	110	525
9	565	low	368	297
10	824	high	425	621
11	601	low	1925	326
12	840	high	215	328
13	648	low	2875	898
14	618	high	210	1220
15	653	high	92	425
16	734	low	6987	492
17	953	high	5620	512
18	961	low	4752	490
19	941	low	3800	185
20	948	low	6820	297
21	738	high	259	980
22	903	low	1500	382

High and Low refer to the level of membrane reactivity for both MMP-2 and MT1-MMP. and indicate >50% and <50% reactivity, respectively.
*Values are of Integrated density.

References

Aberle, H., Schwartz, H. & Kemler, R. (1996). Cadherin-catenin complex: Protein interactions and their implications for Cadherin function. *Journal of Cellular Biochemistry*, **61**, 514-523.

Adams, J.C., Watt, F.M. (1993). Regulation of Development and Differentiation by the Extracellular Matrix. *Development*, **117**, 1183-1198.

Ahmed, A. (1974). The Myoepithelium in Human Breast Carcinoma. *J. Pathology*, **113**, 129-135.

Akiyama, S.K., Yamada, S.S., Chen, W.T. & Yamada, K.M. (1989). Analysis of Fibronectin Receptor Function with monoclonal-antibodies - Roles in Cell-Adhesion, Migration, Matrix Assembly, and Cytoskeletal Organization. *Journal of Cell Biology*, **109**, 863-875.

Alard, P., Lantz, O., Sebagh, M., Calvo, C.F., Weill, D., Chavanel, G., Senik, A. & Charpentier, B. (1993). A Versatile Elisa-PCR Assay For Messenger-RNA Quantitation from a few cells. *Biotechniques*, **15**, 730.

Albelda, S.M., Mette, S.A., Elder, D.E., Stewart, R.M., Damjanovich, L., Herlyn, M. & Buck, C.A. (1990). Integrin Distribution in Malignant-Melanoma - Association of the Beta-3-Subunit with Tumor Progression. *Cancer Research*, **50**, 6757-6764.

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M. & McEwan, R.N. (1987). A Rapid In-vitro Assay For Quantitating the Invasive Potential of Tumor-Cells. *Cancer Research*, **47**, 3239-3245.

Alessandro, R., Minafra, S., Pucciminafra, I., Onisto, M., Garbisa, S., Melchiori, A., Tetlow, L. & Woolley, D.E. (1993). Metalloproteinase and TIMP Expression by the Human Breast-Carcinoma Cell-Line 8701-BC. *International Journal of Cancer*, **55**, 250-255.

Allen, M.V., Smith, G.J., Juliano, R., Maygarden, S.J. & Mohler, J.L. (1998). Downregulation of the Beta 4 Integrin Subunit in Prostatic Carcinoma and Prostatic Intraepithelial Neoplasia. *Human Pathology*, **29**, 311-318.

Alvarez, O.A., Carmichael, D.F. & Declerck, Y.A. (1990). Inhibition of Collagenolytic Activity and Metastasis of Tumor-Cells by a Recombinant Human Tissue Inhibitor of Metalloproteinases. *Journal of the National Cancer Institute*, **82**, 589-595.

Anderson J A (1974). Lobular Carcinoma In-Situ : a long term follow-up in 52 cases. *Acta Pathol. Microbiol. Scand.*, **82**,519-533.

Andra, K., Nikolic, B., Stocher, M., Drenckhahn, D. & Wiche, G. (1998). Not Just Scaffolding: Plectin Regulates Actin Dynamics in Cultured Cells. *Genes & Development*, **12**, 3442-3451.

Angel P, Karin M. (1992). Specific members of the Jun protein family regulate Collagenase expression in response to various extracellular stimuli. *Matrix Suppl*, **1**, 156-164.

Anzai, H., Kitadai, Y., Bucana, C.D., Sanchez, R., Omoto, R. & Fidler, I.J. (1996). Intratumoral heterogeneity and inverse correlation between expression of E-Cadherin and Collagenase type IV in human gastric carcinomas. *Differentiation*, **60**, 119-127.

Aplin, J.D. (1991). Implantation, Trophoblast Differentiation and Haemochorial Placentation - Mechanistic Evidence In-vivo and In-vitro. *Journal of Cell Science*, **99**, 681-692.

Appella, E., Robinson, E.A., Ullrich, S.J., Stoppelli, M.P., Corti, A., Cassani, G. & Blasi, F. (1987). The Receptor-Binding Sequence of Urokinase - a Biological Function for the Growth-Factor Module of Proteases. *Journal of Biological Chemistry*, **262**, 4437-4440.

Apte, S.S., Hayashi, K., Seldin, M.F., Mattei, M.G., Hayashi, M., Olsen, B.R. (1994). Gene encoding a novel murine Tissue Inhibitor of Metalloproteinases (TIMP), TIMP-3, is expressed in developing

mouse epithelia, cartilage and muscle, and is located on Chromosome 10. *Development Dyn.*, **200**, 177-197.

Apte, S.S., Olsen, B.R. & Murphy, G. (1995). The Gene Structure of Tissue Inhibitor of Metalloproteinases (TIMP)-3 and its Inhibitory Activities Define the Distinct TIMP Gene Family. *Journal of Biological Chemistry*, **270**, 14313-14318.

Amoletti, J.P., Albo, D., Granick, M.S., Solomon, M.P., Castiglioni, A., Rothman, V.L. & Tuszynski, G.P. (1995). Thrombospondin and Transforming Growth-Factor-Beta-1 Increase Expression of Urokinase-Type Plasminogen-Activator and Plasminogen- Activator Inhibitor-1 in Human MDA-MB-231 Breast-Cancer Cells. *Cancer*, **76**, 998-1005.

Atherton, A.J., Monaghan, P., Warburton, M.J. & Gusterson, B.A. (1992). Immunocytochemical Localization of the Ectoenzyme Aminopeptidase-IV in the Human Breast. *Journal of Histochemistry & Cytochemistry*, **40**, 705-710.

Atherton, A.J., Ohare, M.J., Buluwela, L., Titley, J., Monaghan, P., Paterson, H.F., Warburton, M.J. & Gusterson, B.A. (1994). Ectoenzyme Regulation by Phenotypically Distinct Fibroblast Subpopulations Isolated from the Human Mammary-Gland. *Journal of Cell Science*, **107**, 2931-2939.

Azzam, H.S., Arand, G., Lippman, M.E. & Thompson, E.W. (1993). Association of MMP-2 Activation Potential With Metastatic Progression in Human Breast-Cancer Cell-Lines Independent of MMP-2 Production. *Journal of the National Cancer Institute*, **85**, 1758-1764.

Azzam, H.S. & Thompson, E.W. (1992). Collagen-Induced Activation of the Mr 72,000 Type-IV Collagenase in Normal and Malignant Human Fibroblastoid Cells. *Cancer Research*, **52**, 4540-4544.

Bafetti, L.M., Young, T.N., Itoh, Y. & Stack, M.S. (1998). Intact vitronectin induces Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinases-2 expression and enhanced cellular invasion by melanoma cells. *Journal of Biological Chemistry*, **273**, 143-149.

Baker, S.E., Hopkinson, S.B., Fitchmun, M., Andreason, G.L., Frasier, F., Plopper, G., Quaranta, V. & Jones, J.C.R. (1996). Laminin-5 and Hemidesmosomes: Role of the alpha 3 chain subunit in Hemidesmosome stability and assembly. *Journal of Cell Science*, **109**, 2509-2520.

Balsamo, J., Leung, T.C., Ernst, H., Zanin, M.K.B., Hoffman, S. & Lilien, J. (1996). Regulated binding of a PTP1B-like phosphatase to N-Cadherin: Control of Cadherin-mediated adhesion by dephosphorylation of beta-catenin. *Journal of Cell Biology*, **134**, 801-813.

Bani, D., Riva, A., Bigazzi, M. & Sacchi, T.B. (1994). Differentiation of Breast Cancer Cells in-vitro is promoted by the concurrent influence of Myoepithelial Cells and Relaxin. *British Journal of Cancer*, **70**, 900-904.

Barcellos-Hoff, M.H., Aggeler, J., Ram, T.G. & Bissell, M.J. (1989). Functional-Differentiation and Alveolar Morphogenesis of Primary Mammary Cultures on Reconstituted Basement Membrane. *Development*, **105**, 223.

Barrett, A.J. (1987). The Cystatins - a New Class of Peptidase Inhibitors. *Trends in Biochemical Sciences*, **12**, 193-196.

Barsky, S.H., Nguyen, M., Grossman, D.A. & Huaman, J.A. (1997). Myoepithelial cells limit DCIS metastasis by blocking invasion and angiogenesis. *Laboratory Investigation*, **76**, 72.

287

Bartek, J., Taylorpapadimitriou, J., Miller, N. & Millis, R. (1985). Patterns of Expression of Keratin-19 as detected with Monoclonal- Antibodies in Human Breast Tissues and Tumors. *International Journal of Cancer*, **36,** 299-306.

Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. & Chambon, P. (1990). A Novel Metalloproteinase Gene Specifically Expressed in Stromal Cells of Breast Carcinomas. *Nature*, **348,** 699-704.

Beaucage, S.L. & Caruthers, M.H. (1981). Deoxynucleoside Phosphoramidites - a New Class of Key Intermediates for Deoxypolynucleotide Synthesis. *Tetrahedron Letters*, **22,** 1859-1862.

Becker, K.F., Atkinson, M.J., Reich, U., Becker, I., Nekarda, H., Siewert, J.R. & Hofler, H. (1994). E-Cadherin Gene-Mutations Provide Clues to Diffuse-Type Gastric Carcinomas. *Cancer Research*, **54,** 3845-3852.

Behrens, J., Mareel, M.M., Vanroy, F.M. & Birchmeier, W. (1989). Dissecting Tumor-Cell Invasion – Epithelial cells acquire invasive properties after the loss of Uvomorulin-Mediated Cell-CellAdhesion. *Journal of Cell Biology*, **108,** 2435-2447.

Behrens, J., vonKries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. & Birchmeier, W. (1996). Functional interaction of beta-Catenin with the transcription factor LEF-1. *Nature*, **382,** 638-642.

Beral, V., Hermon, C., Reeves, G. & Peto, R. (1995). Sudden Fall in Breast-Cancer Death Rates in England and Wales. *Lancet*, **345,** 1642-1643.

Berdichevsky, F., Gilbert, C., Shearer, M. & Taylorpapadimitriou, J. (1992). Collagen-Induced Rapid Morphogenesis of Human Mammary Epithelial- Cells - the role of the alpha-2-beta-1 integrin. *Journal of Cell Science*, **102,** 437-446.

Bergstraesser, L.M., Srinivasan, G., Jones, J.C.R., Stahl, S. & Weitzman, S.A. (1995). Expression of Hemidesmosomes and Component Proteins is lost by Invasive Breast Cancer Cells. *American Journal of Pathology*, **147,** 1823-1839.

Berndorff, D., Gessner, R., Kreft, B., Schnoy, N., Lajouspetter, A.M., Loch, N., Reutter, W., Hortsch, M. & Tauber, R. (1994). Liver-Intestine Cadherin - Molecular-Cloning and Characterization of a Novel Ca2+-Dependent Cell-Adhesion Molecule Expressed in Liver and Intestine. *Journal of Cell Biology*, **125,** 1353-1369.

Berx, G., CletonJansen, A.M., Strumane, K., deLeeuw, W.J.F., Nollet, F., van Roy, F. & Cornelisse, C. (1996). E-Cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene*, **13,** 1919-1925.

Bigg, H.F., Shi, Y.E., Liu, Y.L.E., Steffensen, B. & Overall, C.M. (1997). Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP4) to the COOH-terminal hemopexin-like domain of human gelatinase A - TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. *Journal of Biological Chemistry*, **272,** 15496-15500.

Birkedal-Hansen, H. (1995). Proteolytic Remodeling of Extracellular-Matrix. *Current Opinion in Cell Biology*, **7,** 728-735.

Birkedal-Hansen, H., Moore, W.G.I., Bodden, M.K., Windsor, L.J., Birkedalhansen, B., Decarlo, A. & Engler, J.A. (1993). Matrix Metalloproteinases - a Review. *Critical Reviews in Oral Biology & Medicine*, **4,** 197-250.

Biswas, C., Zhang, Y., Decastro, R., Guo, H.M., Nakamura, T., Kataoka, H. & Nabeshima, K. (1995). Human Tumor Cell-Derived Collagenase Stimulatory Factor (Renamed Emmprin) Is a Member of the Immunoglobulin Superfamily. *Cancer Research*, **55**, 434-439.

Blaschuk, O.W., Sullivan, R., David, S. & Pouliot, Y. (1990). Identification of a Cadherin Cell-Adhesion Recognition Sequence. *Developmental Biology*, **139**, 227-229.

Bloom H. J. G., Richardson W. W. (1957). Histological Grading and Prognosis in Breast Cancer. A study of 1409 cases of which 359 have been follwed up for 15 years. *Br J Cancer*, **11**, 359-377.

Bond, J.S. & Butler, P.E. (1987). Intracellular Proteases. *Annual Review of Biochemistry*, **56**, 333-364.

Boring, C.C., Squires, T.S. & Tong, T. (1993). Cancer Statistics, 1993. *Cancer Journal For Clinicians*, **43**, 7-26.

Borradori, L. & Sonnenberg, A. (1996). Hemidesmosomes: Roles in Adhesion, Signaling and Human Diseases. *Current Opinion in Cell Biology*, **8**, 647-656.

Boudreau, N., Sympson, C.J., Werb, Z. & Bissell, M.J. (1995). Suppression of Ice and Apoptosis in Mammary Epithelial-Cells by Extracellular-Matrix. *Science*, **267**, 891-893.

Boyd, R.S. & Balkwill, F.R. (1999). MMP-2 release and activation in ovarian carcinoma: the role of fibroblasts. *British Journal of Cancer*, **80**, 315-321.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein with respect to the principle of protein dye binding. *Annal. Biochem.* **72**, 248-254.

Bramhall, S.R., Stamp, G.W.H., Dunn, J., Lemoine, N.R. & Neoptolemos, J.P. (1996). Expression of Collagenase (MMP2), Stromelysin (MMP3) and Tissue Inhibitor of the Metalloproteinases (TIMP1) in Pancreatic and Ampullary disease. *British Journal of Cancer*, **73**, 972-978.

Breathnach, R., Matrisian, L.M., Gesnel, M.C., Staub, A. & Leroy, P. (1987). Sequences Coding for part of Oncogene-Induced Transin are highly conserved in a related rat gene. *Nucleic Acids Research*, **15**, 1139-1151.

Brenner, D.A., Ohara, M., Angel, P., Chojkier, M. & Karin, M. (1989). Prolonged Activation of Jun and Collagenase Genes by Tumor Necrosis Factor-Alpha. *Nature*, **337**, 661-663.

Bringuier, P.P., Umbas, R., Schaafsma, H.E., Karthaus, H.F.M., Debruyne, F.M.J. & Schalken, J.A. (1993). Decreased E-Cadherin Immunoreactivity Correlates With Poor Survival in Patients With Bladder-Tumors. *Cancer Research*, **53**, 3241-3245.

Brooks, P.C., Clark, R.A.F. & Cheresh, D.A. (1994). Requirement of Vascular Integrin Alpha(V)Beta(3) For Angiogenesis. *Science*, **264**, 569-571.

Brown, P.J. & Juliano, R.L. (1985). Selective-Inhibition of Fibronectin-Mediated Cell-Adhesion By Monoclonal-Antibodies to a Cell-Surface Glycoprotein. *Science*, **228**, 1448-1451.

Brown, P.D., Bloxidge, R.E., Anderson, E., Howell, A. (1993). Expression of activated Gelatinase in Human Invasive Breast Carcinoma. *Clin. Exp. Metastasis*, **11**, 183-189.

Bukholm, I.K., Nesland, J.M., Karesen, R., Jacobsen, U. & BorresenDale, A.L. (1998). E-Cadherin and alpha-, beta-, and gamma-Catenin protein expression in relation to metastasis in human breast carcinoma. *Journal of Pathology*, **185**, 262-266.

Burbelo, P.D., Miyamoto, S., Utani, A., Brill, S., Yamada, K.M., Hall, A. & Yamada, Y. (1995). p190-B, a new member of the Rho GAP family, and Rho are induced to cluster after integrin cross-linking. *Journal of Biological Chemistry*, **270**, 30919-30926.

Burns, J., Redfern, D.R.M., Esiri, M.M. & McGee, J.O. (1986). Human and Viral Gene Detection in Routine Paraffin Embedded Tissue By In-situ Hybridization With Biotinylated Probes - Viral Localization in Herpes-Encephalitis. *Journal of Clinical Pathology*, **39**, 1066-1073.

Burridge, K., Molony, L. & Kelly, T. (1987). Adhesion Plaques - Sites of Transmembrane Interaction Between the Extracellular-Matrix and the Actin Cytoskeleton. *Journal of Cell Science*, 211-229.

Butler, G.S., Will, H., Atkinson, S.J. & Murphy, G. (1997). Membrane-type-2 matrix metalloproteinase can initiate the processing of progelatinase A and is regulated by the tissue inhibitors of metalloproteinases. *European Journal of Biochemistry*, **244**, 653-657.

Butler, G.S., Butler, M.J., Atkinson, S.J., Will, H., Tamura, T., vanWestrum, S.S., Crabbe, T., Clements, J., dOrtho, M.P. & Murphy, G. (1998). The TIMP2- Membrane Type 1 Metalloproteinase "receptor" regulates the concentration and efficient activation of Progelatinase A - A kinetic study. *Journal of Biological Chemistry*, **273**, 871-880.

Cailleau, R., Young, R., Olive, M., Reeves, W.J.,Jnr. (1974). Beast tumour cell lines from pleural effusions. *J. National Cancer Institute*, **53**, 661-674.

Camps, J.L., Chang, S.M., Hsu, T.C., Freeman, M.R., Hong, S.J., Zhau, H.E., Voneschenbach, A.C. & Chung, L.W.K. (1990). Fibroblast-Mediated Acceleration of Human Epithelial Tumor-Growth In-vivo. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 75-79.

Candidus, S., Bischoff, P., Becker, K.F. & Hofler, H. (1996). No evidence for mutations in the alpha- and beta-Catenin genes in human gastric and breast carcinomas. *Cancer Research*, **56**, 49-52.

Canetesoler, R., Litzky, L., Lubensky, I. & Muschel, R.J. (1994). Localization of the 92 Kd Gelatinase Messenger-RNA in Squamous-Cell and Adenocarcinomas of the Lung using in-situ hybridization. *American Journal of Pathology*, **144**, 518-527.

Cao, J., Sato, H., Takino, T. & Seiki, M. (1995). The C-Terminal Region of Membrane Type Matrix Metalloproteinase is a functional Transmembrane Domain required for Pro-Gelatinase-A Activation. *Journal of Biological Chemistry*, **270**, 801-805.

Capco, D.G., Wan, K.M. & Penman, S. (1982). The Nuclear Matrix 3-Dimensional Architecture and Protein Composition. *Cell*, **29**, 847-858.

Carico, E., French, D., Bucci, B., Falcioni, R., Vecchione, A., Mariani-Constantini, R. (1993). Integrin beta 4 expression in the Neoplastic Progression of Cervical Epithelium. *Gynecol. Oncol.*, **49**, 61-66.

Carmichael, D.F., Sommer, A., Thompson, R.C., Anderson, D.C., Smith, C.G., Welgus, H.G. & Stricklin, G.P. (1986). Primary Structure and cDNA Cloning of Human Fibroblast Collagenase Inhibitor. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 2407-2411.

Carter, C.L., Allen, C. & Henson, D.E. (1989). Relation of Tumor Size, Lymph-Node Status, and Survival in 24,740 Breast-Cancer Cases. *Cancer*, **63**, 181-187.

Carter, W.G., Kaur, P., Gil, S.G., Gahr, P.J. & Wayner, E.A. (1990). Distinct Functions For Integrins alpha-3-beta-1 in Focal Adhesions and alpha-6-beta-4 and Bullous Pemphigoid Antigen in a New Stable Anchoring Contact (Sac) of Keratinocytes - Relation to Hemidesmosomes. *Journal of Cell Biology*, **111**, 3141-3154.

Carter, W.G., Ryan, M.C. & Gahr, P.J. (1991). Epiligrin, a New Cell-Adhesion Ligand for Integrin alpha-3-beta-1 in Epithelial Basement Membranes. *Cell*, **65**, 599-610.

Cattoretti, G., Andreola, S., Clemente, C., Damato, L. & Rilke, F. (1988). Vimentin and p53 Expression on Epidermal Growth-Factor Receptor- Positive, Estrogen Receptor-Negative Breast Carcinomas. *British Journal of Cancer*, **57**, 353-357.
Cawston, T.E., Galloway, W.A., Mercer, E., Murphy, G. & Reynolds, J.J. (1981). Purification of Rabbit Bone Inhibitor of Collagenase. *Biochemical Journal*, **195**, 159-165.

Cepek, K.L., Shaw, S.K., Parker, C.M., Russell, G.J., Morrow, J.S., Rimm, D.L. & Brenner, M.B. (1994). Adhesion Between Epithelial-Cells and T-Lymphocytes Mediated By E- Cadherin and the alpha(E)beta(7) Integrin. *Nature*, **372**, 190-193.

Chen, W.C. & Obrink, B. (1991). Cell Cell Contacts Mediated By E-Cadherin (Uvomorulin) Restrict Invasive Behavior of L-Cells. *Journal of Cell Biology*, **114**, 319-327.

Chen, L.C., Noelken, M.E. & Nagase, H. (1993). Disruption of the Cysteine-75 and Zinc Ion Coordination is not sufficient to activate the precursor of Human Matrix Metalloproteinase-3 (Stromelysin-1). *Biochemistry*, **32**, 10289-10295.

Chen, Q.M., Kinch, M.S., Lin, T.H., Burridge, K. & Juliano, R.L. (1994). Integrin-Mediated Cell-Adhesion activates Mitogen-Activated Protein- Kinases. *Journal of Biological Chemistry*, **269**, 26602-26605.

Cheresh, D.A. (1991). Structure, Function and Biological Properties of Integrin alpha-V- beta-3 on Human-Melanoma Cells. *Cancer and Metastasis Reviews*, **10**, 3-10.

Chin, J.R., Murphy, G. & Werb, Z. (1985). Stromelysin, a Connective Tissue-Degrading Metalloendopeptidase secreted by stimulated Rabbit Synovial Fibroblasts in parallel with Collagenase - Biosynthesis, Isolation, Characterization, and Substrates. *Journal of Biological Chemistry*, **260**, 2367-2376.

Chintala, S.K., Sawaya, R., Aggarwal, B.B., Majumder, S., Giri, D.K., Kyritsis, A.P., Gokaslan, Z.L. & Rao, J.S. (1998). Induction of Matrix Metalloproteinase-9 requires a Polymerized Actin Cytoskeleton in human malignant glioma cells. *Journal of Biological Chemistry*, **273**, 13545-13551.

Clark, E.A. & Brugge, J.S. (1995). Integrins and Signal-Transduction Pathways - the Road Taken. *Science*, **268**, 233-239.

Clark, I.M. & Cawston, T.E. (1989). Fragments of Human Fibroblast Collagenase - Purification and Characterization. *Biochemical Journal*, **263**, 201-206.

Clarke, C., Titley, J., Davies, S., O'Hare, M.J. (1994). An Immunomagnetic Separation Method using Superparamagnetic (MACS) beads for large scale purification of Human Mammary Luminal and Myoepithelial Cells. *Epithelial Cell Biology*, **3**, 38-46.

Clarke A, Lotz M et al (1995). Activation of the p21 pathway of growth arrest and apoptosis by the β4 integrin cytoplasmic domain. *Journal Biological Chemistry* **270**, 22673-22676.

Coghlan, J.P., Aldred, P., Haralambidis, J., Niall, H.D., Penschow, J.D. & Tregear, G.W. (1985). Hybridization Histochemistry. *Analytical Biochemistry*, **149**, 1.

Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant, G.A., Seltzer, J.L., Kronberger, A., He, C.S., Bauer, E.A. & Goldberg, G.I. (1988). H-Ras Oncogene-Transformed Human Bronchial Epithelial-Cells (Tbe-1) secrete a single Metalloprotease capable of degrading Basement Membrane Collagen. *Journal of Biological Chemistry*, **263**, 6579-6587.

Cook, A.F., Vuocolo, E. & Brakel, C.L. (1988). Synthesis and Hybridization of a series of Biotinylated Oligonucleotides. *Nucleic Acids Research*, **16**, 4077-4095.

Coppolino, M.G., Demaurex, N., Grinstein, S., StArnoud, R. & Dedhar, S. (1996). Calreticulin-knockout embryonic stem cells are deficient in integrin- mediated adhesion but not calcium stores. *Molecular Biology of the Cell*, **7**, 1419.

Corcoran, M.L., Kibbey, M.C., Kleinman, H.K. & Wahl, L.M. (1995). Laminin Sikvav Peptide Induction of Monocyte-Macrophage Prostaglandin E(2) and Matrix Metalloproteinases. *Journal of Biological Chemistry*, **270**, 10365-10368.

Cornelius, L.A., Nehring, L.C., Roby, J.D., Parks, W.C. & Welgus, H.G. (1995). Human Dermal Microvascular Endothelial-Cells produce Matrix Metalloproteinases in response to Angiogenic Factors and Migration. *Journal of Investigative Dermatology*, **105**, 170-176.

Cornil, I. & Kerbel, R.S. (1991). Mesenchymal Interactions with Human-Melanoma Cells affecting Tumor Cell Growth are a function of tumor progression. *British Journal of Cancer*, **63**, 828.

Cossins, J., Dudgeon, T.J., Catlin, G., Gearing, A.J.H. & Clements, J.M. (1996). Identification of MMP-18, a putative novel human Matrix Metalloproteinase. *Biochemical and Biophysical Research Communications*, **228**, 494-498.

Cowell, S., Knauper, V., Stewart, M.L., dOrtho, M.P., Stanton, H., Hembry, R.M., Lopez-Otin, C., Reynolds, J.J. & Murphy, G. (1998). Induction of Matrix Metalloproteinase activation cascades based on Membrane-Type 1 Matrix Metalloproteinase: associated activation of gelatinase A, gelatinase B and collagenase 3. *Biochemical Journal*, **331**, 453-458.

Cox, K.H., Deleon, D.V., Angerer, L.M. & Angerer, R.C. (1984). Detection of Messenger-RNAs in Sea-Urchin Embryos By In-situ Hybridization using Asymmetric RNA Probes. *Developmental Biology*, **101**, 485-502.

Crawford, H.C., Fingleton, B.M., RudolphOwen, L.A., Goss, K.J.H., Rubinfeld, B., Polakis, P. & Matrisian, L.M. (1999). The Metalloproteinase Matrilysin is a target of beta-Catenin transactivation in intestinal tumors. *Oncogene*, **18**, 2883-2891.

Cubellis, M.V., Nolli, M.L., Cassani, G. & Blasi, F. (1986). Binding of Single-Chain Pro-urokinase to the Urokinase Receptor of Human U937-Cells. *Journal of Biological Chemistry*, **261**, 5819-5822.

Curry, V.A., Clark, I.M., Bigg, H. & Cawston, T.E. (1992). Large Inhibitor of Metalloproteinases (Limp) Contains Tissue Inhibitor of Metalloproteinases (TIMP)-2 Bound to 72000-Mr Progelatinase. *Biochemical Journal*, **285**, 143-147.

Curschellas, E., Regenass, U. & Matter, A. (1987). Expression of Intermediate Filament Proteins in Human-Breast Tissue Derived Cell-Cultures. *Experientia*, **43**, 683.

D'Ardenne, J., Richman, P.I., Horton, M.A., McAuley, A.E., Jordan, S. (1992). Co-ordinate expression of the alpha-6 integrin laminin receptor and laminin in the breast. *Journal Pathology*, **165**, 213-220.

Damjanovich, L., Albelda, S.M., Mette, S.A. & Buck, C.A. (1992). Distribution of Integrin Cell-Adhesion Receptors in Normal and Malignant Lung-Tissue. *American Journal of Respiratory Cell and Molecular Biology*, **6**, 197-206.

Damsky C H, & Werb Z (1992) Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.*, **4**, 772-781.

Damsky, C.H., Fitzgerald, M.L. & Fisher, S.J. (1992). Distribution Patterns of Extracellular-Matrix Components and Adhesion Receptors are intricately modulated during 1st Trimester Cytotrophoblast Differentiation along the invasive pathway, in-vivo. *Journal of Clinical Investigation*, **89**, 210-222.

Damsky, C., Sutherland, A. & Fisher, S. (1993). Extracellular Matrix-5 - Adhesive Interactions in Early Mammalian Embryogenesis, Implantation, and Placentation. *Faseb Journal*, **7**, 1320-1329.

Daniel, J.M. & Reynolds, A.B. (1995). The Tyrosine Kinase Substrate P120(Cas) Binds Directly to E-Cadherin But Not to the Adenomatous Polyposis-Coli Protein or Alpha-Catenin. *Molecular and Cellular Biology*, **15**, 4819-4824.

Dano, K., Andreasen, P.A., Grondahlhansen, J., Kristensen, P., Nielsen, L.S. & Skriver, L. (1985). Plasminogen Activators, Tissue Degradation and Cancer. *Anticancer Research*, **5**, 605.

Dantzig, A.H., Hoskins, J., Tabas, L.B., Bright, S., Shepard, R.L., Jenkins, I.L., Duckworth, D.C., Sportsman, J.R., Mackensen, D., Rosteck, P.R. & Skatrud, P.L. (1994). Association of Intestinal Peptide-Transport with a protein related to the Cadherin Superfamily. *Science*, **264**, 430-433.

Davies, B., Miles, D.W., Happerfield, L.C., Naylor, M.S., Bobrow, L.G., Rubens, R.D. & Balkwill, F.R. (1993a). Activity of Type-Iv Collagenases in Benign and Malignant Breast Disease. *British Journal of Cancer*, **67**, 1126-1131.

Davies, B., Waxman, J., Wasan, H., Abel, P., Williams, G., Krausz, T., Neal, D., Thomas, D., Hanby, A. & Balkwill, F. (1993b). Levels of Matrix Metalloproteases in Bladder-Cancer Correlate with Tumor Grade and Invasion. *Cancer Research*, **53**, 5365-5369.

De Bruin, P.A.F., Griffioen, G., Verspaget, H.W., Verheijen, J.H., Dooijewaard, G., Vanderingh, H. & Lamers, C. (1988). Plasminogen Activators in Human Colonic Carcinogenesis. *Gastroenterology*, **94**, A90.

Declerck, Y.A., Yean, T.D., Chan, D., Shimada, H. & Langley, K.E. (1991). Inhibition of Tumor Invasion of Smooth-Muscle cell layers by recombinant Human Metalloproteinase Inhibitor. *Cancer Research*, **51**, 2151-2157.

De Clerck Y A, Perez N, et al (1992). Inhibition of Invasion and Metastasis in cells transfected with an Inhibitor of Metalloproteinases. *Cancer Research*, **52**, 701-708.

DeClerck, Y.A. & Laug, W.E. (1996). Cooperation between matrix metalloproteinases and the plasminogen activator-plasmin system in tumor progression. *Enzyme & Protein*, **49**, 72-84.

Dedhar, S., Saulnier, R., Nagle, R. & Overall, C.M. (1993). Specific Alterations in the Expression of alpha-3-beta-1 and alpha-6- beta-4 Integrins in highly Invasive and Metastatic Variants of Human Prostate Carcinoma-Cells selected by in-vitro invasion through Reconstituted Basement-Membrane. *Clinical & Experimental Metastasis*, **11**, 391-400.

Dedhar, S. & Hannigan, G.E. (1996). Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Current Opinion in Cell Biology*, **8**, 657-669.

DeLeeuw, W.J.F., Berx, G., Vos, C.B.J., Peterse, J.L., VandeVijver, M.J., Litvinov, S., VanRoy, F., Cornelisse, C.J. & CletonJansen, A.M. (1997). Simultaneous loss of E-Cadherin and Catenins in invasive lobular breast cancer and lobular carcinoma in situ. *Journal of Pathology*, **183**, 404-411.

Deng, G. & Wu, R. (1981). An Improved Procedure for utilizing Terminal Transferase to add Homopolymers to the 3' Termini of DNA. *Nucleic Acids Research*, **9**, 4173-4188.

Dike, L.E. & Farmer, S.R. (1988). Cell-Adhesion induces expression of Growth-Associated Genes in Suspension-Arrested Fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 6792-6796.

Dimanche-Boitrel, M.T., Vakaet, L., Pujuguet, P., Chauffert, B., Martin, M.S., Hammann, A., Vanroy, F., Mareel, M. & Martin, F. (1994). In-vivo and in-vitro invasiveness of a Rat Colon-Cancer Cell-Line maintaining E-Cadherin Expression - an Enhancing Role of Tumor- Associated Myofibroblasts. *International Journal of Cancer*, **56**, 512-521.

Docherty, A.J.P., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J.R., Murphy, G. & Reynolds, J.J. (1985). Sequence of Human-Tissue Inhibitor of Metalloproteinases and its identity to Erythroid-Potentiating Activity. *Nature*, **318**, 66-69.

Dogic, D., Rousselle, P. & Aumailley, M. (1998). Cell adhesion to laminin 1 or 5 induces isoform-specific clustering of integrins and other focal adhesion components. *Journal of Cell Science*, **111**, 793-802.

Domagala, W., Lasota, J., Bartkowiak, J., Weber, K. & Osborn, M. (1990a). Vimentin Is Preferentially Expressed in Human Breast Carcinomas with low Oestrogen Receptor and high Ki-67 growth fraction. *American Journal of Pathology*, **136**, 219-227.

Domagala, W., Lasota, J., Dukowicz, A. et al. (1990b). Vimentin expression appears to be associated with poor prognosis in Node-Negative Ductal NOS Breast Carcinomas. *American Journal of Pathology*, **137**, 1299-1304.

Domloge-Hultsch, N., Gammon, W.R., Briggaman, R.A., Gil, S.G., Carter, W.G. & Yancey, K.B. (1992). Epiligrin, the Major Human Keratinocyte Integrin Ligand, is a target in both an Acquired Autoimmune and an Inherited Subepidermal Blistering Skin-Disease. *Journal of Clinical Investigation*, **90**, 1628-1633.

D'Ortho, M.P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith, B., Timpl, R., Zardi, L. & Murphy, G. (1997). Membrane-Type Matrix Metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many Matrix Metalloproteinases. *European Journal of Biochemistry*, **250**, 751-757.

Dukas, K., Sarfati, P., Vaysse, N. & Pradayrol, L. (1993). Quantitation of Changes in the Expression of Multiple Genes by Simultaneous Polymerase Chain Reaction. *Analytical Biochemistry*, **215**, 66-72.

Durko, M., Navab, R., Shibata, H.R. & Brodt, P. (1997). Suppression of basement membrane type IV collagen degradation and cell invasion in human melanoma cells expressing an antisense RNA for MMP-1. *Biochimica et Biophysica Acta-Molecular Cell Research*, **1356**, 271-280.

Elices, M.J., Urry, L.A. & Hemler, M.E. (1991). Receptor Functions For the Integrin VLA-3 - Fibronectin, Collagen, and Laminin Binding are differentially influenced by Arg-Gly-Asp Peptide and by Divalent-Cations. *Journal of Cell Biology*, **112**, 169-181.

Elston, C.W. & Ellis, I.O. (1991). Pathological Prognostic Factors in Breast-Cancer .1. The Value of Histological Grade in Breast-Cancer - Experience From a Large Study With Long-Term Follow-Up. *Histopathology*, **19**, 403-410.

Emonard, H.P., Remacle, A.G., Noel, A.C., Grimaud, J.A., Stetlerstevenson, W.G. & Foidart, J.M. (1992). Tumor-Cell Surface-Associated Binding-Site For the M(R)72,000 Type-IV Collagenase. *Cancer Research*, **52**, 5845-5848.

Eyden, B.P., Watson, R.J., Harris, M. & Howell, A. (1986). Intralobular Stromal Fibroblasts in the Resting Human Mammary Gland - Ultrastructural Properties and Intercellular Relationships. *Journal of Submicroscopic Cytology and Pathology*, **18**, 397-408.

Fabris, G., Marchetti, E., Marzola, A., Bagni, A., Querzoli, P. & Nenci, I. (1987). Patho-physiology of Estrogen-Receptors in Mammary Tissue by monoclonal-antibodies. *Journal of Steroid Biochemistry and Molecular Biology*, **27**, 171-176.

Felding-Habermann, B., Mueller, B.M., Romerdahl, C.A. & Cheresh, D.A. (1992). Involvement of Integrin alpha-V gene-expression in Human-Melanoma Tumorigenicity. *Journal of Clinical Investigation*, **89**, 2018-2022.

Ferguson, D.J.P. & Anderson, T.J. (1981). Morphological Evaluation of Cell Turnover in Relation to the Menstrual-Cycle in the Resting Human-Breast. *British Journal of Cancer*, **44**, 177-181.

Ferguson, D.J., Meier, P., Karrison, T., Dawson, P.J., Straus, F.H. & Lowenstein, F.E. (1982). Staging of Breast-Cancer and Survival Rates - an Assessment Based on 50 Years of Experience with Radical-Mastectomy. *Jama-Journal of the American Medical Association*, **248**, 1337-1341.

Fessler, L.I., Duncan, K.G., Fessler, J.H., Salo, T. & Tryggvason, K. (1984). Characterization of the Procollagen-IV Cleavage Products produced by a specific Tumor Collagenase. *Journal of Biological Chemistry*, **259**, 9783-9789.

Festuccia, C., Bologna, M., Vicentini, C., Tacconelli, A., Miano, R., Violini, S. & Mackay, A.R. (1996). Increased Matrix Metalloproteinase-9 secretion in short-term tissue cultures of Prostatic Tumor Cells. *International Journal of Cancer*, **69**, 386-393.

Fey, E.G., Wan, K.M. & Penman, S. (1984). Epithelial Cytoskeletal Framework and Nuclear Matrix Intermediate Filament Scaffold: 3-Dimensional Organization and Protein Composition. *Journal of Cell Biology*, **98**, 1973-1984.

Fields, G.B., Netzelarnett, S.J., Windsor, L.J., Engler, J.A., Birkedalhansen, H. & Vanwart, H.E. (1990). Proteolytic Activities of Human Fibroblast Collagenase –

Fingleton, B.M., Goss, K.J.H., Crawford, H.C. & Matrisian, L.M. (1999). Matrilysin in early stage intestinal tumorigenesis. *Apmis*, **107**, 102-110.

Fisher, S.J., Cui, T.Y., Li, Z., Hartman, L., Grahl, K., Zhang, G.Y., Tarpey, J. & Damsky, C.H. (1989). Adhesive and Degradative Properties of Human Placental Cytotrophoblast Cells in-vitro. *Journal of Cell Biology*, **109**, 891-902.

Fontao, L., Dirrig, S., Owaribe, K., Kedinger, M. & Launay, J.F. (1997). Polarized expression of HD1: Relationship with the cytoskeleton in cultured human colonic carcinoma cells *Experimental Cell Research*, **231**, 319-327.

Foote F, Stewart F (1941). Lobular carcinoma in-situ: a rare form of mammary carcinoma *American Journal Pathology*, **17**, 491-496.

Forsyth, P.A., Wong, H., Laing, T.D., Rewcastle, N.B., Morris, D.G., Muzik, H., Leco, K.J., Johnston, R.N., Brasher, P.M.A., Sutherland, G. & Edwards, D.R. (1999). Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and Membrane Type Matrix Metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. *British Journal of Cancer*, **79**, 1828-1835.

Freake, H.C., Marcocci, C., Iwasaki, J. & Macintyre, I. (1981). 1,25-Dihydroxyvitamin D-3 specifically binds to a Human-Breast Cancer Cell-Line (T47D) and stimulates growth. *Biochemical and Biophysical Research Communications*, **101**, 1131-1138.

Freije, J.M.P., Diezitza, I., Balbin, M., Sanchez, L.M., Blasco, R., Tolivia, J. & Lopezotin, C. (1994). Molecular-Cloning and Expression of Collagenase-3, a Novel Human Matrix Metalloproteinase Produced by Breast Carcinomas. *Journal of Biological Chemistry*, **269**, 16766-16773.

Freimark, B.D., Feeser, W.S. & Rosenfeld, S.A. (1994). Multiple Sites of the Propeptide Region of Human Stromelysin-1 are required for maintaining a latent form of the enzyme. *Journal of Biological Chemistry*, **269**, 26982-26987.

Fridman, R., Fuerst, T.R., Bird, R.E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L.A., Berman, M.L. & Stetlerstevenson, W.G. (1992). Domain-Structure of Human 72-Kda Gelatinase Type-IV Collagenase: Characterization of Proteolytic Activity and Identification of the Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) Binding Regions. *Journal of Biological Chemistry*, **267**, 15398-15405.

Fridman, R., Toth, M., Pena, D. & Mobashery, S. (1995). Activation of Progelatinase-B (MMP-9) by Gelatinase-A (MMP-2). *Cancer Research*, **55**, 2548-2555.

Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H.J. & Imhof, B.A. (1995). High Expression Level of alpha-6 Integrin in Human Breast-Carcinoma is correlated with reduced survival. *Cancer Research*, **55**, 901-906.

Frisch, S.M., Clark, E.J., Werb, Z. (1987). Co-ordinate regulation of Stromelysin and Collagenase genes determined with cDNA probes. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 2600-2604.

Frisch, S.M., Vuori, K., Ruoslahti, E. & ChanHui, P.Y. (1996). Control of adhesion-dependent cell survival by Focal Adhesion Kinase. *Journal of Cell Biology*, **134**, 793-799.

Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D. & Birchmeier, W. (1991). E-Cadherin-Mediated Cell Cell-Adhesion prevents invasiveness of Human Carcinoma Cells. *Journal of Cell Biology*, **113**, 173-185.

Frixen, U.H. & Nagamine, Y. (1993). Stimulation of Urokinase-Type Plasminogen-Activator Expression by Blockage of E-Cadherin-Dependent Cell-Cell Adhesion. *Cancer Research*, **53**, 3618-3623.

Fukushima, Y., Ohnishi, T., Arita, N., Hayakawa, T. & Sekiguchi, K. (1998). Integrin alpha 3 beta 1-mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. *International Journal of Cancer*, **76**, 63-72.

Gabbert, H. (1985). Mechanisms of Tumor Invasion - Evidence from in-vivo observations. *Cancer and Metastasis Reviews*, **4**, 293-309.

Gaire, M., Magbanua, Z., McDonnell, S., McNeil, L., Lovett, D.H. & Matrisian, L.M. (1994). Structure and Expression of the Human Gene for the Matrix Metalloproteinase Matrilysin. *Journal of Biological Chemistry*, **269**, 2032-2040.

Garbi, C., Colletta, G., Cirafici, A.M., Marchisio, P.C. & Nitsch, L. (1990). Transforming Growth-Factor-Beta Induces Cytoskeleton and Extracellular-Matrix Modifications in Frtl-5 Thyroid Epithelial-Cells. *European Journal of Cell Biology*, **53**, 281-289.

Garbisa, S., Pozzatti, R., Muschel, R.J., Saffiotti, U., Ballin, M., Goldfarb, R.H., Khoury, G. & Liotta, L.A. (1987). Secretion of Type-IV Collagenolytic Protease and Metastatic Phenotype: Induction by transfection with c-Ha-Ras but not c-Ha-Ras plus Ad2- E1a. *Cancer Research*, **47**, 1523-1528.

Gasson, J.C., Golde, D.W., Kaufman, S.E., Westbrook, C.A., Hewick, R.M., Kaufman, R.J., Wong, G.G., Temple, P.A., Leary, A.C., Brown, E.L., Orr, E.C. & Clark, S.C. (1985). Molecular Characterization and Expression of the Gene Encoding Human Erythroid-Potentiating Activity. *Nature*, 315, 768-771.

Giancotti, F.G. & Ruoslahti, E. (1990). Elevated Levels of the alpha-5-beta-1-Fibronectin Receptor Suppress the Transformed Phenotype of Chinese Hamster Ovary Cells. *Cell*, 60, 849-859.

Giannelli, G., FalkMarzillier, J., Schiraldi, O., StetlerStevenson, W.G. & Quaranta, V. (1997). Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science*, 277, 225-228.

Gil, S.G., Brown, T.A., Ryan, M.C. & Carter, W.G. (1994). Junctional Epidermolysis Bullosis - Defects in Expression of Epiligrin/Nicein/Kalinin and Integrin Beta-4 That Inhibit Hemidesmosome Formation. *Journal of Investigative Dermatology*, 103, S31-S38.

Gilles, C., Polette, M., Piette, J., Munaut, C., Thompson, E.W., Birembaut, P. & Foidart, J.M. (1996). High level of MT-MMP expression is associated with invasiveness of cervical cancer cells. *International Journal of Cancer*, 65, 209-213.

Gilles, C., Polette, M., Seiki, M., Birembaut, P. & Thompson, E.W. (1997). Implication of collagen type 1-induced membrane-type 1 matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. *Laboratory Investigation*, 76, 651-660.

Ginsberg, M.H., Du, X. & Plow, E.F. (1992). Inside-out integrin signalling. *Curr. Opin. Cell Biology*, 4, 766-771.

Glukhova, M., Kpteliansky, V., Sastre, X., Thiery, J.P. (1995). Adhesion Systems in Normal Breast and in Invasive Breast Carcinoma. *Am. J. Pathology*, 146, 706-716.

Going, J.J., Anderson, T.J., Battersby, S. & Macintyre, C.C.A. (1988). Proliferative and Secretory Activity in Human-Breast During Natural and Artificial Menstrual Cycles. *American Journal of Pathology*, 130, 193-204.

Goldberg, G.I., Marmer, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S. & He, C.S. (1989). Human 72-Kilodalton Type-Iv Collagenase Forms a Complex With a Tissue Inhibitor of Metalloproteases Designated Timp-2. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 8207-8211.

Goldberg, G.I., Strongin, A., Collier, I.E., Genrich, L.T. & Marmer, B.L. (1992). Interaction of 92-Kda Type-Iv Collagenase With the Tissue Inhibitor of Metalloproteinases Prevents Dimerization, Complex-Formation With Interstitial Collagenase, and Activation of the Proenzyme With Stromelysin. *Journal of Biological Chemistry*, 267, 4583-4591.

Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Grant, G.A., Bauer, E.A. & Eisen, A.Z. (1986). Human Fibroblast Collagenase - Primary Structure and Tissue Specific Levels of Enzyme-Synthesis. *Journal of Investigative Dermatology*, 87, 141.

Gomez, M., Navarro, P., Quintanilla, M. & Cano, A. (1992). Expression of Alpha-6-Beta-4 Integrin Increases During Malignant Conversion of Mouse Epidermal-Keratinocytes - Association of Beta-4 Subunit to the Cytokeratin Fraction. *Experimental Cell Research*, 201, 250-261.

Gomm, J.J., Smith, J., Ryall, G.K., Baillie, R., Turnbull, L. & Coombes, R.C. (1991). Localization of Basic Fibroblast Growth-Factor and Transforming Growth Factor-Beta-1 in the Human Mammary-Gland. *Cancer Research*, 51, 4685-4692.

Gomm, J.J., Browne, P.J. Coope, R.C., Liu, Q.Y., Buluwela, L., & Coombes, R.C. (1997). Isolation of Pure Populations of Epithelial and Myoepithelial cells from the Normal Human Mammary Gland using Immunomagnetic Separation with Dynabeads. *Analytical Biochemistry*, **226**, 91-99.

Gong, J.G., Ko, T.C. & Brattain, M.G. (1998). Disruption of fibronectin binding to the alpha(5)beta(1) integrin stimulates the expression of Cyclin-Dependent Kinases and DNA synthesis through activation of Extracellular Signal-Regulated Kinase. *Journal of Biological Chemistry*, **273**, 1662-1669.

Gonzalez, M.A., Pinder, S.E., Wencyk, P.M., Bell, J.A., Elston, C.W., Nicholson, R.I., Robertson, J.F.R., Blamey, R.W. & Ellis, I.O. (1999). An immunohistochemical examination of the expression of E-cadherin, alpha- and beta/gamma-catenins, and alpha 2- and beta 1-integrins in invasive breast cancer. *Journal of Pathology*, **187**, 523-529.

Gouon, V., Tucker, G.C., KrausBerthier, L., Atassi, G. & Kieffer, N. (1996). Up-regulated expression of the beta 3 integrin and the 92-kDA gelatinase in human HT-134 melanoma cell tumors grown in nude mice. *International Journal of Cancer*, **68**, 650-662.

Graff, J.R., Herman, J.G., Lapidus, R.G., Chopra, H., Xu, R., Jarrard, D.F., Isaacs, W.B., Pitha, P.M., Davidson, N.E. & Baylin, S.B. (1995). E-Cadherin Expression is silenced by DNA Hypermethylation in Human Breast and Prostate Carcinomas. *Cancer Research*, **55**, 5195-5199.

Grant, G.A., Eisen, A.Z., Marmer, B.L., Roswit, W.T. & Goldberg, G.I. (1987). The Activation of Human-Skin Fibroblast Procollagenase - Sequence Identification of the Major Conversion Products. *Journal of Biological Chemistry*, **262**, 5886-5889.

Green, K.J. & Jones, J.C.R. (1996). Desmosomes and Hemidesmosomes: Structure and function of molecular components. *Faseb Journal*, **10**, 871-881.

Greene, J., Wang, M.S., Liu, Y.L.E., Raymond, L.A., Rosen, C. & Shi, Y.N.E. (1996). Molecular cloning and characterization of human Tissue Inhibitor of Metalloproteinase 4. *Journal of Biological Chemistry*, **271**, 30375-30380

Greenhough R B. (1925).Varying degrees of malignancy in cancer of the breast. *J. Cancer Research*, **9**, 452-463.

Gregoire, M.& Lieubeau, B. (1995). The role of Fibroblasts in tumour behaviour. *Cancer Met. Rev.* **14**, 339-350.

Grossman, D.A., Shao, Z.M. & Barsky, S.H. (1999). Myoepithelial cells inhibit DCIS progression through ER-beta mediated maspin and nitric oxide secretion. *Laboratory Investigation*, **79**, 109.

Guan, J.L. & Hynes, R.O. (1990). Lymphoid-Cells Recognize an Alternatively Spliced Segment of Fibronectin via the integrin receptor-alpha-4-beta-1. *Cell*, **60**, 53-61.

Guerin, E., Ludwig, M.G., Basset, P. & Anglard, P. (1997). Stromelysin-3 induction and interstitial collagenase repression by retinoic acid: Therapeutical implication of receptor-selective retinoids dissociating transactivation and AP-1-mediated transrepression. *Journal of Biological Chemistry*, **272**, 11088-11095.

Gui, G.P.H., Puddefoot, J.R., Vinson, G.P., Wells, C.A. & Carpenter, R. (1997). Altered cell-matrix contact: A prerequisite for breast cancer metastasis? *British Journal of Cancer*, **75**, 623-633.

Gui, G.P.H., Wells, C.A., Browne, P.D., Yeomans, P., Jordan, S., Puddefoot, J.R., Vinson, G.P. & Carpenter, R. (1995). Integrin Expression in Primary Breast-Cancer and its relation to Axillary Nodal Status. *Surgery*, **117**, 102-108.

Gum, R., Lengyel, E., Juarez, J., Chen, J.H., Sato, H., Seiki, M. & Boyd, D. (1996). Stimulation of 92-kDa gelatinase B promoter activity by ras is Mitogen-Activated Protein Kinase Kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. *Journal of Biological Chemistry*, **271**, 10672-10680.

Gusterson, B.A., Warburton, M.J., Mitchell, D., Ellison, M., Neville, A.M. & Rudland, P.S. (1982). Distribution of Myoepithelial Cells and Basement-Membrane Proteins in the Normal Breast and in Benign and Malignant Breast Diseases. *Cancer Research*, **42**, 4763-4770.

Gusterson, B.A., Monaghan, P., Mahendran, R., Ellis, J. & Ohare, M.J. (1986). Identification of Myoepithelial Cells in Human and Rat Breasts by Anti-Common Acute Lymphoblastic-Leukemia Antigen-Antibody A12. *Journal of the National Cancer Institute*, **77**, 343-349.

Gutman, A. & Wasylyk, B. (1990). The Collagenase gene promoter contains a TPA and Oncogene-Responsive Unit encompassing the PEA3 and AP-1 binding sites. *Embo Journal*, **9**, 2241-2246.

Hall, P.A., Coates, P., Lemoine, N.R. & Horton, M.A. (1991). Characterization of Integrin Chains in Normal and Neoplastic Human Pancreas. *Journal of Pathology*, **165**, 33-41.

Ham A W, Cormack D H (1979).The Breast in : *Histology*, 8th Ed. Philadelphia : J B Lippincott, pp 866-874.

Hanby, A.M., Gillett, C.E., Pignatelli, M. & Stamp, G.W.H. (1993). Beta-1 and Beta-4 Integrin Expression in Methacarn and Formalin-Fixed Material from in-situ Ductal Carcinoma of the Breast. *Journal of Pathology*, **171**, 257-262.

Hart, I.R. & Saini, A. (1992). Biology of Tumor-Metastasis. *Lancet*, **339**, 1453-1457.

Hashimoto, M., Niwa, O., Nitta, Y., Takeichi, M. & Yokoro, K. (1989). Unstable Expression of E-Cadherin Adhesion Molecules in Metastatic Ovarian Tumor-Cells. *Japanese Journal of Cancer Research*, **80**, 459-463.

Hasty, K.A., Pourmotabbed, T.F., Goldberg, G.I., Thompson, J.P., Spinella, D.G., Stevens, R.M. & Mainardi, C.L. (1990). Human Neutrophil Collagenase - a Distinct Gene-Product with homology to other Matrix Metalloproteinases. *Journal of Biological Chemistry*, **265**, 11421-11424.

Hayakawa, T., Yamashita, K., Ohuchi, E. & Shinagawa, A. (1994). Cell Growth-Promoting activity of Tissue Inhibitor of Metalloproteinases-2 (TIMP-2). *Journal of Cell Science*, **107**, 2373-2379.

Hayakawa, T., Yamashita, K., Tanzawa, K., Uchijima, E. & Iwata, K. (1992). Growth-Promoting Activity of Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) for a wide range of cells - a possible new growth-factor in serum. *Febs Letters*, **298**, 29-32.

Hayashi, S., Gillam, I.C., Delaney, A.D., Tener, G.M. (1978). Acetylation of chromosome squashes of Drosophila Melanogaster decreases the background in autoradiographs from hybridisation with ^{125}I-labelled RNA. *J. Histochem. Cytochem.*, **26**, 677-679.

He, C.S., Wilhelm, S.M., Pentland, A.P., Marmer, B.L., Grant, G.A., Eisen, A.Z. & Goldberg, G.I. (1989). Tissue Cooperation in a Proteolytic Cascade activating Human Interstitial Collagenase. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2632-2636.

Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B. & Fidler, I.J. (1987). A Simple Quantitative Assay for studying the invasive potential of High and Low Human Metastatic Variants. *Cancer Letters*, **38**, 137-147.

Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B., Misiorowski, R.L., Saba, P.Z., Sundareshan, P. & Welch, D.R. (1989). Comparison of Tumor-Cell Invasion Assays - Human Amnion versus Reconstituted Basement-Membrane Barriers. *Invasion & Metastasis*, **9**, 278-297.

Husbey R A, Thomas L B. (1954). Histological and histochemical alterations in the normal breast tissues of patients with advanced breast cancer being treated with oestrogenic hormones. *Cancer*, **7**, 54-74.
Heppner, K.J., Matrisian, L.M., Jensen, R.A. & Rodgers, W.H. (1996). Expression of most Matrix Metalloproteinase family members in Breast Cancer represents a tumor-induced host response. *American Journal of Pathology*, **149**, 273-282.

Hermiston, M.L. & Gordon, J.I. (1995). In-Vivo Analysis of Cadherin Function in the Mouse Intestinal Epithelium - Essential Roles in Adhesion, Maintenance of Differentiation, and Regulation of Programmed Cell-Death. *Journal of Cell Biology*, **129**, 489-506.

Hermon, C. & Beral, V. (1996). Breast cancer mortality rates are leveling off or beginning to decline in many western countries: Analysis of time trends, age- cohort and age-period models of breast cancer mortality in 20 countries. *British Journal of Cancer*, **73**, 955-960.

Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M. & Kemler, R. (1991). The Uvomorulin-Anchorage Protein Alpha-Catenin Is a Vinculin Homolog. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 9156-9160.

Hertig, A.T., Rock, J., Adams, J.(1956). A description of 34 human ova within the first 17 days of development. *American Journal of Anatomy*, **98**, 435-494.

Hessle, H., Sakai, L.Y., Hollister, D.W., Burgeson, R.E. & Engvall, E. (1984). Basement-Membrane Diversity Detected By Monoclonal-Antibodies. *Differentiation*, **26**, 49-54.

Heussen, C & Dowdle, E.B. (1980). Electrophoretic analysis of Plasminogen Activators in polyacrylamide gels containing sodium dodecyl sulphate and co-polymerised substrates. *Analytical Biochemistry*, **102**, 196-202.

Hieda, Y., Nishizawa, Y., Uematsu, J. & Owaribe, K. (1992). Identification of a new Hemidesmosomal Protein, HD1 - a major, high molecular mass component of isolated Hemidesmosomes. *Journal of Cell Biology*, **116**, 1497-1506.

Himelstein, B.P., Canetesoler, R., Bernhard, E.J. & Muschel, R.J. (1994). Induction of Fibroblast 92 Kda Gelatinase Type-IV Collagenase Expression By Direct-Contact With Metastatic Tumor-Cells. *Journal of Cell Science*, **107**, 477-486.

Himelstein, B.P., Canetesoler, R., Bernhard, E.J., Dilks, D.W. & Muschel, R.J. (1995). Metalloproteinases in Tumor Progression - the Contribution of MMP-9. *Invasion & Metastasis*, **14**, 246-258.

Himelstein, B.P. & Muschel, R.J. (1996). Induction of Matrix Metalloproteinase 9 expression in Breast Carcinoma cells by a soluble factor from fibroblasts. *Clinical & Experimental Metastasis*, **14**, 197-208.

Hinck, L., Nathke, I.S., Papkoff, J. & Nelson, W.J. (1994). Dynamics of Cadherin/Catenin Complex-Formation - Novel Protein Interactions and Pathways of Complex Assembly. *Journal of Cell Biology*, **125**, 1327-1340.

Hiraguri, S., Godfrey, T., Nakamura, H., Graff, J., Collins, C., Shayesteh, L., Doggett, N., Johnson, K., Wheelock, M., Herman, J., Baylin, S., Pinkel, D. & Gray, J. (1998). Mechanisms of inactivation of E-Cadherin in Breast Cancer cell lines. *Cancer Research*, **58**, 1972-1977.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. & Takeichi, M. (1992). Identification of a Neural Alpha-Catenin as a key regulator of Cadherin Function and Multicellular Organization. *Cell*, **70**, 293-301.

Holland, R., Peterse, J.L., Millis, R.R., Eusebi, V., Faverly, D., Vandevijver, M.J. & Zafrani, B. (1994). Ductal Carcinoma in-Situ - a Proposal For a New Classification. *Seminars in Diagnostic Pathology*, **11**, 167-180.

Hormia, M., Falkmarzillier, J., Plopper, G., Tamura, R.N., Jones, J.C.R. & Quaranta, V. (1995). Rapid Spreading and mature Hemidesmosome formation in Hacat Keratinocytes induced by incubation with soluble Laminin-5r. *Journal of Investigative Dermatology*, **105**, 557-561.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C. & Burridge, K. (1986). Interaction of Plasma-Membrane Fibronectin Receptor with Talin - a Transmembrane Linkage. *Nature*, **320**, 531-533.

Hoschuetzky, H., Aberle, H. & Kemler, R. (1994). Beta-Catenin mediates the interaction of the Cadherin Catenin Complex with Epidermal Growth-Factor Receptor. *Journal of Cell Biology*, **127**, 1375-1380.

Hoyhtya, M., Fridman, R., Komarek, D., Porterjordan, K., Stetlerstevenson, W.G., Liotta, L.A. & Liang, C.M. (1994). Immunohistochemical Localization of Matrix Metalloproteinase 2 and its specific inhibitor TIMP-2 in Neoplastic Tissues with monoclonal antibodies. *International Journal of Cancer*, **56**, 500-505.

Hsu, S., Raine, L., Fanger, H. (1981a) Use of Avidin-Biotin_peroxidase Complex, ABC, an immunoperoxidase technique. *J. Histochem. Cytochem.*, **29**, 577-580.

Hsu, S.& Raine, L. (1981b). Protein A, avidin and biotin in immunohistochemistry. *J. Histochem. Cytochem.*, **29**, 1349-1353.

Huhtala, P., Chow, L.T. & Tryggvason, K. (1990). Structure of the Human Type-IV Collagenase Gene. *Journal of Biological Chemistry*, **265**, 11077-11082.

Huhtala, P., Tuuttila, A., Chow, L.T., Lohi, J., Keskioja, J. & Tryggvason, K. (1991). Complete Structure of the Human Gene For 92-Kda Type-IV Collagenase - Divergent Regulation of Expression for the 92-Kilodalton and 72- Kilodalton enzyme genes in HT-1080 cells. *Journal of Biological Chemistry*, **266**, 16485-16490.

Huhtala, P., Humphries, M.J., McCarthy, J.B., Tremble, P.M., Werb, Z. & Damsky, C.H. (1995). Cooperative Signaling by alpha-5-beta-1 and alpha-4-beta-1 integrins regulates Metalloproteinase gene-expression in Fibroblasts adhering to Fibronectin. *Journal of Cell Biology*, **129**, 867-879.

Hujanen, E.S., Vaisanen, A., Zheng, A.P., Tryggvason, K. & Turpeenniemihujanen, T. (1994). Modulation of M(R)-72,000 and M(R)-92,000 Type-IV Collagenase (Gelatinase-A and Gelatinase-B) gene expression by Interferon-alpha and Interferon-gamma in Human-Melanoma. *International Journal of Cancer*, **58**, 582-586.

Hulsken, J., Behrens, J. & Birchmeier, W. (1994). Tumor-Suppressor Gene-Products in Cell Contacts - the Cadherin-APC- Armadillo Connection. *Current Opinion in Cell Biology*, **6**, 711-716.

Hurley, R.W., McCarthy, J.B. & Verfaillie, C.M. (1995). Direct Adhesion to Bone-Marrow Stroma Via Fibronectin Receptors Inhibits Hematopoietic Progenitor Proliferation. *Journal of Clinical Investigation*, **96**, 511-519.

Hurskainen, T., Hoyhtya, M., Tuuttila, A., Oikarinen, A., Autio-Harmainen, H. (1996). mRNA expression of TIMP-1, 2, and 3 and 92kDa type IV collagenase in early human placenta and decidual membrane as studied by in-situ hybridisation. *J. Histochem. Cytochem.*, **44**, 1379-1387.

Hurskainen, T., Seiki, M., Apte, S.S., SyrjakallioYlitalo, M., Sorsa, T., Oikarinen, A. & AutioHarmainen, H. (1998). Production of Membrane-Type Matrix Metalloproteinase-1 (MT-MMP-1) in early human placenta: A possible role in placental implantation? *Journal of Histochemistry & Cytochemistry*, **46**, 221-229.

Hynes, R.O. (1987). Integrins - a Family of Cell-Surface Receptors. *Cell*, **48**, 549-554.

Hynes, R.O. (1992). Integrins - Versatility, Modulation, and Signaling in Cell-Adhesion. *Cell*, **69**, 11-25.

Imhof B A, Vollmers H P. et al (1983) Cell-Cell interactions and Polarity of Epithelial cells: specific perturbation using a monoclonal antibody. *Cell* **35**, 667-675.

Imren, S., Kohn, D.B., Shimada, H., Blavier, L. & DeClerck, Y.A. (1996). Overexpression of Tissue Inhibitor of Metalloproteinases-2 by retroviral-mediated gene transfer in vivo inhibits tumor growth and invasion. *Cancer Research*, **56**, 2891-2895.

Irigoyen, J.P., Besser, D. & Nagamine, Y. (1997). Cytoskeleton reorganization induces the Urokinase-Type Plasminogen Activator gene via the Ras/Extracellular Signal-Regulated Kinase (ERK) signaling pathway. *Journal of Biological Chemistry*, **272**, 1904-1909.

Ito, A., Nakajima, S., Sasaguri, Y., Nagase, H. & Mori, Y. (1995). Coculture of Human Breast Adenocarcinoma MCF-7 Cells and Human Dermal Fibroblasts enhances the production of Matrix Metalloproteinase-1, Metalloproteinase-2 and Metalloproteinase-3 in Fibroblasts. *British Journal of Cancer*, **71**, 1039-1045.

Itoh,T., Tanioka, M., Yoshida, H., Nishimoto, H., Itohara, S. (1998). Reduced Angiogenesis and Tumour Progression in Gelatinase-A deficient mice. *Cancer Research*, **58**, 1048-1051.

Iwata, H., Kobayashi, S., Iwase, H., Masaoka, A., Fujimoto, N. & Okada, Y. (1996). Production of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in human Breast Carcinomas. *Japanese Journal of Cancer Research*, **87**, 602-611.

Jankowski, J.A., Bedford, F.K. & Kim, Y.S. (1997). Changes in gene structure and regulation of E-Cadherin during Epithelial Development, Differentiation, and Disease. *Progress in Nucleic Acid Research and Molecular Biology*, **57**, 187-215.

Jones, J.C.R., Asmuth, J., Baker, S.E., Langhofer, M., Roth, S.I. & Hopkinson, S.B. (1994). Hemidesmosomes - Extracellular-Matrix Intermediate Filament Connectors. *Experimental Cell Research*, **213**, 1-11.

Jones, J.L., Critchley, D.R. & Walker, R.A. (1992). Alteration of Stromal Protein and Integrin Expression in Breast - a Marker of Premalignant Change. *Journal of Pathology*, **167**, 399-406.

Jones, J.L., Royall, J.E., Critchley, D.R. & Walker, R.A. (1997). Modulation of Myoepithelial-associated alpha 6 beta 4 integrin in a Breast Cancer cell line alters invasive potential. *Experimental Cell Research*, **235**, 325-333.

Jones, J.L., Royall, J.E. & Walker, R.A. (1996). E-Cadherin relates to EGFR expression and lymph node metastasis in Primary Breast Carcinoma. *British Journal of Cancer*, **74**, 1237-1241.

Juliano, R.L. & Haskill, S. (1993). Signal Transduction from the Extracellular-Matrix. *Journal of Cell Biology*, **120**, 577-585.

Kadowaki, T., Shiozaki, H., Inoue, M., Tamura, S., Oka, H., Doki, Y., Iihara, K., Matsui, S., Iwazawa, T., Nagafuchi, A., Tsukita, S. & Mori, T. (1994). E-Cadherin and alpha-Catenin Expression in Human Esophageal Cancer. *Cancer Research*, **54**, 291-296.

Karin, M., Liu, Z.G. & Zandi, E. (1997). AP-1 function and regulation. *Current Opinion in Cell Biology*, **9**, 240-246.

Kataoka, H., Decastro, R., Zucker, S. & Biswas, C. (1993). Tumor Cell-Derived Collagenase-Stimulatory Factor increases expression of Interstitial Collagenase, Stromelysin, and 72-Kda Gelatinase. *Cancer Research*, **53**, 3154-3158.

Kataoka, H., Meng, J.Y., Uchino, H., Nabeshima, K., Kihira, Y., Matuo, Y. & Koono, M. (1997). Modulation of Matrix Metalloproteinase-7 (Matrilysin) secretion in coculture of human Colon Carcinoma cells with fibroblasts from orthotopic and ectopic organs. *Oncology Research*, **9**, 101-109.

Kedeshian, P., Sternlicht, M.D., Nguyen, M., Shao, Z.M. & Barsky, S.H. (1998). Humatrix, a novel Myoepithelial matrical gel with unique biochemical and biological properties. *Cancer Letters*, **123**, 215-226.

Keely, P.J., Fong, A.M., Zutter, M.M. & Santoro, S.A. (1995). Alteration of Collagen-Dependent Adhesion, Motility, and Morphogenesis by the expression of antisense alpha(2) Integrin messenger-RNA in Mammary Cells. *Journal of Cell Science*, **108**, 595-607.

Kenny, A.J., Ohare, M.J. & Gusterson, B.A. (1989). Cell-Surface Peptidases as Modulators of Growth and Differentiation. *Lancet*, **2**, 785-787.

Kerr, L.D., Miller, D.B. & Matrisian, L.M. (1990). TGF-beta-1 inhibition of Transin /Stromelysin gene-expression is mediated through a Fos Binding Sequence. *Cell*, **61**, 267-278.

Khan, K.M.F. & Falcone, D.J. (1997). Role of Laminin in matrix induction of macrophage Urokinase-Type Plasminogen Activator and 92-kDa Metalloproteinase expression. *Journal of Biological Chemistry*, **272**, 8270-8275.

Kinch, M.S., Clark, G.J., Der, C.J. & Burridge, K. (1995). Tyrosine Phosphorylation regulates the Adhesions of Ras-transformed Breast Epithelia. *Journal of Cell Biology*, **130**, 461-471.

Kinsella, A.R., Green, B., Lepts, G.C., Hill, C.L., Bowie, G. & Taylor, B.A. (1993). The Role of the Cell-Cell Adhesion Molecule E-Cadherin in Large-Bowel Tumor-Cell Invasion and Metastasis. *British Journal of Cancer*, **67**, 904-909.

Kirby, D.R.S. (1965). The Invasiveness of the Trophoblast. In: W.W.Park (ed). The early conceptus, normal and abnormal. University of St. Andrews Press, Edinburgh, pp 68-74.

Klein, C.E., Steinmayer, T., Kaufmann, D., Weber, L. & Brocker, E.B. (1991a). Identification of a Melanoma Progression Antigen as Integrin VLA-2. *Journal of Investigative Dermatology*, **96**, 281-284.

Klein, C.E., Dressel, D., Steinmayer, T., et al. (1991b). Integrin alpha-2 beta-1 is up-regulated in Fibroblasts and highly aggressive Melanoma cells in three-dimensional collagen lattices and mediates the re-organisation of Collagen I fibrils. *Journal of Cell Biology*, **115**, 1427-1436.

Kleiner, D.E., Unsworth, E.J., Krutzsch, H.C. & Stetlerstevenson, W.G. (1992). Higher-Order Complex-Formation Between the 72-Kilodalton Type-IV Collagenase and Tissue Inhibitor of Metalloproteinases-2. *Biochemistry*, **31**, 1665-1672.

Kleiner, D.E. & Stetlerstevenson, W.G. (1994). Quantitative Zymography - Detection of Picogram Quantities of Gelatinases. *Analytical Biochemistry*, **218**, 325-329.

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K. & Martin, G.R. (1982). Isolation and Characterization of Type-Iv Procollagen, Laminin, and Heparan-Sulfate Proteoglycan From the Ehs Sarcoma. *Biochemistry*, **21**, 6188-6193.

Knauper, V., Will, H., LopezOtin, C., Smith, B., Atkinson, S.J., Stanton, H., Hembry, R.M. & Murphy, G. (1996). Cellular mechanisms for human Procollagenase-3 (MMP-13) activation - evidence that MT1-MMP (MMP-14) and Gelatinase A (MMP-2) are able to generate active enzyme. *Journal of Biological Chemistry*, **271**, 17124-17131.

Knauper, V., Cowell, S., Smith, B., LopezOtin, C., Oshea, M., Morris, H., Zardi, L. & Murphy, G. (1997a). The role of the c-terminal domain of human Collagenase-3 (MMP-13) in the activation of Procollagenase-3, substrate specificity, and Tissue Inhibitor of Metalloproteinase interaction. *Journal of Biological Chemistry*, **272**, 7608-7616.

Knauper, V., Smith, B., LopezOtin, C. & Murphy, G. (1997b). Activation of Progelatinase B (proMMP-9) by active Collagenase-3 (MMP-13). *European Journal of Biochemistry*, **248**, 369-373.

Knudsen, K.A. & Wheelock, M.J. (1992). Plakoglobin, or an 83 Kd Homolog distinct from beta-Catenin, interacts with E-Cadherin and N-Cadherin. *Molecular Biology of the Cell*, **3**, A215.

Kolkenbrock, H., Orgel, D., Heckerkia, A., Noack, W. & Ulbrich, N. (1991). The complex between a Tissue Inhibitor of Metalloproteinases (TIMP-2) and 72-Kda Progelatinase is a Metalloproteinase Inhibitor. *European Journal of Biochemistry*, **198**, 775-781.

Kolkenbrock, H., HeckerKia, A., Orgel, D., Ulbrich, N. & Will, H. (1997). Activation of Progelatinase A and Progelatinase A -TIMP-2 complex by Membrane Type 2 Matrix Metalloproteinase. *Biological Chemistry*, **378**, 71-76.

Kondapaka, S.B., Fridman, R. & Reddy, K.B. (1997). Epidermal Growth Factor and Amphiregulin up-regulate Matrix Metalloproteinase-9 (MMP-9) in human Breast Cancer cells. *International Journal of Cancer*, **70**, 722-726.

Koretz, K., Schlag, P., Boumsell, L. & Moller, P. (1991). Expression of VLA-alpha-2, VLA-alpha-6, and VLA-beta-1 chains in normal Mucosa and Adenomas of the Colon, and in Colon Carcinomas and their Liver Metastases. *American Journal of Pathology*, **138**, 741-750.

Korhonen, M., Laitinen, L., Ylanne, J., Koukoulis, G.K., Quaranta, V., Juusela, H., Gould, V.E. & Virtanen, I. (1992). Integrin Distributions in Renal-Cell Carcinomas of Various Grades of Malignancy. *American Journal of Pathology*, **141**, 1161-1171.

Koukoulis, G.K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V. & Gould, V.E. (1991). Immunohistochemical Localization of Integrins in the Normal, Hyperplastic, and Neoplastic Breast -

correlations with their functions as Receptors and Cell-Adhesion Molecules. *American Journal of Pathology*, **139**, 787-799.

Koukoulis, G.K., Virtanen, I., Moll, R., Quaranta, V. & Gould, V.E. (1993). Immunolocalization of Integrins in the Normal and Neoplastic Colonic Epithelium. *Virchows Archiv B-Cell Pathology Including Molecular Pathology*, **63**, 373-383.

Kozlowski, J.M., Fidler, I.J., Campbell, D., Xu, Z.L., Kaighn, M.E. & Hart, I.R. (1984). Metastatic Behaviour of Human Tumor Cell Lines grown in the nude mouse. *Cancer Research*, **44**, 3522-3529.

Kramer, R.H., Bensch, K.G. & Wong, J. (1986). Invasion of Reconstituted Basement-Membrane Matrix by Metastatic Human Tumor Cells. *Cancer Research*, **46**, 1980-1989.

Kuniyasu, H., Ellis, L.M., Evans, D.B., Abbruzzese, J.L., Fenoglio, C.J., Bucana, C.D., Cleary, K.R., Tahara, E. & Fidler, I.J. (1999). Relative expression of E-Cadherin and type IV Collagenase genes predicts disease outcome in patients with resectable Pancreatic Carcinoma. *Clinical Cancer Research*, **5**, 25-33.

Kurpakus, M.A., Quaranta, V. & Jones, J.C.R. (1991). Surface Relocation of alpha 6 beta 4 Integrins and Assembly of Hemidesmosomes in an in-vitro model of Wound-Healing. *Journal of Cell Biology*, **115**, 1737-1750.

Kurschat, P., Zigrino, P., Nischt, R., Breitkopf, K., Steurer, P., Klein, C.E., Krieg, T. & Mauch, C. (1999). Tissue Inhibitor of Matrix Metalloproteinase-2 regulates Matrix Metalloproteinase-2 activation by modulation of Membrane-Type 1 Matrix Metalloproteinase activity in high and low invasive Melanoma cell lines. *Journal of Biological Chemistry*, **274**, 21056-21062.

Kypta, R.M., Su, H. & Reichardt, L.F. (1996). Association between a transmembrane protein tyrosine phosphatase and the Cadherin-Catenin complex. *Journal of Cell Biology*, **134**, 1519-1529.

Kyriazis, A.A. & Kyriazis, A.P. (1980). Preferential sites of growth of human tumours in nude mice following subcutaneous transplantation. *Cancer Research*, **40**, 4509-4511.

Lafyatis, R., Kim, S.J., Angel, P., Roberts, A.B., Sporn, M.B., Karin, M. & Wilder, R.L. (1990). Interleukin-1 stimulates and All-Trans-Retinoic Acid inhibits Collagenase gene expression through its 5' Activator Protein-1- Binding Site. *Molecular Endocrinology*, **4**, 973. 980.

Lala,P.K. & Graham, C.H. (1990). Mechanisms of Trophoblast invasiveness and their control: the role of Proteases and Protease Inhibitors. *Cancer Metastasis Reviews*, **9**, 369-379.

Lambird P A, Shelley W M (1969). The spatial distribution of lobular in-situ mammary carcinoma *JAMA*, **210**, 689-693.

Langhofer, M., Hopkinson, S.B. & Jones, J.C.R. (1993). The Matrix Secreted By 804G cells contains Laminin-Related components that participate in Hemidesmosome assembly in-vitro. *Journal of Cell Science*, **105**, 753-764.

Larjava, H., Salo, T., Haapasalmi, K., Kramer, R.H. & Heino, J. (1993). Expression of Integrins and Basement-Membrane Components by Wound Keratinocytes. *Journal of Clinical Investigation*, **92**, 1425-1435.

Larue, L., Ohsugi, M., Hirchenhain, J. & Kemler, R. (1994). E-Cadherin Null Mutant Embryos fail to form a Trophectoderm Epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 8263-8267.

Lazard, D., Sastre, X., Frid, M.G., Glukhova, M.A., Thiery, J.P. & Koteliansky, V.E. (1993). Expression of Smooth Muscle-Specific Proteins in Myoepithelium and Stromal Myofibroblasts of Normal and Malignant Human Breast-Tissue. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 999-1003.

Leco, K.J., Apte, S.S., Taniguchi, G.T., Hawkes, S.P., Khokha, R., Schultz, G.A. & Edwards, D.R. (1997). Murine Tissue Inhibitor of Metalloproteinases-4 (TIMP-4): cDNA isolation and expression in adult mouse tissues. *Febs Letters*, **401**, 213-217.

Lehmann, J.M., Sers, C., Riethmuller, G. & Johnson, J.P. (1988). Muc18 a marker of Tumor Progression in Melanoma is a member of the Ig Superfamily. *Anticancer Research*, **8**, 1136.

Leisi, P., Julien, J.P., Vilja, P., Grarveld, F., Rechardt, L. (1986) Specific detection of neuronal ell bodies – in-situ hybridisation with a biotin-labelled neurofilament cDNA probe. *J. Histochem. Cytochem.*, 923-926.

Lengyel, E., Gum, R., Juarez, J., Clayman, G., Seiki, M., Sato, H. & Boyd, D. (1995). Induction of M(R)-92,000 Type-IV Collagenase expression in a Squamous Cell Carcinoma cell line by Fibroblasts. *Cancer Research*, **55**, 963-967.

Lengyel, E., Gum, R., Stepp, E., Juarez, J., Wang, H. & Boyd, D. (1996). Regulation of Urokinase-Type Plasminogen Activator expression by an ERK1-dependent signaling pathway in a Squamous Cell Carcinoma cell line. *Journal of Cellular Biochemistry*, **61**, 430-443.

Lennington, W.J., Jensen, R.A., Dalton, L.W. & Page, D.L. (1994). Ductal Carcinoma in-Situ of the Breast - Heterogeneity of Individual Lesions. *Cancer*, **73**, 118-124.

Lewis, M.E., Sherman, T.G. & Watson, S.J. (1985). In-situ Hybridization Histochemistry with Synthetic Oligonucleotides - Strategies and Methods. *Peptides*, **6**, 75-87.

Li, J.Z., Avraham, H., Rogers, R.A., Raja, S. & Avraham, S. (1996). Characterization of RAFTK, a novel Focal Adhesion Kinase, and its integrin-dependent phosphorylation and activation in Megakaryocytes. *Blood*, **88**, 417-428.

Li, P., Barraclough, R., Fernig, D.G., Smith, J.A. & Rudland, P.S. (1998). Stem cells in breast epithelia *International Journal of Experimental Pathology*, **79**, 193-206.

Librach, C.L., Werb, Z., Fitzgerald, M.L., Chiu, K., Corwin, N.M., Esteves, R.A., Grobelny, D., Galardy, R., Damsky, C.H. & Fisher, S.J. (1991). 92-Kd Type-IV Collagenase mediates invasion of Human Cytotrophoblasts. *Journal of Cell Biology*, **113**, 437-449.

Librach, C.L., Feigenbaum, S.L., Bass, K.E., Cui, T.Y., Verastas, N., Sadovsky, Y., Quigley, J.P., French, D.L. & Fisher, S.J. (1994). Interleukin-1-beta regulates Human Cytotrophoblast Metalloproteinase Activity and Invasion in-vitro. *Journal of Biological Chemistry*, **269**, 17125-17131.

Lindsay, C.K., Thorgeirsson, U.P., Tsuda, H. & Hirohashi, S. (1997). Expression of Tissue Inhibitor of Metalloproteinase-1 and Type IV Collagenase/Gelatinase messenger RNAs in human breast cancer. *Human Pathology*, **28**, 359-366.

Liotta, L.A., Rao, C.N. & Wewer, U.M. (1986). Biochemical Interactions of Tumor Cells with the Basement Membrane. *Annual Review of Biochemistry*, **55**, 1037-1057.

Lipponen, P., Saarelainen, E., Ji, H., Aaltomaa, S. & Syrjanen, K. (1994). Expression of E-Cadherin (E-Cd) as related to other Prognostic Factors and Survival in Breast Cancer. *Journal of Pathology*, **174**, 101-109.

Liu, Q.Y., Niranjan, B., Gomes, P., Gomm, J.J., Davies, D., Coombes, R.C. & Buluwela, L. (1996). Inhibitory effects of Activin on the Growth and Morphogenesis of primary and transformed Mammary Epithelial cells. *Cancer Research*, **56**, 1155-1163.

Llano, E., Pendas, A.M., Knauper, V., Sorsa, T., Salo, T., Salido, E., Murphy, G., Simmer, J.P., Bartlett, J.D. & LopezOtin, C. (1997). Identification and structural and functional characterization of human Enamelysin (MMP-20). *Biochemistry*, **36**, 15101-15108.

Llano, P., Pendas, A.M., Freije, J.P., Nakano, A., Knauper, V., Murphy, G. & LopezOtin, C. (1999). Identification and characterization of human MT5-MMP, a new membrane- bound activator of Progelatinase A overexpressed in brain tumors. *Cancer Research*, **59**, 2570-2576.

Llorens, A., Rodrigo, I., LopezBarcons, L., GonzalezGarrigues, M., Lozano, E., Vinyals, A., Quintanilla, M., Cano, A. & Fabra, A. (1998). Down-regulation of E-Cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to Matrix Metalloproteinase-9 Gelatinase expression. *Laboratory Investigation*, **78**, 1131-1142.

Lohi, J., Lehti, K., Westermarck, J., Kahari, V.M. & KeskiOja, J. (1996). Regulation of Membrane-Type Matrix Metalloproteinase-1 expression by Growth Factors and Phorbol 12-Myristate 13-Acetate. *European Journal of Biochemistry*, **239**, 239-247.

Longacre, T.A. & Bartow, S.A. (1986). A Correlative Morphological study of Human Breast and Endometrium in the Menstrual Cycle. *American Journal of Surgical Pathology*, **10**, 382-393.

Lonn, U., Lonn, S., Nylen, U. & Stenkvist, B. (1992). Appearance and Detection of Multiple Copies of the Mdr-1 Gene in clinical samples of Mammary Carcinoma. *International Journal of Cancer*, **51**, 682-686.

Lu, X.Q., Levy, M., Weinstein, I.B. & Santella, R.M. (1991). Immunological Quantitation of Levels of Tissue Inhibitor of Metalloproteinase-1 in Human Colon Cancer. *Cancer Research*, **51**, 6231-6235.

Lund, L.R., Ronne, E., Roldan, A.L., Behrendt, N., Romer, J., Blasi, F. & Dano, K. (1991). Urokinase Receptor messenger-RNA level and gene transcription are strongly and rapidly increased by Phorbol-Myristate Acetate in Human Monocyte-like U937 cells. *Journal of Biological Chemistry*, **266**, 5177-5181.

Lyons, R.M., Keskioja, J. & Moses, H.L. (1988). Proteolytic Activation of Latent Transforming Growth Factor-beta from Fibroblast Conditioned Medium. *Journal of Cell Biology*, **106**, 1659-1665.

Manes, S., Mira, E., Barbacid, M.D., Cipres, A., FernandezResa, P., Buesa, J.M., Merida, I., Aracil, M., Marquez, G. & Martinez, C. (1997). Identification of Insulin-like Growth Factor-Binding Protein-1 as a potential physiological substrate for Human Stromelysin-3. *Journal of Biological Chemistry*, **272**, 25706-25712.

Marcy, A.I., Eiberger, L.L., Harrison, R., Chan, H.K., Hutchinson, N.I., Hagmann, W.K., Cameron, P.M., Boulton, D.A. & Hermes, J.D. (1991). Human Fibroblast Stromelysin Catalytic Domain - expression, purification, and characterization of a C-terminally truncated form. *Biochemistry*, **30**, 6476-6483.

Mareel, M.M., Behrens, J., Birchmeier, W., Debruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C. & Vanroy, F.M. (1991). Down-Regulation of E-Cadherin expression in Madin Darby Canine Kidney (MDCK) cells inside Tumors of Nude Mice. *International Journal of Cancer*, 47, 922-928.

Marti, H.P., Lee, L., Kashgarian, M. & Lovett, D.H. (1994). Transforming Growth-Factor-beta-1 stimulates Glomerular Mesangial cell synthesis of the 72-Kd Type-IV Collagenase. *American Journal of Pathology*, 144, 82-94.

Martinez-Hernandez, A. Fink, L.M., Pierce, G.B. (1976). Removal of Basement Membrane in the Involuting Breast. *Laboratory Investigations*, 31, 455-462.

Martorana, A.M., Zheng, G.P., Crowe, T.C., Ogrady, R.L. & Lyons, J.G. (1998). Epithelial cells up-regulate Matrix Metalloproteinases in cells within the same Mammary Carcinoma that have undergone an Epithelial- Mesenchymal transition. *Cancer Research*, 58, 4970-4979.

Masure, S., Billiau, A., Vandamme, J. & Opdenakker, G. (1990). Human Hepatoma cells produce an 85 Kda Gelatinase regulated by Phorbol 12-Myristate 13-Acetate. *Biochimica Et Biophysica Acta*, 1054, 317-325.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M.C. & Breathnach, R. (1985). Epidermal Growth-Factor and Oncogenes induce transcription of the same cellular messenger-RNA in Rat Fibroblasts. *Embo Journal*, 4, 1435-1440.

Mauviel, A., Chung, K.Y., Agarwal, A., Tamai, K. & Uitto, J. (1996). Cell-specific induction of distinct Oncogenes of the Jun family is responsible for differential regulation of Collagenase gene expression by Transforming Growth Factor-beta in Fibroblasts and Keratinocytes. *Journal of Biological Chemistry*, 271, 10917-10923.

Mayer, B., Johnson, J.P., Leitl, F., Jauch, K.W., Heiss, M.M., Schildberg, F.W., Birchmeier, W. & Funke, I. (1993). E-Cadherin expression in Primary and Metastatic Gastric Cancer - down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Research*, 53, 1690-1695.

McDonnell, S.E., Kerr, L.D. & Matrisian, L.M. (1990). Epidermal Growth-Factor stimulation of Stromelysin messenger-RNA in Rat Fibroblasts requires induction of Protooncogenes c-Fos and c-Jun and activation of Protein Kinase-C. *Molecular and Cellular Biology*, 10, 4284-4293.

Meredith, J.E., Fazeli, B. & Schwartz, M.A. (1993). The Extracellular-Matrix as a Cell Survival Factor. *Molecular Biology of the Cell*, 4, 953-961.

Messent, A.J., Tuckwell, D.S., Knauper, V., Humphries, M.J., Murphy, G. & Gavrilovic, J. (1998). Effects of Collagenase cleavage of type I Collagen on alpha(2)beta(1) integrin mediated cell adhesion. *Journal of Cell Science*, 111, 1127-1135.

Migita, K., Eguchi, K., Kawabe, Y., Ichinose, Y., Tsukada, T., Aoyagi, T., Nakamura, H. & Nagataki, S. (1996). TNF-alpha-mediated expression of Membrane-Type Matrix Metalloproteinase in Rheumatoid Synovial Fibroblasts. *Immunology*, 89, 553-557.

Mignatti, P. & Rifkin, D.B. (1993). Biology and Biochemistry of Proteinases in Tumor Invasion. *Physiological Reviews*, 73, 161-195.

Miller E J, Harris E D., Chung, E., et al (1976). Cleavage of type II and type III Collagens with Mammalian Collagenase : site of cleavage and primary structure of the NH_2 terminal portion of the smaller fragment released from both Collagens. *Biochemistry*, 15, 787-792

Miller, F.R., McEachern, D. & Miller, B.E. (1989). Growth-Regulation of Mouse Mammary Tumor cells in Collagen gel cultures by diffusible factors produced by Normal Mammary Gland Epithelium and Stromal Fibroblasts. *Cancer Research*, 49, 6091-6097.

308

Miller, A.B. (1992). Incidence and Demographics. In Harris, J.R., Hellman, S., Henderson, I.C., Kinne, D.W. *Breast Diseases. 2nd Edition Philadelphia: J.B. Lippincott, pp119-126.*

Miyazaki, K., Hattori, Y., Umenishi, F., Yasumitsu, H. & Umeda, M. (1990). Purification and Characterization of Extracellular Matrix-Degrading Metalloproteinase, Matrin (Pump-1), secreted from Human Rectal Carcinoma cell line. *Cancer Research,* **50,** 7758-7764.

Moll, U.M., Youngleib, G.L., Rosinski, K.B. & Quigley, J.P. (1990). Tumor Promoter stimulated Mr92,000 Gelatinase secreted by Normal and Malignant Human cells - isolation and characterization of the enzyme from Ht1080 tumor cells. *Cancer Research,* **50,** 6162-6170.

Moll, R., Mitze, M., Frixen, U.H. & Birchmeier, W. (1993). Differential loss of E-Cadherin expression in Infiltrating Ductal and Lobular Breast Carcinomas. *American Journal of Pathology,* **143,** 1731-1742.

Monaghan, P. Clarke, C.L., Perusinghe, N.P., Omerod, M.G., O'Hare, M.J. (1995) Epidermal Growth Factor Receptor expression on Human Breast Luminal and Basal cells in vitro. *Epithelial Cell Biology,* **4,** 52-62.

Monteagudo, C., Merino, M.J., Sanjuan, J., Liotta, L.A. & Stetlerstevenson, W.G. (1990). Immunohistochemical Distribution of Type-IV Collagenase in Normal, Benign, and Malignant Breast-Tissue. *American Journal of Pathology,* **136,** 585-592.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K.W. (1997). Activation of beta-Catenin-Tcf signaling in Colon Cancer by mutations in beta-Catenin or APC. *Science,* **275,** 1787-1790.

Mukaida, H., Hirabayashi, N., Hirai, T., Iwata, T., Saeki, S. & Toge, T. (1991). Significance of freshly cultured Fibroblasts from different tissues in promoting cancer cell growth. *International Journal of Cancer,* **48,** 423-427.

Muller, D., Quantin, B., Gesnel, M.C., Milloncollard, R., Abecassis, J. & Breathnach, R. (1988). The Collagenase Gene Family in Humans consists of at least 4 members. *Biochemical Journal,* **253,** 187-192.

Muller, D., Wolf, C.,Abecassis, J., Millon, R., Engelmann, A., Bronner, G., et al. (1993). Increased Stromelysin-3 gene expression is associated with increased local invasiveness in Head and Neck Squamous Carcinomas. *Cancer Research,* **53,** 165-169.

Murphy, G., Allan, J.A., Willenbrock, F., Cockett, M.I., Oconnell, J.P. & Docherty, A.J.P. (1992). The Role of the C-Terminal Domain in Collagenase and Stromelysin Specificity. *Journal of Biological Chemistry,* **267,** 9612-9618.

Murphy, G., Cockett, M.I., Ward, R.V. & Docherty, A.J.P. (1991). Matrix Metalloproteinase Degradation of Elastin, Type-IV Collagen and Proteoglycan - a quantitative comparison of the activities of 95 Kda and 72 Kda Gelatinases, Stromelysin-1 and Stromelysin-2 and Punctuated Metalloproteinase (Pump). *Biochemical Journal,* **277,** 277-279.

Murphy, G., Willenbrock, F., Ward, R.V., Cockett, M.I., Eaton, D. & Docherty, A.J.P. (1992). The C-terminal domain of 72-Kda Gelatinase-a is not required for catalysis, but is essential for membrane activation and modulates interactions with Tissue Inhibitors of Metalloproteinases. *Biochemical Journal,* **283,** 637-641.

Murray, G.I., Duncan, M.E., Oneil, P., McKay, J.A., Melvin, W.T. & Fothergill, J.E. (1998). Matrix Metalloproteinase-1 is associated with poor prognosis in Oesophageal Cancer. *Journal of Pathology,* **185,** 256-261.

Murray, G.I., Duncan, M.E., Oneil, P., Melvin, W.T. & Fothergill, J.E. (1996). Matrix Metalloproteinase-1 is associated with poor prognosis in Colorectal Cancer. *Nature Medicine*, **2**, 461-462.

Nabeshima, K., Kishi, J., Kurogi, T., Komada, N., Kataoka, H., Okada, Y. & Koono, M. (1994). Stimulation of TIMP-1 and Metalloproteinase production in cocultures of Human Tumor Cells and Human Fibroblasts. *Cancer Letters*, **78**, 133-140.

Nagafuchi, A. & Takeichi, M. (1989). Transmembrane Control of Cadherin-Mediated cell adhesion - a 94 Kda protein functionally associated with a specific region of the cytoplasmic domain of E-Cadherin. *Cell Regulation*, **1**, 37-44.

Nagafuchi, A., Takeichi, M. & Tsukita, S. (1991). The 102 Kd Cadherin-Associated Protein - Similarity to Vinculin and Posttranscriptional Regulation of Expression. *Cell*, **65**, 849-857.

Nagase, H., Suzuki, K., Enghild, J.J. & Salvesen, G. (1991). Stepwise Activation Mechanisms of the Precursors of Matrix Metalloproteinases-1 (Tissue Collagenase) and 3 (Stromelysin). *Biomedica Biochimica Acta*, **50**, 749-754.

Nagato T, Yoshida H, et al (1980). A Scanning Electron Microscope study of Myoepithelial Cells in Exocrine Glands. *Cell Tissue Research*, **209**, 1-10.

Nagle, R.B., Hao, J.S., Knox, J.D., Dalkin, B.L., Clark, V. & Cress, A.E. (1995). Expression of Hemidesmosomal and Extracellular Matrix Proteins by Normal and Malignant Human Prostate Tissue. *American Journal of Pathology*, **146**, 1498-1507.

Natali, P.G., Nicotra, M.R., Botti, C., Mottolese, M., Bigotti, A. & Segatto, O. (1992). Changes in expression of alpha-6-beta-4 Integrin heterodimer in Primary and Metastatic Breast Cancer. *British Journal of Cancer*, **66**, 318-322.

Natali, P.G., Nicotra, M.R., Bartolazzi, A., Cavaliere, R. & Bigotti, A. (1993). Integrin Expression in Cutaneous Malignant-Melanoma - association of the alpha-3/beta-1 heterodimer with Tumor Progression. *International Journal of Cancer*, **54**, 68-72.

Nawrocki, B., Polette, M., VanHengel, J., Tournier, J.M., VanRoy, F. & Birembaut, P. (1998). Cytoplasmic redistribution of E-Cadherin-Catenin adhesion complex is associated with down-regulated Tyrosine Phosphorylation of E-Cadherin in Human Bronchopulmonary Carcinomas. *American Journal of Pathology*, **153**, 1521-1530.

Nebe, B., Sanftleben, H., Pommerenke, H., Peters, A. & Rychly, J. (1998). Hepatocyte Growth Factor enables enhanced Integrin-Cytoskeleton linkage by affecting Integrin expression in Subconfluent Epithelial Cells. *Experimental Cell Research*, **243**, 263-273.

Nesbit, M. & Herlyn, M. (1994). Adhesion Receptors in Human Melanoma Progression. *Invasion & Metastasis*, **14**, 131-146.

Nielsen, B.S., Timshel, S., Kjeldsen, L., Sehested, M., Pyke, C., Borregaard, N. & Dano, K. (1996). 92 kDa type IV Collagenase (MMP-9) is expressed in Neutrophils and Macrophages but not in Malignant Epithelial cells in Human Colon Cancer. *International Journal of Cancer*, **65**, 57-62.

Nievers, M.G., Schaapveld, R.Q.J., Oomen, L., Fontao, L., Geerts, D. & Sonnenberg, A. (1998). Ligand-independent role of the beta(4) integrin subunit in the formation of Hemidesmosomes. *Journal of Cell Science*, **111**, 1659-1672.

310

Nip, J., Shibata, H., Loskutoff, D.J., Cheresh, D.A. & Brodt, P. (1992). Human Melanoma cells derived from Lymphatic Metastases use Integrin alpha-V-beta-3 to adhere to Lymph Node Vitronectin. *Journal of Clinical Investigation*, **90**, 1406-1413.

Niu, J., Gu, X., Turton, J., Meldrum, C., Howard, E.W. & Agrez, M. (1998). Integrin mediated signalling of Gelatinase B secretion in Colon Cancer cells. *Biochemical and Biophysical Research Communications*, **249**, 287-291.

Nobes, C.D. & Hall, A. (1995). Rho, Rac, and Cdc42 GTPases Regulate the Assembly of Multimolecular Focal Complexes associated with Actin Stress Fibers, Lamellipodia, and Filopodia. *Cell*, **81**, 53-62.

Noe, V., Willems, J., Vandekerckhove, J., VanRoy, F., Bruyneel, E. & Mareel, M. (1999). Inhibition of adhesion and induction of Epithelial Cell invasion by HAV-containing E-Cadherin specific peptides. *Journal of Cell Science*, **112**, 127-135.

Noel, A., Hajitou, A., Lhoir, C., Maquoi, E., Baramova, E., Lewalle, J.M., Remacle, A., Kebers, F., Brown, P., CalbergBacq, C.M. & Foidart, J.M. (1998). Inhibition of stromal Matrix Metalloproteases: Effects on Breast Tumor promotion by Fibroblasts. *International Journal of Cancer*, **76**, 267-273.

Noel, A.C., Calle, A., Emonard, H.P., Nusgens, B.V., Simar, L., Foidart, J., Lapiere, C.M. & Foidart, J.M. (1991). Invasion of Reconstituted Basement Membrane Matrix is not correlated to the Malignant Metastatic Cell Phenotype. *Cancer Research*, **51**, 405-414.

Nomura, H., Fujimoto, N., Seiki, M., Mai, M. & Okada, Y. (1996). Enhanced production of Matrix Metalloproteinases and activation of Matrix Metalloproteinase 2 (Gelatinase A) in Human Gastric Carcinomas. *International Journal of Cancer*, **69**, 9-16.

Nomura, H., Sato, H., Seiki, M., Mai, M. & Okada, Y. (1995). Expression of Membrane-Type Matrix Metalloproteinase in Human Gastric Carcinomas. *Cancer Research*, **55**, 3263-3266.

Norton, A.J. (1993). Microwave-Oven Heating for Antigen Unmasking in Routinely Processed Tissue-Sections. *Journal of Pathology*, **171**, 79-80.

Nose, A., Tsuji, K. & Takeichi, M. (1990). Localization of Specificity Determining Sites in Cadherin Cell- Adhesion Molecules. *Cell*, **61**, 147-155.

Ogata, Y., Itoh, Y. & Nagase, H. (1995). Steps Involved in Activation of the Pro-Matrix Metalloproteinase-9 (Progelatinase-B)-Tissue Inhibitor of Metalloproteinases-1 complex by 4-Aminophenylmercuric Acetate and Proteinases. *Journal of Biological Chemistry*, **270**, 18506-18511.

Ohare, M.J., Ormerod, M.G., Monaghan, P., Lane, E.B. & Gusterson, B.A. (1991). Characterization in-vitro of Luminal and Myoepithelial Cells isolated from the Human Mammary Gland by Cell Sorting. *Differentiation*, **46**, 209-221.

Ohtani, H., Motohashi, H., Sato, H., Seiki, M. & Nagura, H. (1996). Dual over-expression pattern of Membrane-Type Metalloproteinase-1 in Cancer and Stromal cells in Human Gastrointestinal Carcinoma revealed by in situ hybridization and immunoelectron microscopy. *International Journal of Cancer*, **68**, 565-570.

Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. & Okada, Y. (1997). Membrane Type 1 Matrix Metalloproteinase digests Interstitial Collagens and other Extracellular Matrix Macromolecules. *Journal of Biological Chemistry*, **272**, 2446-2451.

Oka, H., Shiozaki, H., Kobayashi, K., Tahara, H., Tamura, S., Miyata, M., Doki, Y., Iihara, K., Matsuyoshi, N., Hirano, S., Takeichi, M. & Mori, T. (1992). Immunohistochemical evaluation of E-Cadherin adhesion molecule expression in Human Gastric Cancer. *Virchows Archiv a-Pathological Anatomy and Histopathology*, **421**, 149-156.

Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M. & Mori, T. (1993). Expression of E-Cadherin cell adhesion molecules in Human Breast Cancer tissues and its relationship to Metastasis. *Cancer Research*, **53**, 1696-1701.

Okada, Y., Nagase, H. & Harris, E.D. (1986). A Metalloproteinase from Human Rheumatoid Synovial Fibroblasts that digests Connective Tissue Matrix Components - Purification and Characterization. *Journal of Biological Chemistry*, **261**, 4245-4255.

Okada, Y., Tsuchiya, H., Shimizu, H., Tomita, K., Nakanishi, I., Sato, H., Seiki, M., Yamashita, K. & Hayakawa, T. (1990). Induction and Stimulation of 92-Kda Gelatinase Type-IV Collagenase Production in Osteosarcoma and Fibrosarcoma cell lines by Tumor Necrosis Factor-alpha. *Biochemical and Biophysical Research Communications*, **171**, 610-617.

Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M.T., Iwata, K. & Nagase, H. (1992). Localization of Matrix Metalloproteinase-3 (Stromelysin) in Osteoarthritic Cartilage and Synovium. *Laboratory Investigation*, **66**, 680-690.

Okada, A., Bellocq, J.P., Rouyer, N., Chenard, M.P., Rio, M.C., Chambon, P. & Basset, P. (1995). Membrane-Type Matrix Metalloproteinase (MT-MMP) gene is expressed in Stromal Cells of Human Colon, Breast, and Head and Neck Carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 2730-2734.

Okumura, Y., Sato, H., Seiki, M. & Kido, H. (1997). Proteolytic activation of the precursor of Membrane Type 1 Matrix Metalloproteinase by Human Plasmin - A possible cell surface activator. *Febs Letters*, **402**, 181-184.

Onisto, M., Riccio, M.P., Scannapieco, P., Caenazzo, C., Griggio, L., Spina, M., Stetlerstevenson, W.G. & Garbisa, S. (1995). Gelatinase A/TIMP-2 imbalance in Lymph Node Positive Breast Carcinomas, as measured by RT-PCR. *International Journal of Cancer*, **63**, 621-626.

Ornstein, D.L., MacNab, J. & Cohn, K.H. (1999). Evidence for Tumor-Host cooperation in regulating MMP-2 expression in Human Colon Cancer. *Clinical & Experimental Metastasis*, **17**, 205-212.

Oshea, M., Willenbrock, F., Williamson, R.A., Cockett, M.I., Freedman, R.B., Reynolds, J.J., Docherty, A.J.P. & Murphy, G. (1992). Site-Directed Mutations that alter the inhibitory activity of the Tissue Inhibitor of Metalloproteinases-1 - importance of the N-terminal region between Cysteine-3 and Cysteine-13. *Biochemistry*, **31**, 10146-10152.

Osin, P.P., Anbazhagan, R., Bartkova, J., Nathan, B. & Gusterson, B.A. (1998). Breast development gives insights into Breast Disease. *Histopathology*, **33**, 275-283.

O'Toole, T.E., Katagiri, Y., Faull, R.J., Peter, K., Tamura, R., Quaranta, V., Loftus, J.C., Shattil, S.J. & Ginsberg, M.H. (1994). Integrin Cytoplasmic domains mediate Inside-Out Signal Transduction. *Journal of Cell Biology*, **124**, 1047-1059.

Overall, C.M., Wrana, J.L. & Sodek, J. (1989). Transforming Growth Factor-beta regulation of Collagenase, 72 Kda- Progelatinase, TIMP and PAI-1 expression in Rat Bone cell populations and Human Fibroblasts. *Connective Tissue Research*, **20**, 289-294.

Overduin, M., Harvey, T.S., Bagby, S., Tong, K.I., Yau, P., Takeichi, M. & Ikura, M. (1995). Solution Structure of the Epithelial Cadherin domain responsible for selective cell adhesion. *Science*, **267**, 386-389.

Owaribe, K., Nishizawa, Y. & Franke, W.W. (1991). Isolation and Characterization of Hemidesmosomes from Bovine Corneal Epithelial cells. *Experimental Cell Research*, **192**, 622-630.

Ozawa, M., Baribault, H. & Kemler, R. (1989). The Cytoplasmic Domain of the cell adhesion molecule Uvomorulin associates with 3 independent proteins structurally related in different species. *Embo Journal*, **8**, 1711-1717.

Ozawa, M. & Kemler, R. (1992). Molecular-Organization of the Uvomorulin-Catenin Complex. *Journal of Cell Biology*, **116**, 989-996.

Page, D.L. & Dupont, W.D. (1991). Proliferative Breast Disease - Diagnosis and Implications. *Science*, **253**, 915.

Pannetier, C., Delassus, S., Darche, S., Saucier, C. & Kourilsky, P. (1993). Quantitative Titration of Nucleic-Acids by Enzymatic Amplification Reactions run to Saturation. *Nucleic Acids Research*, **21**, 577-583.

Pechoux, C., Gudjonsson, T., RonnovJessen, L., Bissell, M.J. & Petersen, O.W. (1999). Human Mammary Luminal Epithelial cells contain progenitors to Myoepithelial cells. *Developmental Biology*, **206**, 88-99.

Pei, D.Q. & Weiss, S.J. (1995). Furin-Dependent Intracellular Activation of the Human Stromelysin-3 Zymogen. *Nature*, **375**, 244-247.

Pei, D.Q. (1999). Identification and characterization of the fifth Membrane-Type Matrix Metalloproteinase MT5-MMP. *Journal of Biological Chemistry*, **274**, 8925-8932.

Peifer, M. (1997). Cancer beta-Catenin as Oncogene: The Smoking Gun. *Science*, **275**, 1752-1753.

Pendas, A.M., Knauper, V., Puente, X.S., Llano, E., Mattei, M.G., Apte, S., Murphy, G. & LopezOtin, C. (1997). Identification and characterization of a novel Human Matrix Metalloproteinase with unique structural characteristics, chromosomal location, and tissue distribution. *Journal of Biological Chemistry*, **272**, 4281-4286.

Pennisi, E. (1998). How a Growth Control Path takes a wrong turn to Cancer. *Science*, **281**, 1809.

Peterson, O.W. & van Deurs, B. (1988) Growth Factor control of Myoepithelial cell differentiation in cultures of Human Mammary Gland. *Differentiation*, **39**, 197-215.

Petersen, L.C., Lund, L.R., Nielsen, L.S., Dano, K. & Skriver, L. (1988). One Chain Urokinase-Type Plasminogen-Activator from Human Sarcoma cells is a Proenzyme with little or no intrinsic activity. *Journal of Biological Chemistry*, **263**, 11189-11195.

Picard, O., Rolland, Y. & Poupon, M.F. (1986). Fibroblast-Dependent Tumorigenicity of Cells in Nude Mice: Implication for Implantation of Metastases. *Cancer Research*, **46**, 3290-3294.

Pierceall, W.E., Woodard, A.S., Morrow, J.S., Rimm, D. & Fearon, E.R. (1995). Frequent Alterations in E-Cadherin and alpha-Catenin and beta-Catenin expression in Human Breast Cancer cell lines. *Oncogene*, **11**, 1319-1326.

Pierschbacher, M.D., Hayman, E.G. & Ruoslahti, E. (1981). Location of the Cell Attachment Site in Fibronectin. *Oncodevelopmental Biology and Medicine*, **2**, 16.

Pierschbacher, M.D. & Ruoslahti, E. (1984). Variants of the Cell Recognition Site of Fibronectin that retain Attachment-Promoting Activity. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, **81,** 5985-5988.

Pignatelli, M., Hanby, A.M. & Stamp, G.W.H. (1990). Expression of Integrin Cell Adhesion Molecules in Malignant Epithelial Tumors. *Journal of Pathology*, **161,** A339.

Pignatelli, M., Hanby, A.M. & Stamp, G.W.H. (1991). Low Expression of beta-1, alpha-2 and alpha-3 subunits of VLA Integrins in Malignant Mammary Tumors. *Journal of Pathology*, **165,** 25-32.

Pignatelli, M., Liu, D., Nasim, M.M., Stamp, G.W.H., Hirano, S. & Takeichi, M. (1992). Morphoregulatory Activities of E-Cadherin and beta-1 Integrins in Colorectal Tumor Cells. *British Journal of Cancer*, **66,** 629-634.

Pignatelli, M. & Vessey, C.J. (1994). Adhesion Molecules - Novel Molecular Tools in Tumor Pathology. *Human Pathology*, **25,** 849-856.

Pilcher, B.K., Dumin, J.A., Sudbeck, B.D., Krane, S.M., Welgus, H.G. & Parks, W.C. (1997). The activity of Collagenase-1 is required for Keratinocyte migration on a Type I Collagen matrix. *Journal of Cell Biology*, **137,** 1445-1457.

Pileri, S.A., Roncador, G., Ceccarelli, C., Piccioli, M., Briskomatis, A., Sabattini, E., Ascani, S., Santini, D., Piccaluga, P.P., Leone, O., Damiani, S., Ercolessi, C., Sandri, F., Pieri, F., Leoncini, L. & Falini, B. (1997). Antigen retrieval techniques in immunohistochemistry: Comparison of different methods. *Journal of Pathology*, **183,** 116-123.

Plow, E.F., Freaney, D.E., Plescia, J. & Miles, L.A. (1986). The Plasminogen System and Cell-Surfaces - Evidence for Plasminogen and Urokinase Receptors on the same cell type. *Journal of Cell Biology*, **103,** 2411-2420.

Polakis, P. (1997). The Adenomatous Polyposis Coli (APC) Tumor Suppressor. *Biochimica Et Biophysica Acta-Reviews On Cancer*, **1332,** F127-F147.

Pijnenborg, R., Dixon, G., Robertson, W,B., Brosens, I.. (1980). Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta,* **1,** 3-19.

Polette, M., Gilbert, N., Stas, I., Nawrocki, B., Noel, A., Remacle, A., Stetlerstevenson, W.G., Birembaut, P. & Foidart, M. (1994). Gelatinase-A expression and localization in Human Breast Cancers - an in-situ hybridization study and immunohistochemical detection using Confocal Microscopy. *Virchows Archiv-an International Journal of Pathology*, **424,** 641-645.

Polette, M., Nawrocki, B., Gilles, C., Seiki, M., Sato, H., Tournier, J.M., Birembaut, P. (1996). MT-MMP expression and localisation in Human Breast and Lung Carcinomas. *Virchows Archives,* **428,** 29-35.

Polette, M., Gilles, C., Marchand, V., Seiki, M., Tournier, J.M. & Birembaut, P. (1997). Induction of Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) expression in Human Fibroblasts by Breast Adenocarcinoma cells. *Clinical & Experimental Metastasis*, **15,** 157-163.

Poller D N, Silverstain M J et al. (1994).Ideas in pathology. Ductal Carcinoma in-situ of the Breast :a proposal for a new simplified classification. *Mod Pathol,* 7, 257-262.

Popowicz, P., Engel, G., Wilking, N. & Linder, S. (1995). Differential effects of Fibroblast Coculture on MCF-7 and MDA-MB -231 Breast Carcinoma cell invasion through Matrigel. *Oncology Reports,* **2,** 303-306.

Poulsom, R., Pignatelli, M., Stetlerstevenson, W.G., Liotta, L.A., Wright, P.A., Jeffery, R.E., Longcroft, J.M., Rogers, L. & Stamp, G.W.H. (1992). Stromal expression of 72 Kda Type-IV Collagenase (MMP-2) and TIMP-2 messenger-RNAs in Colorectal Neoplasia. *American Journal of Pathology*, **141**, 389-396.

Poulsom, R., Hanby, A.M., Pignatelli, M., Jeffery, R.E., Longcroft, J.M., Rogers, L. & Stamp, G.W.H. (1993). Expression of Gelatinase-A and TIMP-2 messenger-RNAs in Desmoplastic Fibroblasts in both Mammary Carcinomas and Basal Cell Carcinomas of the Skin. *Journal of Clinical Pathology*, **46**, 429-436.

Price, J.E. (1996). Metastasis from Human Breast Cancer cell lines. *Breast Cancer Research and Treatment*, **39**, 93-102.

Pringle, J.H., Homer, C.E., Warford, A., Kendall, C.H. & Lauder, I. (1987). In-situ Hybridization: Alkaline-Phosphatase Visualization of Biotinylated Probes in Cryostat and Paraffin Sections. *Histochemical Journal*, **19**, 488-496.

Pringle, J.H., Primrose, L., Kind, C.N., Talbot, I.C. & Lauder, I. (1989). In-situ Hybridization Demonstration of Poly-Adenylated RNA sequences in Formalin-Fixed Paraffin Sections using a Biotinylated Oligonucleotide Poly d(T) Probe. *Journal of Pathology*, **158**, 279.

Puente, X.S., Pendas, A.M., Llano, E., Velasco, G. & LopezOtin, C. (1996). Molecular cloning of a novel Membrane-Type Matrix Metalloproteinase from a Human Breast Carcinoma. *Cancer Research*, **56**, 944-949.

Pulyaeva, H., Bueno, J., Polette, M., Birembaut, P., Sato, H., Seiki, M. & Thompson, E.W. (1997). MT1-MMP correlates with MMP-2 activation potential seen after Epithelial to Mesenchymal transition in Human Breast Carcinoma cells. *Clinical & Experimental Metastasis*, **15**, 111-120.

Pyke, C., Ralfkiaer, E., Huhtala, P., Hurskainen, T., Dano, K. & Tryggvason, K. (1992). Localization of messenger-RNA for Mr 72,000 and 92,000 Type-IV Collagenases in Human Skin Cancers by In-situ Hybridization. *Cancer Research*, **52**, 1336-1341.

Pyke, C., Ralfkiaer, E., Tryggvason, K. & Dano, K. (1993). Messenger-RNA For 2 Type-IV Collagenases is located in Stromal Cells in Human Colon Cancer. *American Journal of Pathology*, **142**, 359-365.

Quantin, B., Murphy, G. & Breathnach, R. (1989). Pump-1 cDNA codes for a protein with characteristics similar to those of classical Collagenase family members. *Biochemistry*, **28**, 5327-5334.

Rabinovitz, I. & Mercurio, A.M. (1997). The integrin alpha 6 beta 4 functions in carcinoma cell migration on Laminin-1 by mediating the formation and stabilization of actin- containing motility structures. *Molecular Biology of the Cell*, **8**, 1533.

Ramosdesimone, N., Moll, U.M., Quigley, J.P. & French, D.L. (1993). Inhibition of Matrix Metalloproteinase-9 Activation By a Specific Monoclonal-Antibody. *Hybridoma*, **12**, 349-363.

RamosDeSimone, N., HahnDantona, E., Sipley, J., Nagase, H., French, D.L. & Quigley, J.P. (1999). Activation of Matrix Metalloproteinase-9 (MMP-9) via a converging Plasmin/Stromelysin-1 cascade enhances tumor cell invasion. *Journal of Biological Chemistry*, **274**, 13066-13076.

Ranscht, B. (1994). Cadherins and Catenins - Interactions and Functions in Embryonic Development. *Current Opinion in Cell Biology*, **6**, 740-746.

Ravanti, L., Heino, J., LopezOtin, C. & Kahari, V.M. (1999). Induction of Collagenase-3 (MMP-13) expression in Human Skin Fibroblasts by three-dimensional Collagen is mediated by p38 Mitogen-Activated Protein Kinase. *Journal of Biological Chemistry*, **274**, 2446-2455.

Rice, G.E., Bevilacqua, M.P. (1989). An inducible Endothelial cell surface Glycoprotein mediates Melanoma adhesion. *Science*, **246**, 1303-1306.

Riikonen, T., Koivisto, L., Vihinen, P. & Heino, J. (1995). Transforming Growth-Factor-beta regulates Collagen gel contraction by increasing alpha-2-beta-1 integrin expression in Osteogenic Cells. *Journal of Biological Chemistry*, **270**, 376-382.

Rimm, D.L., Sinard, J.H. & Morrow, J.S. (1995). Reduced alpha-Catenin and E-Cadherin expression in Breast Cancer. *Laboratory Investigation*, **72**, 506-512.

Risinger, J.I., Berchuck, A., Kohler, M.F. & Boyd, J. (1994). Mutations of the E-Cadherin gene in Human Gynecologic Cancers. *Nature Genetics*, **7**, 98-102.

Roberts, A.B., McCurie, B., Sporn, M.B. (1992). TGFβ: Regulation of Extracellular Matrix. *Kidney International*, **41**, 557-559.

Robinson, M.J. & Cobb, M.H. (1997). Mitogen-Activated Protein Kinase pathways. *Current Opinion in Cell Biology*, **9**, 180-186.

Ronco, M., Chen, Y., Goldfarb, S., Ziyadeh, F. (1992) Elevated glucose stimulates TGF beta gene expression and bioactivity in proximal tubule. *Kidney International*, **41**, 107-114.

RonnovJessen, L., Petersen, O.W. & Bissell, M.J. (1996). Cellular changes involved in conversion of Normal to Malignant Breast: Importance of the Stromal Reaction. *Physiological Reviews*, **76**, 69-125.

Roskelley, C.D., Desprez, P.Y. & Bissell, M.J. (1994). Extracellular Matrix-Dependent Tissue-Specific Gene-Expression in Mammary Epithelial Cells requires both Physical and Biochemical Signal-Transduction. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 12378-12382.

Roskelley, C.D., Srebrow, A. & Bissell, M.J. (1995). A Hierarchy of ECM-Mediated Signaling Regulates Tissue-Specific Gene Expression. *Current Opinion in Cell Biology*, **7**, 736-747.

Rougier, J.P., Moullier, P., Piedagnel, R. & Ronco, P.M. (1997). Hyperosmolality suppresses but TGF beta 1 increases MMP9 in human peritoneal Mesothelial cells. *Kidney International*, **51**, 337-347.

Rubinfeld, B., Robbins, P., ElGamil, M., Albert, I., Porfiri, E. & Polakis, P. (1997). Stabilization of beta-Catenin by genetic defects in Melanoma cell lines. *Science*, **275**, 1790-1792.

Rudland, P.S. & Hughes, C.M. (1989). Immunocytochemical Identification of Cell-Types in Human Mammary- Gland - Variations in Cellular Markers Are Dependent On Glandular Topography and Differentiation. *Journal of Histochemistry & Cytochemistry*, **37**, 1087-1100.

Rudland, P.S. (1993). Epithelial Stem-Cells and their possible role in the development of the Normal and Diseased Human Breast. *Histology and Histopathology*, **8**, 385-404.

Ryan, M.C., Tizard, R., Vandevanter, D.R. & Carter, W.G. (1994). Cloning of the Lama3 gene encoding the alpha-3-chain of the adhesive ligand Epiligrin: expression in wound repair. *Journal of Biological Chemistry*, **269**, 22779-22787.

Saarialho-kere, U.K., Kovacs, S.O., Pentland, A.P., Olerud, J.E., Welgus, H.G. & Parks, W.C. (1993). Cell-Matrix interactions modulate Interstitial Collagenase expression by Human Keratinocytes actively Involved in wound healing. *Journal of Clinical Investigation*, **92**, 2858-2866.

Saffell, J.L., Williams, E.J., Mason, I.J., Walsh, F.S. & Doherty, P. (1997). Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. *Neuron*, **18**, 231-242.

Sager, R., Anisowicz, A., Neveu, M., Liang, P. & Sotiropoulou, G. (1993). Identification by Differential Display of alpha-6 integrin as a candidate Tumor-Suppressor Gene. *Faseb Journal*, **7**, 964-970.

Saika, S., Kawashima, Y., Okada, Y., Tanaka, S., Yamanaka, O., Ohnishi, Y. & Ooshima, A. (1998). Recombinant TIMP-1 and -2 enhance the proliferation of Rabbit Corneal Epithelial cells in vitro and the spreading of Rabbit Corneal Epithelium in situ. *Current Eye Research*, **17**, 47-52.

Saiki, I., Murata, J., Yoneda, J., Kobayashi, H. & Azuma, I. (1994). Influence of Fibroblasts on the Invasion and Migration of Highly or Weakly Metastatic B16 Melanoma Cells. *International Journal of Cancer*, **56**, 867-873.

Salazar H, Tobon, et al (1975). Developmental, Gestational and Postgestational modifications of the Human Breast. *Clin Obstet Gynaecol.*, **18**, 113-137.

Salo, T., Liotta, L.A. & Tryggvason, K. (1983). Purification and Characterization of a Murine Basement-Membrane Collagen-degrading enzyme secreted by Metastatic Tumor Cells. *Journal of Biological Chemistry*, **258**, 3058-3063.

Sanchez-lopez, R., Alexander, C.M., Behrendtsen, O., Breathnach, R. & Werb, Z. (1993). Role of Zinc-Binding-Encoded and Hemopexin Domain-Encoded sequences in the substrate specificity of Collagenase and Stromelysin-2 as revealed by Chimeric Proteins. *Journal of Biological Chemistry*, **268**, 7238-7247.

Sanes, J.R. (1989). Extracellular-Matrix Molecules that influence Neural Development. *Annual Review of Neuroscience*, **12**, 491-516.

Sang, Q.X., Birkedalhansen, H. & Vanwart, H.E. (1995). Proteolytic and Nonproteolytic Activation of Human Neutrophil Progelatinase-B. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*, **1251**, 99-108.

Sato, H., Kita, M. & Seiki, M. (1993). V-Src activates the expression of 92-Kda Type-IV Collagenase gene through the AP-1 site and the GT Box homologous to Retinoblastoma Control Elements - a mechanism regulating gene expression independent of that by inflammatory Cytokines. *Journal of Biological Chemistry*, **268**, 23460-23468.

Sato, H. & Seiki, M. (1993). Regulatory Mechanism of 92-Kda Type-IV Collagenase gene expression which is associated with invasiveness of Tumor cells. *Oncogene*, **8**, 395-405.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. & Seiki, M. (1994). A Matrix Metalloproteinase expressed on the surface of invasive Tumor cells. *Nature*, **370**, 61-65.

Sato, H., Kinoshita, T., Takino, T., Nakayama, K. & Seiki, M. (1996). Activation of a recombinant Membrane Type 1-Matrix Metalloproteinase (MT1-MMP) by Furin and its interaction with Tissue Inhibitor of Metalloproteinases (TIMP)-2. *Febs Letters*, **393**, 101-104.

Savoia, P., Trusolino, L., Pepino, E., Cremona, O. & Marchisio, P.C. (1993). Expression and Topography of Integrins and Basement-Membrane Proteins in Epidermal Carcinomas - Basal but not Squamous-Cell Carcinomas display loss of alpha-6-beta-4 and BM-600 Nicein. *Journal of Investigative Dermatology*, **101**, 352-358.

Scardigli, R., Soddu, S., Falcioni, R., Crescenzi, M., Cimino, L. & Sacchi, A. (1996). The beta(4) integrin subunit is expressed in Mouse Fibroblasts and modulated by Transforming Growth Factor-beta 1. *Experimental Cell Research*, **227**, 223-229.

Schaapveld, R.Q.J., Borradori, L., Geerts, D., vanLeusden, M.R., Kuikman, I., Nievers, M.G., Niessen, C.M., Steenbergen, R.D.M., Snijders, P.J.F. & Sonnenberg, A. (1998). Hemidesmosome formation is initiated by the beta 4 integrin subunit, requires complex formation of beta 4 and HD1/Plectin, and involves a direct interaction between beta 4 and the Bullous Pemphigoid Antigen 180. *Journal of Cell Biology*, **142**, 271-284.

Schadendorf, D., Gawlik, C., Haney, U., Ostmeier, H., Suter, L. & Czarnetzki, B.M. (1993). Tumor Progression and Metastatic Behavior in-vivo correlates with Integrin expression on Melanocytic Tumors. *Journal of Pathology*, **170**, 429-434.

Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K. & Birchmeier, W. (1991). E-Cadherin expression in Squamous-Cell Carcinomas of Head and Neck - inverse correlation with Tumor dedifferentiation and Lymph-Node Metastasis. *Cancer Research*, **51**, 6328-6337.

Schmid, T.M., Mayne, R., Jeffrey, J.J. & Linsenmayer, T.F. (1986). Type-X Collagen Contains 2 Cleavage Sites for a Vertebrate Collagenase. *Journal of Biological Chemistry*, **261**, 4184-4189.

Schnaper, H.W., Grant, D.S., Stetlerstevenson, W.G., Fridman, R., Dorazi, G., Murphy, A.N., Bird, R.E., Hoythya, M., Fuerst, T.R., French, D.L., Quigley, J.P. & Kleinman, H.K. (1993). Type-IV Collagenase and TIMPs modulate Endothelial Cell Morphogenesis in-vitro. *Journal of Cellular Physiology*, **156**, 235-246.

Schreiner, C.L., Bauer, J.S., Danilov, Y.N., Hussein, S., Sczekan, M.M. & Juliano, R.L. (1989). Isolation and Characterization of Chinese-Hamster Ovary Cell variants deficient in the expression of Fibronectin Receptor. *Journal of Cell Biology*, **109**, 3157-3167.

Schultz, R.M., Silberman, S., Persky, B., Bajkowski, A.S. & Carmichael, D.F. (1988). Inhibition by Human Recombinant Tissue Inhibitor of Metalloproteinases of Human Amnion Invasion and Lung Colonization By Murine B16-F10 Melanoma cells. *Cancer Research*, **48**, 5539-5545.

Schwartz M A, Schaller M D, et al (1995) Integrins: Emerging Paradigms of Signal Transduction. *Annual Review of Cell Biology and Developmental Biology*, **11**, 549-600.

Sciavolino, P.J., Lee, T.H. & Vilcek, J. (1994). Interferon-beta induces Metalloproteinase messenger-RNA expression in Human Fibroblasts - Role of Activator Protein-1. *Journal of Biological Chemistry*, **269**, 21627-21634.

Seftor, R.E.B., Seftor, E.A., Stetlerstevenson, W.G. & Hendrix, M.J.C. (1993). The 72 Kda Type-IV Collagenase is modulated via differential expression of alpha-V-beta-3-Integrin and alpha-5-beta-1-Integrin during Human-Melanoma Cell Invasion. *Cancer Research*, **53**, 3411-3415.

Segain, J.P., Harb, J., Gregoire, M., Meflah, K. & Menanteau, J. (1996). Induction of Fibroblast Gelatinase B expression by direct contact with cell lines derived from Primary Tumor but not from Metastases. *Cancer Research*, **56**, 5506-5512.

Seidah, N.G., Day, R. & Chretien, M. (1993). The Family of Prohormone and Pro-Protein Convertases. *Biochemical Society Transactions*, **21**, 685-691.

Seltzer, J.L., Eisen, A.Z., Bauer, E.A., Morris, N.P., Glanville, R.W. & Burgeson, R.E. (1989). Cleavage of Type-VII Collagen by Interstitial Collagenase and Type-IV Collagenase (Gelatinase) derived from Human Skin. *Journal of Biological Chemistry*, **264**, 3822-3826.

Senior, R.M., Campbell, E.J., Landis, J.A., Cox, F.R., Kuhn, C. & Koren, H.S. (1982). Elastase of U-937 Monocyte-Like Cells - Comparisons with Elastases derived from Human Monocytes and Neutrophils and Murine Macrophage- Like Cells. *Journal of Clinical Investigation*, **69**, 384-393.

Senior, R.M., Connolly, N.L., Cury, J.D., Welgus, H.G. & Campbell, E.J. (1989). Elastin Degradation by Human Alveolar Macrophages - a Prominent Role of Metalloproteinase Activity. *American Review of Respiratory Disease*, **139**, 1251-1256.

Shao, Z.M., Nguyen, M., Alpaugh, M.L., Oconnell, J.T. & Barsky, S.H. (1998). The Human Myoepithelial cell exerts antiproliferative effects on Breast Carcinoma cells characterized by p21(WAF1/CIP1) induction, G(2)/M arrest, and apoptosis. *Experimental Cell Research*, **241**, 394-403.

Shapiro, S.D., Griffin, G.L., Gilbert, D.J., Jenkins, N.A., Copeland, N.G., Welgus, H.G., Senior, R.M. & Ley, T.J. (1992). Molecular Cloning, Chromosomal localization, and Bacterial Expression of a Murine Macrophage Metalloelastase. *Journal of Biological Chemistry*, **267**, 4664-4671.

Shapiro, S.D., Kobayashi, D.K., Pentland, A.P. & Welgus, H.G. (1993). Induction of Macrophage Metalloproteinases by Extracellular-Matrix: Evidence for Enzyme-Specific and Substrate-Specific responses involving Prostaglandin-Dependent Mechanisms. *Journal of Biological Chemistry*, **268**, 8170-8175.

Shapiro, L., Fannon, A.M., Kwong, P.D., Thompson, A., Lehmann, M.S., Grubel, G., Legrand, J.F., Alsnielsen, J., Colman, D.R. & Hendrickson, W.A. (1995). Structural Basis of Cell-Cell Adhesion by Cadherins. *Nature*, **374**, 327-337.

Shaw, L.M., Rabinovitz, I., Wang, H.H.F., Toker, A. & Mercurio, A.M. (1997). Activation of Phosphoinositide 3-OH Kinase by the alpha 6 beta 4 integrin promotes Carcinoma Invasion. *Cell*, **91**, 949-960.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. & Ito, F. (1994). Tyrosine Phosphorylation of beta-Catenin and Plakoglobin enhanced by Hepatocyte Growth-Factor and Epidermal Growth-Factor in Human Carcinoma-Cells. *Cell Adhesion and Communication*, **1**, 295-305.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Miyazawa, K., Kitamura, N., Johnson, K.R., Wheelock, M.J., Matsuyoshi, N., Takeichi, M. & Ito, F. (1995). Association of p120, a Tyrosine Kinase Substrate, with E- Cadherin/Catenin Complexes. *Journal of Cell Biology*, **128**, 949-957.

Shih, I.M., Elder, D.E., Hsu, M.Y. & Herlyn, M. (1994). Regulation of Mel-Cam/Muc18 expression on Melanocytes of Different Stages of Tumor Progression by Normal Keratinocytes. *American Journal of Pathology*, **145**, 837-845.

Shimizu, S., Nishikawa, Y., Kuroda, K., Takagi, S., Kozaki, K., Hyuga, S., Saga, S. & Matsuyama, M. (1996). Involvement of Transforming Growth Factor beta 1 in autocrine enhancement of Gelatinase B secretion by Murine Metastatic Colon Carcinoma cells. *Cancer Research*, **56**, 3366-3370.

Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M. & Abe, O. (1989). Cadherin Cell-Adhesion Molecules in Human Epithelial Tissues and Carcinomas. *Cancer Research*, **49**, 2128-2133.

Shiozaki, H., Iihara, K., Oka, H., Kadowaki, T., Matsui, S., Gofuku, J., Inoue, M., Nagafuchi, A., Tsukita, S. & Mori, T. (1994). Immunohistochemical detection of alpha-Catenin expression in Human Cancers. *American Journal of Pathology*, **144**, 667-674.

Shiozaki, H., Kadowaki, T., Doki, Y., Inoue, M., Tamura, S., Oka, H., Iwazawa, T., Matsui, S., Shimaya, K., Takeichi, M. & Mori, T. (1995). Effect of Epidermal Growth-Factor on Cadherin-Mediated Adhesion in a Human Esophageal Cancer Cell-Line. *British Journal of Cancer*, **71**, 250-258.

Silva, J.S., Georgiade, G.S., Dilley, W.G., McCarty, K.S. & Wells, S.A. (1983). Menstrual Cycle-Dependent Variations of Breast Cyst Fluid Proteins and Sex Steroid-Receptors in the Normal Human-Breast. *Cancer*, **51**, 1297-1302.

Smith, J.W. (1994). The Structural Basis of Integrin-Ligand (RGD) Interaction. In: *Integrins. Molecular and Biological Responses to the Extracellular Matrix. Ed. Cheresh, D.A. &Mecham, R.P.,Academic Press Inc. San Diego. pp1-32.*

Soini, Y., Hurskainen, T., Hoyhtya, M., Oikarinen, A. & Autioharmainen, H. (1994). 72 Kd and 92 Kd Type-IV Collagenase, Type-IV Collagen, and Laminin messenger-RNAs in Breast Cancer – a study by in-situ hybridization. *Journal of Histochemistry & Cytochemistry*, **42**, 945-951.

Sollberg, S., Ryynanen, J., Olsen, D.R. & Uitto, J. (1992). Transforming Growth-Factor-beta up-regulates the expression of the genes for beta-4 integrin and Bullous Pemphigoid Antigens (BPAg1 and BPAg2) in Normal and Transformed Human Keratinocytes. *Journal of Investigative Dermatology*, **99**, 409-414.

Sommers, C.L., Gelmann, E.P., Kemler, R., Cowin, P. & Byers, S.W. (1994). Alterations in beta-Catenin Phosphorylation and Plakoglobin Expression in Human Breast Cancer Cells. *Cancer Research*, **54**, 3544-3552.

Sonnenberg, A., Daams, H., Vandervalk, M.A., Hilkens, J. & Hilgers, J. (1986). Development of Mouse Mammary Gland - Identification of Stages in Differentiation of Luminal and Myoepithelial Cells Using Monoclonal- Antibodies and Polyvalent Antiserum against Keratin. *Journal of Histochemistry & Cytochemistry*, **34**, 1037-1046.

Sonnenberg, A., Linders, C.J.T., Daams, J.H. & Kennel, S.J. (1990). The alpha-6-beta-1 (VLA-6) and alpha-6-beta-4 protein complexes - Tissue Distribution and Biochemical Properties. *Journal of Cell Science*, **96**, 207-217.

Sorsa, T., Salo, T., Koivunen, E., Tyynela, J., Konttinen, Y.T., Bergmann, U., Tuuttila, A., Niemi, E., Teronen, O., Heikkila, P., Tschesche, H., Leinonen, J., Osman, S. & Stenman, U.H. (1997). Activation of Type IV Procollagenases by human Tumor-Associated Trypsin-2. *Journal of Biological Chemistry*, **272**, 21067-21074.

Soule, H.D., Vazquez, J., Long, A., Alberts, S., Brennan, M. (1973). A Human cell line from a pleural effusion derived from a Breast Carcinoma. *J. National Cancer Institute*, **51**, 1409-1416.

Sprengers, E.D. & Kluft, C. (1987). Plasminogen-Activator Inhibitors. *Blood*, **69**, 381-387.

Springman, E.B., Angleton, E.L., Birkedalhansen, H. & Vanwart, H.E. (1990). Multiple-Modes of Activation of Latent Human Fibroblast Collagenase - Evidence for the role of a Cys-73 active-site Zinc complex in latency and a Cysteine Switch Mechanism for Activation. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 364-368.

Stampfer, M., Hallowes, R,C., Hackett, A.J. (1980) Growth of Normal Human Mammary cells in culture. *In Vitro*, **16**, 415-425.

Stanley, P. & Hogg, N. (1998). The I domain of integrin LFA-1 interacts with ICAM-1 domain 1 at residue Glu-34 but not Gln-73. *Journal of Biological Chemistry*, **273**, 3358-3362.

Stanton, H., Gavrilovic, J., Atkinson, S.J., dOrtho, M.P., Yamada, K.M., Zardi, L. & Murphy, G. (1998). The activation of ProMMP-2 (Gelatinase A) by HT1080 Fibrosarcoma cells is promoted by culture on a Fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *Journal of Cell Science*, **111**, 2789-2798.

Starkey, J.R. (1990). Cell-Matrix Interactions during Tumor Invasion. *Cancer and Metastasis Reviews*, **9**, 113-123.

Steiner, D.F., Smeekens, S.P., Ohagi, S. & Chan, S.J. (1992). The New Enzymology of Precursor Processing Endoproteases. *Journal of Biological Chemistry*, **267**, 23435-23438.

Stetler-Stevenson, W.G., Krutzsch, H.C. & Liotta, L.A. (1989). Tissue Inhibitor of Metalloproteinase (TIMP-2) - a New Member of the Metalloproteinase Inhibitor Family. *Journal of Biological Chemistry*, **264**, 17374-17378.

Stetler-Stevenson, W.G., Brown, P.D., Onisto, M., Levy, A.T. & Liotta, L.A. (1990). Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) messenger-RNA expression in Tumor Cell Lines and Human Tumor Tissues. *Journal of Biological Chemistry*, **265**, 13933-13938.

Stetlerstevenson, W.G., Talano, J.A., Gallagher, M.E., Krutzsch, H.C. & Liotta, L.A. (1991). Inhibition of Human Type-IV Collagenase by a highly conserved peptide sequence derived from its Prosegment. *American Journal of the Medical Sciences*, **302**, 163-170.

Stetlerstevenson, W.G., Liotta, L.A. & Kleiner, D.E. (1993). Extracellular Matrix-6: Role of Matrix Metalloproteinases in Tumor Invasion and Metastasis. *Faseb Journal*, **7**, 1434-1441.

Streuli, C.H. & Bissell, M.J. (1990). Expression of Extracellular-Matrix Components is Regulated by Substratum. *Journal of Cell Biology*, **110**, 1405-1415.

Streuli, C.H., Bailey, N. & Bissell, M.J. (1991). Control of Mammary Epithelial Differentiation - Basement-Membrane induces Tissue-Specific gene expression in the absence of Cell Cell Interaction and Morphological Polarity. *Journal of Cell Biology*, **115**, 1383-1395.

Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A.P.N., Roskelley, C. & Bissell, M.J. (1995). Laminin Mediates Tissue-Specific Gene-Expression in Mammary Epithelia. *Journal of Cell Biology*, **129**, 591-603.

Strongin, A.Y., Marmer, B.L., Grant, G.A. & Goldberg, G.I. (1993). Plasma Membrane-Dependent Activation of the 72-Kda Type-IV Collagenase is prevented by complex formation with TIMP-2. *Journal of Biological Chemistry*, **268**, 14033-14039.

Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A. & Goldberg, G.I. (1995). Mechanism of Cell-Surface Activation of 72-Kda Type-IV Collagenase: Isolation of the Activated Form of the Membrane Metalloprotease. *Journal of Biological Chemistry*, **270**, 5331-5338.

Sudbeck, B.D., Saarialhokere, U.K., Welgus, H.G., Pentland, A.P. & Parks, W.C. (1994). Collagenase Induction by Basal Keratinocytes requires contact with native Type-1 Collagen and is mediated Bb y Tyrosine Kinase-Dependent and PKC-Dependent Pathways. *Journal of Investigative Dermatology*, **102**, 550.

Sugimoto, K., Honda, S., Yamamoto, T., Ueki, T., Monden, M., Kaji, A., Matsumoto, K. & Nakamura, T. (1996). Molecular cloning and characterization of a newly identified member of the Cadherin family, PB-Cadherin. *Journal of Biological Chemistry*, **271**, 11548-11556.

Sun, H., Santoro, S.A. & Zutter, M.M. (1998). Downstream events in Mammary Gland Morphogenesis mediated by re-expression of the alpha(2)beta(1) integrin: The role of the alpha(6) and beta(4) integrin subunits. *Cancer Research*, **58**, 2224-2233.

Suzuki, K., Nagase, H., Ito, A., Enghild, J.J. & Salvesen, G. (1990). The Role of Matrix Metalloproteinase-3 in the Stepwise Activation of Human Rheumatoid Synovial Procollagenase. *Biological Chemistry Hoppe-Seyler*, **371**, 305-310.

Tagliabue, E., Ghirelli, C., Squicciarini, P., Aiello, P., Colnaghi, M.I. & Menard, S. (1998). Prognostic value of alpha 6 beta 4 integrin expression in breast carcinomas is affected by laminin production from tumor cells. *Clinical Cancer Research*, **4**, 407-410.

Takada, Y., Elices, M.J., Crouse, C, Hemler, M.E. (1989). The primary structure of the alpha 4 subunit of VLA-4: Homology to other integrins and a possible cell-cell adhesion function. *Embo Journal*, **8**, 1361-1368.

Takayama, T., Shiozaki, H., Shibamoto, S., Oka, H., Kimura, Y., Tamura, S., Inoue, M., Monden, T., Ito, F. & Monden, M. (1996). beta-Catenin expression in Human Cancers. *American Journal of Pathology*, **148**, 39-46.

Takeichi, M. (1995). Morphogenetic Roles of Classic Cadherins. *Current Opinion in Cell Biology*, **7**, 619-627.

Takino, T., Sato, H., Shinagawa, A. & Seiki, M. (1995). Identification of the 2nd Membrane-Type Matrix Metalloproteinase (MT- MMP-2) gene from a Human Placenta cDNA Library – MT-MMPs form a unique Membrane-Type Subclass in the MMP Family. *Journal of Biological Chemistry*, **270**, 23013-23020.

Talhouk, R.S., Bissell, M.J. & Werb, Z. (1992). Coordinated Expression of Extracellular Matrix-Degrading Proteinases and their Inhibitors Regulates Mammary Epithelial Function during Involution. *Journal of Cell Biology*, **118**, 1271-1282.

Tanaka, H., Mori, Y., Ishii, H. & Akedo, H. (1988). Enhancement of Metastatic Capacity of Fibroblast-Tumor Cell Interaction in Mice. *Cancer Research*, **48**, 1456-1459.

Tang, A., Eller, M.S., Hara, M., Yaar, M., Hirohashi, S. & Gilchrest, B.A. (1994). E-Cadherin is the major mediator of Human Melanocyte Adhesion to Keratinocytes in-vitro. *Journal of Cell Science*, **107**, 983-992.

Taylo-Papadimitriou, J., Berdichevsky, F., Dsouza, B. & Burchell, J. (1993). Human Models of Breast-Cancer. *Cancer Surveys*, **16**, 59-78.

Terranova, V.P., Hujanen, E.S., Loeb, D.M., Martin, G.R., Thornburg, L. & Glushko, V. (1986). Use of a Reconstituted Basement-Membrane to Measure Cell Invasiveness and Select for Highly Invasive Tumor Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 465-469.

Teti, A., Farina, A.R., Villanova, I., Tiberio, A., Sciortino, G., Chambers, A.F., Gulino, A., Mackay, A.R. (1998). Activation of MMP-2 by Human GCT23 Giant cell tumour cells induced by Osteopontin, Bone Sialoprotein and GRGDSP peptides is RGD and cell shape-change dependent. *International Journal Cancer*, **77**, 82-93.

Tetu, B., Brisson, J., Lapointe, H. & Bernard, P. (1998). Prognostic significance of Stromelysin 3, Gelatinase A, and Urokinase expression in Breast Cancer. *Human Pathology*, **29**, 979-985.

Thompson, E.W., Paik, S.M., Brunner, N., Sommers, C.L., Zugmaier, G., Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., Martin, G.R. & Dickson, R.B. (1992). Association of Increased Basement-Membrane Invasiveness with absence of Estrogen-Receptor and expression of Vimentin in Human Breast Cancer Cell Lines. *Journal of Cellular Physiology*, **150**, 534-544.

Tokuraku, M., Sato, H., Murakami, S., Okada, Y., Watanabe, Y. & Seiki, M. (1995). Activation of the Precursor of Gelatinase a/72 Kda Type-IV Collagenase/MMP-2 in Lung Carcinomas correlates with the expression of Membrane-Type Matrix Metalloproteinase (MT-MMP) and with Lymph- Node Metastasis. *International Journal of Cancer*, **64**, 355-359.

Tomasek, J.J., Halliday, N.L., Updike, D.L., AhernMoore, J.S., Vu, T.K.H., Liu, R.W. & Howard, E.W. (1997). Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. *Journal of Biological Chemistry*, **272**, 7482-7487.

Topper Y J, Freeman C S. (1980). Multiple hormone interactions in the Developmental Biology of the Mammary Gland. *Physiological Reviews*, **60**, 1049-1106.

Tremble, P., Chiquetehrismann, R. & Werb, Z. (1994). The Extracellular-Matrix Ligands Fibronectin and Tenascin collaborate in regulating Collagenase gene expression in Fibroblasts. *Molecular Biology of the Cell*, **5**, 439-453.

Tsubura, A., Shikata, N., Inui, T., Morii, S., Hatano, T., Oikawa, T. & Matsuzawa, A. (1988). Immunohistochemical localization of Myoepithelial Cells and Basement- Membrane in Normal, Benign and Malignant Human Breast Lesions. *Virchows Archiv a-Pathological Anatomy and Histopathology*, **413**, 133-139.

Tsuruo, T., Yamori, T., Naganuma, K., Tsukagoshi, S. & Sakurai, Y. (1983). Characterization of Metastatic Clones derived from a Metastatic Variant of Mouse Colon Adenocarcinoma-26. *Cancer Research*, **43**, 5437-5442.

Uematsu, J., Nishizawa, Y., Sonnenberg, A. & Owaribe, K. (1994). Demonstration of Type-II Hemidesmosomes in a Mammary-Gland Epithelial-Cell Line, BMGE-H. *Journal of Biochemistry*, **115**, 469-476.

Ueno,H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M., Okada,Y. (1997). Expression and tissue localisation of Membrane Types 1, 2, and 3 Matrix Metalloproteinases in Human Invasive Breast Carcinomas. *Cancer Research*, **57**, 3159-3167.

Umbas, R., Schalken, J.A., Aalders, T.W., Carter, B.S., Karthaus, H.F.M., Schaafsma, H.E., Debruyne, F.M.J. & Isaacs, W.B. (1992). Expression of the Cellular Adhesion Molecule E-Cadherin is reduced or absent in High-Grade Prostate Cancer. *Cancer Research*, **52**, 5104-5109.

Uria, J.A., Ferrando, A.A., Velasco, G., Freije, J.M.P. & Lopezotin, C. (1994). Structure and Expression in Breast Tumors of Human TIMP-3, a new member of the Metalloproteinase Inhibitor Family. *Cancer Research*, **54**, 2091-2094.

Uria, J.A., Jimenez, M.G., Balbin, M., Freije, J.M.P. & LopezOtin, C. (1998). Differential effects of Transforming Growth Factor-beta on the expression of Collagenase-1 and Collagenase-3 in Human Fibroblasts. *Journal of Biological Chemistry*, **273**, 9769-9777.

Valagussal P, Bonadonna G et al. (1978). Patterns of Relapse and Survival following Radical Mastectomy.*Cancer,* **41,** 1170-1178.

van-Duinen, C.M., Vandenbroek, L., Vermeer, B.J. & Bruijn, J.A. (1994). The Distribution of Cellular Adhesion Molecules in Pigmented Skin Lesions. *Cancer,* **73,** 2131-2139.

Van-Lamsweerde, A.L., Henry, N. & Vaes, G. (1983). Metastatic Heterogeneity of Cells from Lewis Lung Carcinoma. *Cancer Research,* **43,** 5314-5320.

Van-Waes, C., Kozarsky, K.F., Warren, A.B., Kidd, L., Paugh, D., Liebert, M. & Carey, T.E. (1991). The A9 Antigen associated with aggressive Human Squamous Carcinoma is Structurally and Functionally similar to the newly defined Integrin alpha-6-beta-4. *Cancer Research,* **51,** 2395-2402.

Van-Wart, H.E. & Birkedalhansen, H. (1990). The Cysteine Switch - a Principle of Regulation of Metalloproteinase Activity with Potential Applicability to the Entire Matrix Metalloproteinase Gene Family. *Proceedings of the National Academy of Sciences of the United States of America,* **87,** 5578-5582.

Varner, J.A., Emerson, D.A. & Juliano, R.L. (1995). Integrin alpha-5-beta-1 expression negatively regulates Cell-Growth: Reversal by Attachment to Fibronectin. *Molecular Biology of the Cell,* **6,** 725-740.

Vassalli, J.D., Sappino, A.P. & Belin, D. (1991). The Plasminogen-Activator Plasmin System. *Journal of Clinical Investigation,* **88,** 1067-1072.

Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W. & Vanroy, F. (1991). Genetic Manipulation of E-Cadherin expression by Epithelial Tumor Cells reveals an Invasion Suppressor Role. *Cell,* **66,** 107-119.

Vos, C.B.J., CletonJansen, A.M., Berx, G., deLeeuw, W.J.F., terHaar, N.T., vanRoy, F., Cornelisse, C.J., Peterse, J.L. & vandeVijver, M.J. (1997). E-Cadherin inactivation in Lobular Carcinoma in situ of the Breast: an early event in tumorigenesis. *British Journal of Cancer,* **76,** 1131-1133.

Walker, R.A. (1988). Breast Myoepithelium - the Ignored Cell. *Journal of Pathology,* **156,** 5-6.

Wang, M.S., Liu, Y.L.E., Greene, J., Sheng, S.J., Fuchs, A., Rosen, E.M. & Shi, Y.E. (1997). Inhibition of tumor growth and metastasis of Human Breast Cancer cells transfected with Tissue Inhibitor of Metalloproteinase -4. *Oncogene,* **14,** 2767-2774.

Ward, R.V., Atkinson, S.J., Reynolds, J.J. & Murphy, G. (1994). Cell Surface-Mediated Activation of Progelatinase-A: Demonstration of the Involvement of the C-Terminal Domain of Progelatinase-A in Cell-Surface Binding and Activation of Progelatinase-A by Primary Fibroblasts. *Biochemical Journal,* **304,** 263-269.

Watanabe, M., Takahashi, Y., Ohta, T., Mai, M., Sasaki, T. & Seiki, M. (1996). Inhibition of metastasis in Human Gastric Cancer cells transfected with Tissue Inhibitor of Metalloproteinase-1 gene in Nude Mice. *Cancer,* **77,** 1676-1680.

Weaver, V.M., Howlett, A.R., Langtonwebster, B., Petersen, O.W. & Bissell, M.J. (1995). The Development of a Functionally Relevant Cell-Culture Model of Progressive Human Breast Cancer. *Seminars in Cancer Biology,* **6,** 175-184.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C. & Bissell, M.J. (1997). Reversion of the Malignant Phenotype of Human Breast cells in three- dimensional culture and in-vivo by integrin blocking antibodies. *Journal of Cell Biology*, **137**, 231-245.

Wei, Y., Lukashev, M., Simon, D.I., Bodary, S.C., Rosenberg, S., Doyle, M.V. & Chapman, H.A. (1996). Regulation of integrin function by the Urokinase Receptor. *Science*, **273**, 1551-1555.

Weiss, S.J. (1989). Tissue Destruction by Neutrophils. *New England Journal of Medicine*, **320**, 365-376.

Wellings S R, Jensen H M, et al. (1975). An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J. Nat Cancer Inst.*, **55**, 231-273.

Werb Z, Banda M J, et al. (1980) Degradation of Connective Tissue Matrices by Macrophages I. Proteolysis of Elastin, Glycoproteins and Collagen by Proteinases isolated from Macrophages. *Journal Exp Med*, **152**, 1340-1357.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E. & Damsky, C.H. (1989). Signal Transduction through the Fibronectin Receptor induces Collagenase and Stromelysin gene expression. *Journal of Cell Biology*, **109**, 877-889.

Westermarck, J. & Kahari, V.M. (1999). Regulation of Matrix Metalloproteinase expression in Tumour Invasion. *Faseb Journal*, **13**, 781-792.

Westermarck, J., Seth, A. & Kahari, V.M. (1997). Differential regulation of Interstitial Collagenase (MMP-1) gene expression by ETS transcription factors. *Oncogene*, **14**, 2651-2660.

Wetzels, R.H.W., Hollan, R., van Haelst, U.J.G.M., Lane, E.B., Leigh, I.M., Ramaekers, F.C.S. (1990). Detection of Basement Membrane components and Basal Cell keratin 14 in noninvasive and invasive carcinomas of the Breast. *American Journal of Pathology*, **134**, 571-579.

Wheeler J E, Enterline H T, et al (1974). Lobular Carcinoma in-situ of the Breast : a long term follow up. *Cancer*, **34**, 554-563

Wicha, M.S., Liotta, L.A., Vonderhaar, B.K., Kidwell, W.R. (1980). Effects of inhibition of Basement Membrane Collagen deposition on Rat Mammary Gland development. *Dev. Biol.* **80**, 253-266.

Wilhelm, S.M., Collier, I.E., Kronberger, A., Eisen, A.Z., Marmer, B.L., Grant, G.A., Bauer, E.A. & Goldberg, G.I. (1987). Human Skin Fibroblast Stromelysin: Structure, Glycosylation, Substrate-Specificity, and Differential Expression in Normal and Tumorigenic Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 6725-6729.

Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A. & Goldberg, G.I. (1989). Sv40-Transformed Human-Lung Fibroblasts secrete a 92-Kda Type-IV Collagenase which is identical to that secreted by Normal Human Macrophages. *Journal of Biological Chemistry*, **264**, 17213-17221.

Will, H. & Hinzmann, B. (1995). cDNA Sequence and messenger-RNA Tissue Distribution of a Novel Human Matrix Metalloproteinase with a Potential Transmembrane Segment. *European Journal of Biochemistry*, **231**, 602-608

Will, H., Atkinson, S.J., Butler, G.S., Smith, B. & Murphy, G. (1996). The soluble catalytic domain of Membrane Type 1 Matrix Metalloproteinase cleaves the propeptide of Progelatinase A and initiates autoproteolytic activation: Regulation by TIMP-2 and TIMP- 3. *Journal of Biological Chemistry*, **271**, 17119-17123.

Willenbrock, F., Crabbe, T., Slocombe, P.M., Sutton, C.W., Docherty, A.J.P., Cockett, M.I., Oshea, M., Brocklehurst, K., Phillips, I.R. & Murphy, G. (1993). The Activity of the Tissue Inhibitors of Metalloproteinases is regulated by C-Terminal Domain Interactions - a Kinetic-Analysis of the Inhibition of Gelatinase-A. *Biochemistry*, **32**, 4330-4337.

Williamson, R.A., Marston, F.A.O., Angal, S., Koklitis, P., Panico, M., Morris, H.R., Carne, A.F., Smith, B.J., Harris, T.J.R. & Freedman, R.B. (1990). Disulfide Bond Assignment in Human Tissue Inhibitor of Metalloproteinases (TIMP). *Biochemical Journal*, **268**, 267-274.

Witty, J.P., Lempka, T., Coffey, R.J. & Matrisian, L.M. (1995). Decreased Tumor Formation in 7,12-Dimethylbenzanthracene-Treated Stromelysin-1 Transgenic Mice is associated with alterations in Mammary Epithelial-Cell Apoptosis. *Cancer Research*, **55**, 1401-1406.

Woessner, J.F. (1991). Matrix Metalloproteinases and their Inhibitors in Connective-Tissue Remodeling. *Faseb Journal*, **5**, 2145-2154.

Wong, H., Anderson, W.D., Cheng, T. & Riabowol, K.T. (1994). Monitoring messenger-RNA Expression by Polymerase Chain-Reaction - the Primer-Dropping Method. *Analytical Biochemistry*, **223**, 251-258.

Xiao, G.W., Liu, Y.E., Gentz, R., Sang, Q.A., Ni, J., Goldberg, I.D. & Shi, Y.E. (1999). Suppression of Breast Cancer growth and metastasis by a Serpin Myoepithelium-derived Serine Proteinase Inhibitor expressed in the Mammary Myoepithelial Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 3700-3705.

Xie, B., Bucana, C.D. & Fidler, I.J. (1994). Density-Dependent Induction of 92-Kd Type-IV Collagenase Activity in Cultures of A431 Human Epidermoid Carcinoma Cells. *American Journal of Pathology*, **144**, 1058-1067.

Xie, B., Laouar, A., Huberman, E. (1998). Fibronectin-mediated cell adhesion is required for induction of 92 kDa Type IV Collagenase/Gelatinase (MMP-9) gene expression during Macrophage differentiation. *Journal Biological Chemistry*, **273**, 11576-11582.

Xu, L.H., Owens, L.V., Sturge, G.C., Yang, X.H., Liu, E.T., Craven, R.J. & Cance, W.G. (1996). Attenuation of the expression of the Focal Adhesion Kinase induces apoptosis in tumor cells. *Cell Growth & Differentiation*, **7**, 413-418.

Xue, W., Mizukami, I., Todd, R.F. & Petty, H.R. (1997). Urokinase-type Plasminogen Activator Receptors associate with beta 1 and beta 3 integrins of Fibrosarcoma cells: Dependence on Extracellular Matrix components. *Cancer Research*, **57**, 1682-1689.

Yamamoto, M., Mohanam, S., Sawaya, R., Fuller, G.N., Seiki, M., Sato, H., Gokaslan, Z.L., Liotta, L.A., Nicolson, G.L. & Rao, J.S. (1996). Differential expression of Membrane-Type Matrix Metalloproteinase and its correlation with Gelatinase A activation in Human Malignant Brain Tumors in vivo and in vitro. *Cancer Research*, **56**, 384-392.

Yauch, R.L., Felsenfeld, D.P., Kraeft, S.K., Chen, L.B., Sheetz, M.P. & Hemler, M.E. (1997). Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. *Journal of Experimental Medicine*, **186**, 1347-1355.

Yebra, M., Parry, G.C.N., Stromblad, S., Mackman, N., Rosenberg, S., Mueller, B.M. & Cheresh, D.A. (1996). Requirement of receptor-bound Urokinase-type Plasminogen Activator for integrin alpha v beta 5-directed cell migration. *Journal of Biological Chemistry*, **271**, 29393-29399.

Yoshiji, H., Gomez, D.E. & Thorgeirsson, U.P. (1996). Enhanced RNA expression of Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) in Human Breast Cancer. *International Journal of Cancer*, **69**, 131-134.

Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T. & Hirohashi, S. (1995). Silencing of the E-Cadherin Invasion-Suppressor gene by Cpg Methylation in Human Carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 7416-7419.

Yoshizaki, T., Sato, H., Maruyama, Y., Murono, S., Furukawa, M., Park, C.S. & Seiki, M. (1997). Increased expression of Membrane Type 1-Matrix Metalloproteinase in Head and Neck Carcinoma. *Cancer*, **79**, 139-144.

Yu, M., Sato, H., Seiki, M. & Thompson, E.W. (1995). Complex Regulation of Membrane-Type Matrix Metalloproteinase Expression and Matrix Metalloproteinase-2 Activation By Concanavalin A in MDA-MB-231 Human Breast Cancer Cells. *Cancer Research*, **55**, 3272-3277.

Zhu, X.Y. & Assoian, R.K. (1995). Integrin-Dependent Activation of MAP Kinase - a Link to Shape-Dependent Cell Proliferation. *Molecular Biology of the Cell*, **6**, 273-282.

Ziyadeh, F., Sharma, K., Ericksen, M., Wolf, G. (1994). Stimulation of Collagen gene expression and protein synthesis in Murine Mesangial cells by high glucose is mediated by autocrine activation of TGFβ. *Journal Clinical Investigation*, **93**, 536-542.

Zutter, M.M., Mazoujian, G. & Santoro, S.A. (1990). Decreased Expression of Integrin Adhesive Protein Receptors in Adenocarcinoma of the Breast. *American Journal of Pathology*, **137**, 863-870.

Zutter, M.M., Santoro, S.A., Staatz, W.D. & Tsung, Y.L. (1995). Reexpression of the alpha 2 beta 1 Integrin Abrogates the Malignant Phenotype of Breast Carcinoma Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 7411-7415.

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

Publications Relating to Thesis

Original Work:

J.L.Jones, J.E.Royall & R.A.Walker

E-Cadherin expression relates to EGF-R expression and lymph node status in primary breast carcinoma.

British Journal Cancer, 74, 1237-1241, 1996.

J.L.Jones, J.E.Royall, D.R.Critchley & R.A.Walker

Modulation of myoepithelial-associated α6β4 integrin in a breast cancner cell line alters invasive potential

Experimental Cell Research, 235, 325-333, 1997.

J.L.Jones, P. Glynn & R.A.Walker

Expression of Matrix Metalloproteinase (MMP) –2 and MMP-9, their inhibitors and the activator MT1-

MMP in primary breast cancer.

Journal Pathology (in press, October 1999)

Invited Reviews:

J.L.Jones, & R.A.Walker

Mechanisms of control of Matrix Metalloproteinases in cancer.

Journal Pathology, 183, 377-379, 1997.

J.L.Jones, & R.A.Walker

Cell-cell and cell-stromal interactions in breast cancer invasion and metastasis.

International Journal Oncology, 11, 609-616, 1997.

R.A.Walker, J.L.Jones, S. Chappell, T. Walsh & J.A.Shaw

Molecular pathology of breast cancer and its application to clinical management.

Cancer Metastses Reviews, 16, 2-27, 1997.

J.L.Jones, & R.A.Walker

Integrins: a role as cell signalling molecules.

Clinical pathology, molecular pathology (in press)

Abstracts:

J.L.Jones, J.E.Royall & R.A.Walker

Confocal and ultrastructural localisation of α6β4 integrin in normal, benign and malignant breast.

Journal Pathology, 175S; 141, 1995.

J.L.Jones, & R.A.Walker

Normal breast myoepithelial cells modulate expression of MMP-2 and MT1-MMP in fibroblasts and breast

cancer cell lines.

Journal Pathology, 179; 2A, 1996

J.L.Jones, J.A.Shaw, H.J.Pringle & R.A.Walker

E-Cadherin modulates MMP-9 expression in breast cancer cell lines.

Journal Pathology, 2A, 1999.

Manuscripts in Preparation:

J.L.Jones, J.A.Shaw, H.J.Pringle & R.A.Walker

Normal breast myoepithelial cells modulate expression of gelatinases in fibroblasts and breast cancer cell

lines and inhibit invasion.

J.L.Jones, J.A.Shaw, H.J.Pringle & R.A.Walker

E-Cadherin modulates MMP-9 expression in breast cancer cell lines.