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
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 Professor 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

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiii</td>
</tr>
</tbody>
</table>

Chapter 1: Introduction

1.1 Breast cancer - the Problem 1-59

1.2 Development, Structure and Function of the Normal Breast 2

1.2.1 Embryogenesis and Early Breast Development 3

1.2.2 Adolescent Breast Development 3

1.2.3 The Adult Breast 4

1.2.4 Cytical Variations in the Breast 5

1.3 Breast Cancer 6

1.3.1 Classification of Malignant Breast Disease 6

1.3.2 Histological Grading of Tumours 8

1.3.3 Staging of Breast Cancers 9

1.4 Tumour invasion and Metastasis 10

1.5 Proteolytic Remodelling of the Extracellular Matrix 12

1.5.1 Serine Proteinases 12

1.5.2 Cysteine Proteinases 14

1.5.3 Matrix Metalloproteinases 15

1.5.3.1 Domain Structure of MMP Family 18

1.5.3.2 Structural Basis of MMP Activation - the cysteine switch mechanism 20

1.5.3.3 Control of MMP Activity 22

Tissue Inhibitors of Metalloproteinases (TIMPs) 22

Regulation of Gene Transcription 25

Pro-enzyme Activation 28

1.5.4 MT-MMPs-a new family of MMPs 29

1.5.4.1 Regulation of MT-MMP Expression 30

Regulation of Gene Expression 30

Post-translational Regulation 30

1.5.4.2 Mechanism of pro-MMP-2 Activation via MT-MMP 31

1.5.4.3 Interaction of MT1-MMP with other MMPs 32

1.5.5 The Role of MMPs in Cancer 34

1.6 Cell Adhesion Molecules 35

1.6.1 Cell-Cell Adhesion 36

1.6.1.1 Cadherins 36

1.6.1.2 Regulation of Cadherin Function: interaction with catenins 38

1.6.2 Cell-Matrix Adhesion 41

1.6.2.1 Integrins 41

1.6.2.2 Signalling by Integrin Receptors 45

1.6.3 The Role of E-Cadherin and Integrins in Cancer 47

1.6.3.1 E-Cadherin 47
Chapter 2: Expression of Matrix Metalloproteinases and Cell Adhesion Molecules in Normal and Malignant Breast

2.1 Introduction
2.2 Materials
2.2.1 Tissue
2.2.2 Antibodies
2.2.3 Western Blotting
2.2.4 Zymography
2.2.5 Northern Blotting and In-situ Hybridisation
2.2.6 Chemicals and Miscellaneous
2.3 Methods
2.3.1 Western Blotting
2.3.2 Haematoxylin and Eosin staining of Tissue Sections
2.3.3 Immunohistochemistry
2.3.4 Zymographic Analysis of Breast Tissue
2.3.5 In-Situ Hybridisation
2.4 Results
2.4.1 Western Blotting
2.4.2 Immunohistochemistry
2.4.3 Zymographic Analysis of Breast Tissue
2.4.3.1 Determination of Linear Range 110
2.4.3.2 Assessment of Reproducibility 111
2.4.3.3 Correlation between MMP-2 Activity and Immunohistochemical Pattern of Expression of MMP-2 and MT1-MMP 112
2.4.4 In-Situ Hybridisation for MMP-9 and MT1-MMP 114
2.4.4.2 Labelling of Probes and Northern Blot Analysis 114
2.4.4.3 In-Situ Hybridisation 115

2.5 Summary of Results 119

2.6 Discussion 121
2.6.1 Discussion of Techniques 121
2.6.1.1 Antigen Retrieval 121
2.6.1.2 Substrate gel Zymography 122
2.6.1.3 In-Situ Hybridisation 123

2.6.2 Discussion of Results 125
2.6.2.1 Expression of MMPs and TIMPs in Normal Breast Tissue 125
2.6.2.2 Expression of MMP-2, MT1-MMP and TIMP-2 in Breast Carcinomas 125
2.6.2.3 Expression of MMP-9 and TIMP-1 in Breast Carcinomas 127
2.6.2.4 Relationship between Immunohistochemical Expression of MMP-2 and MT1-MMP and Enzyme Activity 128
2.6.2.5 Localisation of MMP-9 and MT1-MMP by In-Situ Hybridisation 129
2.6.2.6 Expression of Cell Adhesion Molecules in Normal/Benign Breast 131
2.6.2.7 Expression of Integrins in Breast Carcinomas 132
2.6.2.8 Expression of E-Cadherin in Breast Carcinomas 136
2.6.2.9 Relationship between expression of Cell Adhesion Molecules and MMP/TIMPs 140

2.7 Conclusions 143

Chapter 3: In-Vitro Studies of the Relationship between MMP Expression, Invasion and Cell Adhesion Molecule expression 145-219

3.1 Introduction 146
3.2 Materials 150
3.2.1 Cell Lines 150
3.2.2 Tissue Culture Reagents 150
3.2.3 Antibodies 151
3.2.4 Oligonucleotide Primers and Probes 152
3.2.5 Chemicals 152
3.2.6 Molecular Biology Reagents 153
3.2.7 Miscellaneous reagents 153

3.3 Methods 155
3.3.1 Culture 155
3.3.1.1 Cell Line Banking 155
3.3.1.2 Maintenance of Cell lines 155
3.3.1.3 Passage of cell Lines 155
3.3.1.4 Cell Freezing 156
3.3.1.5 Culture of Cells under Serum-free Conditions 156

3.3.2 Invasion Assays 156
3.3.3 Reverse-Transcription – Polymerase Chain Reaction 158
3.3.3.1 mRNA Extraction 158
3.3.3.2 Reverse-Transcription – Polymerase Chain Reaction 159
3.3.3.3 ELOSA Quantitation of PCR Products 160

3.3.4 Culture of Cells under Different Conditions 164
3.3.4.1 Culture with TGF-β1 164
3.3.4.2 Culture at Different Cell Density 164
3.3.4.3 Antibody Blocking Studies 165
3.3.5 Immunohistochemistry on Cell Lines
  3.3.5.1 Single-Labeling Immunofluorescence
  3.3.5.2 Dual Staining for Integrins and Actin Cytoskeleton
  3.3.5.3 Cytoskeletal Solubilisation Procedure
3.3.6 Western Blotting
3.3.7 Substrate Gel Zymography
3.3.8 Transmission Electron Microscopy
3.4 Results
  3.4.1 Invasive Behaviour of Breast Cancer Cell Lines
    3.4.1.1 Optimisation of Invasion Assay
    3.4.1.2 Analysis of Invasive Behaviour by Breast Cancer Cell Lines
  3.4.2 Pattern of MMP and TIMP Expression by Breast Cancer Cell Lines
  3.4.3 Characterisation of Breast Cancer Cell Lines
    3.4.3.1 Expression of Cell Adhesion Molecules by Breast Cancer Cell Lines
    3.4.3.2 Expression of Other Markers by Breast Cancer Cell Lines
  3.4.4 Relationship between E-Cadherin Expression, Invasive Behaviour and Expression of MMPs and TIMPs
    3.4.4.1 Analysis of Level of Gene expression by RT-PCR and ELOSA Quantitation
    3.4.4.2 Analysis of Enzyme Activity
    3.4.4.3 Altered E-Cadherin-Mediated Adhesion and MMP-9 Expression is Associated with Enhanced Invasion
  3.4.5 Modulation of α6β4 Integrin
  3.4.6 Changes in Level of β4 Integrin Protein in TGF-β1-Treated cells
  3.4.7 Relationship between α6β4 Integrin Expression and Invasive Behaviour
  3.4.8 Cytoskeletal Interactions of α6β4 Integrin in MDA-MB 231 Cells
  3.4.9 Relationship between Altered Invasion in TGF-β1-Treated Cells and Expression of MMPs, MT1-MMP and TIMPs
3.5 Summary of Results
3.6 Discussion
  3.6.1 Discussion of Techniques
    3.6.1.1 Invasion Assay
    3.6.1.2 RT-PCR-ELOSA
    3.6.1.3 Cytoskeletal Solubilisation Experiments
    3.6.2 Characteristics of Cell Lines
    3.6.2.2 Relationship between E-Cadherin Expression, Expression of MMP-9 and Invasive Behaviour
    3.6.2.3 Modulation of Expression of Cell Adhesion Molecules in Breast Cell Lines
    3.6.2.4 Role of α6β4 Integrin in Invasion
    3.6.2.5 Cytoskeletal Interactions of α6β4 Integrin
    3.6.2.6 What Factors Control Localisation and Function of α6β4 Integrin?
    3.6.2.7 Relationship between Altered Invasion of TGF-β1-Treated Cells and Expression of MMPs and TIMPs
3.7 Conclusions

Chapter 4: The Effect of Cell Interactions on Tumour Cell Invasion and on MMP Expression

4.1 Introduction
4.2 Materials
  4.2.1 Normal Breast Tissue
  4.2.2 Tissue Culture Reagents
  4.2.3 Materials for Cell Separation
List of Figures

Fig. 1.1: Structure of Normal Adult Breast 4
Fig. 1.2: Schematic Model Outlining Putative Steps of Tumour Invasion and Metastasis 11
Fig. 1.3: The Urokinase Plasminogen Activator System. 13
Fig. 1.4: Domain Structure of Matrix Metalloproteinases. 19
Fig. 1.5: Cysteine-Switch Mechanism for Activation of MMPs. 21
Fig 1.6: Interaction of TIMP-2 with Latent MMP-2 and other Active MMPs 24
Fig. 1.7: Regulatory Elements of Promotor Regions of Human MMP Genes. 26
Fig. 1.8: Mechanism of MT1-MMP-mediated activation of pro-MMP-2 32
Fig. 1.9: Matrix Metalloproteinase Activation Cascades 33
Fig. 1.10: Structure of E-Cadherin and Interaction with Catenins and Actin Cytoskeleton 37
Fig. 1.11: Regulation of Cadherin – Catenin Complex. 40
Fig. 1.12: Structure and Cytoskeletal Interactions of Integrin Heterodimers. 44
Fig. 1.13: Integrin Signalling Mechanisms. 46
Fig. 1.14: Physiological Invasion – Placental Implantation 54
Fig. 1.15: Pathological Invasion – Progression of Malignant Melanoma. 57
Fig. 2.1: Western Blot Analysis of MMP/TIMP Antibodies. 87
Fig. 2.2: Expression of MMPs and TIMPs in Normal Breast 92
Fig. 2.3: Expression of MMP-2, MT1-MMP and TIMP-2 in Breast Carcinoma 94
Fig. 2.4: Relationship between membrane MMP-2 and membrane MT1-MMP staining and lymph node status. 95
Fig. 2.5: Relationship between membrane staining for MT1-MMP and MMP-2(os). 96
Fig. 2.6: Relationship between membrane MT1-MMP and membrane MMP-2 (ss). 96
Fig. 2.7: Expression of MMP-9 and TIMP-1 in Breast Carcinoma 97b
Fig. 2.8: Relationship between Tumour Cell Staining for MMP-9 and Tumour Type. 99
Fig. 2.9: Expression of Cell Adhesion Molecules in Breast Carcinoma 100
Fig. 2.10: Expression of Integrins in Breast Carcinoma 102
Fig. 2.11: Relationship between Membrane Reactivity for α2, α3, and β1 Integrins and Tumour Grade. 104
Fig.2.12a: The relationship between membrane and cytoplasmic α6 integrin and tumour grade. 105
Fig. 2.12b: The relationship between membrane and cytoplasmic β4 integrin and tumour grade. 105
Fig. 2.13: Expression of E-Cadherin in Breast Carcinoma 108
Fig 2.14: Relationship between Extent of Tumour Cell Membrane and Tumour Cell Cytoplasmic E-Cadherin and Lymph Node Status. 109
Fig. 2.15: Relationship between Integrated Density (ID) Readings for (i) MMP-2 and (ii) MMP-9 and Concentration of Purified Enzyme. 111
Fig. 2.16: Substrate Gel Zymogram of Primary Breast Carcinomas. 113
Fig. 4.4: Invasion Assay Modified for use with Co-cultures
Fig. 4.5: RT-PCR Analysis of Separated Cell Populations
Fig. 4.6: Characteristics of β4-Integrin Selected Cells
Fig. 4.7: Characteristics of Negatively Selected Cells
Fig. 4.8: Effect of Co-culture with Fibroblasts on Tumour Cell Invasion.
Fig. 4.9: Comparison of Invasion Assays Performed over 24 and 48 hours.
Fig. 4.10: Effect of Tumour Cell-Primary Fibroblast Co-culture on Tumour Cell Expression of MMP-2
Fig. 4.11: Effect of Tumour Cell – Fibroblast Co-culture on Tumour Cell MT1-MMP Expression.
Fig. 4.14: Substrate Gel Zymogram Demonstrating Change in Enzyme Activity on Tumour Cell-Primary Fibroblast Co-culture.
Fig. 4.12: Effect of Tumour Cell-Fibroblast Co-culture on Fibroblast MMP-2 and MT1-MMP Expression.
Fig. 4.13: Effect of Tumour Cell-Fibroblast Co-culture on Fibroblast TIMP Expression.
Fig. 4.15: Effect of Myoepithelial cells on Tumour Cell Invasion
Fig. 4.16: Effect of Myoepithelial Cells on Tumour Cell Invasion in Co-culture with a) F518 dermal fibroblasts and b) primary breast fibroblasts.
Fig 4.17: Effect of Myoepithelial Cells on Tumour Cell Gene Expression
Fig 4.18: Effect of Myoepithelial Cells on Fibroblast Gene Expression.
Fig 4.19: Effect of Myoepithelial Cells on Fibroblast Gene Expression.
Fig. 4.20: Substrate Gel Zymogram on Conditioned Medium from Control
Fig. 4.21: Agarose Gel Electrophoresis for RT-PCR Products from Myoepithelial Cells.
Fig. 4.22: Substrate Gel Zymogram on Conditioned Medium from Myoepithelial Cells.
List of Tables

Table 1.1: Histological Classification of Malignant Breast Disease, Relative Frequency and Survival Rates 7
Table 1.2: Five Year Survival Rates (%) in Relation to Tumour Size and Lymph Node Status 9
Table 1.3: Classification of Matrix Metalloproteinases and their Substrate Specificity 16-17
Table 1.4: Tissue Inhibitors of Metalloproteinases (TIMPs) 23
Table 1.5: Classes of Cell Adhesion Molecules. Their Ligands and Tissue Distribution 36
Table 1.6: The Integrin Family of Receptors and their Ligands 43
Table 2.1: Summary of Different Techniques for Detecting and Measuring Proteolytic Activity 64
Table 2.2: Clinicopathological Features of Carcinomas Studied 65
Table 2.3: Details of oligonucleotide Probes for In-situ Hybridisation 71
Table 2.4: Conditions Tested for In-Situ Hybridisation on HT 1080 Cells and on Primary Tissue 85
Table 2.5: Concentration of Primary Antibodies used for Western Blotting 86
Table 2.6: Concentration of Primary Antibodies used for Immunohistochemistry 90
Table 2.7: Summary of extent and pattern of staining for MMP-2, MT1-MMP and TIMP-2 in Carcinomas 93
Table 2.8: Summary of Extent and Pattern of Staining for MMP-9 and TIMP-1 in Carcinomas 98
Table 2.8b: Summary of Extent and Pattern of Staining for Integrins in Carcinomas 103
Table 2.10: Summary of Reactivity for E-Cadherin in Relation to Tumour Type, Grade and Lymph Node Status 107
Table 2.11: Integrated Density Readings for pro-MMP-2 on Four Separate Substrate Gels 112
Table 2.12: Comparison of Immunohistochemistry and In-Situ Hybridisation for MMP-9 and MT1-MMP 117
Table 3.1: Oligonucleotide Primer and Probe Sequences 153
Table 3.2: Dilutions of Primary Antibodies used for Immunofluorescence 167
Table 3.3: Markers Expressed by Breast Cancer Cell Lines 176
Table 3.4: Assessment of Reproducibility of RT-PCR-ELOSA 183
Table 3.5: Effect of TGF-β1 on Level of MMP and TIMP Gene Expression in MDA-MB 231 and MCF-7 199
Table 4.1: Primer Sequences for PCR 226
Table 4.2: Range of antibody-labelled beads used for cell separation 228
Table 4.3: Details of Antibodies used to characterise Bead-Separated Cell Populations 232
Table 4.4: Combinations of Co-cultures Performed 233
Table 4.5: Effect of Tumour Cell – Fibroblast Co-culture on Tumour Cell MMP and TIMP Expression 245
Table 4.6: Effect of Tumour Cell – Fibroblast Co-culture on Fibroblast Gene Expression 247
Table 4.7: Effect of Tumour Cell Co-culture with Primary Fibroblasts on Enzyme Activity 249
Table 4.8: Effect of Myoepithelial Cells on Tumour Cell Gene Expression in Tumour-Fibroblast Co-cultures 255
Table 4.9: Effect of Myoepithelial Cells on Fibroblast Gene Expression in Tumour Cell – Fibroblast Co-Cultures 259
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidin-Biotin-Complex</td>
</tr>
<tr>
<td>AJ</td>
<td>Alec Jeffries</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein - 1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>Bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum Albumin</td>
</tr>
<tr>
<td>CALLA</td>
<td>Common Acute Lymphoblastic Leukaemia Antigen</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Medium</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CTB</td>
<td>Cytotrophoblast</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-Deoxyadenosine 5'-Triphosphate</td>
</tr>
<tr>
<td>dd</td>
<td>Deionised</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2' Deoxynucleotide 5'-Triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>2' Deoxyuridine 5'Triphosphate</td>
</tr>
<tr>
<td>E-CD</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium Ethylene Diamine Tetraacetate</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EHS</td>
<td>EngelbrothHolm Swarm</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>ELOSA</td>
<td>Enzyme Linked Oligonucleotide Assay</td>
</tr>
<tr>
<td>EMA</td>
<td>Epithelial Membrane Antigen</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>Extracellular MMP Inducer</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen Receptor</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluoro-isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde -3- Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GLB</td>
<td>Gel Loading Buffer</td>
</tr>
<tr>
<td>HBAR</td>
<td>Heat Based Antigen Retrieval</td>
</tr>
<tr>
<td>HD1</td>
<td>Hemidesmosome 1/Plectin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSB</td>
<td>High Salt Buffer</td>
</tr>
<tr>
<td>ID</td>
<td>Integrated Density</td>
</tr>
<tr>
<td>IDC</td>
<td>Infiltrating Ductal Carcinoma</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate Filaments</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ILC</td>
<td>Infiltrating Lobular Carcinoma</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular Carcinoma in-situ</td>
</tr>
<tr>
<td>LMP</td>
<td>Last Menstrual Period</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino] ethanesulfonic acid</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MPC</td>
<td>Magnetic Particle Concentrator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane Type- Metalloproteinase</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NRS</td>
<td>Normal Rabbit Serum</td>
</tr>
<tr>
<td>NSS</td>
<td>Normal Swine Serum</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly Acrylimide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth Muscle Actin</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus Aquaticus</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
</tr>
<tr>
<td>TRITC</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
</tbody>
</table>
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.
Chapter 1: Introduction

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).

![Structure of Normal Adult Breast](image-url)
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
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.

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

NST : No Special Type
NA: Not Appropriate

*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>
<thead>
<tr>
<th>LN STATUS</th>
<th>SIZE (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5</td>
</tr>
<tr>
<td>Negative</td>
<td>99.2</td>
</tr>
<tr>
<td>1-3 nodes positive</td>
<td>95.3</td>
</tr>
<tr>
<td>4+ nodes positive</td>
<td>59.0</td>
</tr>
<tr>
<td>Total</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Taken from: Carter et al, 1989.
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.
Chapter 1: Introduction

Fig. 1.2: Schematic Model Outlining Putative Steps of Tumour Invasion and Metastasis

(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)
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.
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).

![Figure 1.3: The Urokinase Plasminogen Activator System.](image)

**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-antiplasmin 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.
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...
### Table 1.3: Classification of Matrix Metalloproteinases and their Substrate Specificity

<table>
<thead>
<tr>
<th>ENZYME</th>
<th><em>MW(kDa)</em></th>
<th>MMP</th>
<th>SUBSTRATE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial collagenase</td>
<td>52</td>
<td>MMP-1</td>
<td>Collagens I, II, III, VII, X</td>
<td>Goldberg et al, 1986</td>
</tr>
<tr>
<td>Polymorphonuclear collagenase</td>
<td>85</td>
<td>MMP-8</td>
<td>Collagens I, II, III</td>
<td>Hasty et al, 1990</td>
</tr>
<tr>
<td>Collagenase-3</td>
<td>60</td>
<td>MMP-13</td>
<td>Collagen II (I &amp; II), denatured collagens &amp; aggrecan</td>
<td>Freij e et al, 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Knauper et al, 1997</td>
</tr>
<tr>
<td>72kDa Gelatinase (Type IV collagenase)</td>
<td>72</td>
<td>MMP-2</td>
<td>Denatured collagens</td>
<td>Collier et al, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Native collagens IV, V</td>
<td>Seltzer et al, 1989</td>
</tr>
<tr>
<td>92kDa gelatinase (Type IV collagenase)</td>
<td>92</td>
<td>MMP-9</td>
<td>Denatured collagens</td>
<td>Wilhelm et al, 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Native collagens IV</td>
<td></td>
</tr>
<tr>
<td>Stromelysin-1</td>
<td>57</td>
<td>MMP-3</td>
<td>Proteoglycan core protein, fibronectin, laminin, denatured collagens. Collagen IV, V, IX, X</td>
<td>Chin et al, 1985</td>
</tr>
<tr>
<td>Stromelysin-2</td>
<td>53</td>
<td>MMP-10</td>
<td>Denatured collagens</td>
<td>Breathnach et al, 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Collagens III, IV, V (weak)</td>
<td>Muller et al, 1988</td>
</tr>
<tr>
<td>Stromelysin-3</td>
<td>61</td>
<td>MMP-11</td>
<td>Fibronectin &amp; Laminin</td>
<td>Basset et al, 1990</td>
</tr>
<tr>
<td>Matrilysin (PUMP-1)</td>
<td>28</td>
<td>MMP-7</td>
<td>Proteoglycan core protein, fibronectin, laminin, denatured collagens. Collagen IV, V, IX, X</td>
<td>Quantin et al, 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Miyazaki et al, 1990</td>
</tr>
<tr>
<td>Metalloelastase</td>
<td>54</td>
<td>MMP-12</td>
<td>Elastin</td>
<td>Senior et al, 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Senior et al, 1989</td>
</tr>
<tr>
<td>MMP-19</td>
<td>57</td>
<td>MMP-19</td>
<td>Stromelysin-like profile</td>
<td>Pendas et al, 1997</td>
</tr>
<tr>
<td>Enamelysin</td>
<td>54</td>
<td>MMP-20</td>
<td>Amelogenin</td>
<td>Llano et al, 1997</td>
</tr>
</tbody>
</table>
Table 1.3: Classification of Matrix Metalloproteinases and their Substrate Specificity (contd)

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>*MW (kDa)</th>
<th>MMP</th>
<th>SUBSTRATE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Type 2-MMP</td>
<td>64</td>
<td>MMP-15</td>
<td>Activates pro-MMP-2&lt;br&gt; Laminin</td>
<td>Will &amp; Hinzmannnn, 1995&lt;br&gt; dOrtho et al, 1997</td>
</tr>
<tr>
<td>Membrane Type 3-MMP</td>
<td>64</td>
<td>MMP-16</td>
<td>Activates pro-MMP-2</td>
<td>Takino et al, 1995</td>
</tr>
<tr>
<td>Membrane Type 4-MMP</td>
<td>70</td>
<td>MMP-17</td>
<td>?activates pro-MMP-2</td>
<td>Puente et al, 1996</td>
</tr>
<tr>
<td>Membrane Type 5-MMP</td>
<td>65</td>
<td>MMP-21</td>
<td>activates pro-MMP-2</td>
<td>Llano et al, 1999&lt;br&gt; Pei, 1999</td>
</tr>
</tbody>
</table>

et al, 1986; Wilhelm et al, 1987). Although structurally distinct from the stromelysins, *matri ly sin* (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.](image)

Only MMP-2 and MMP-9 possess the fibronectin type II-like repeats, and only MMP-9 has an \( \alpha_2 \)-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
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 $\alpha_2$ 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).

![Diagram of Cysteine-Switch Mechanism for Activation of MMPs]

**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)

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

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.
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,
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
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).

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).
Chapter 1: Introduction

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

<table>
<thead>
<tr>
<th>FAMILY</th>
<th>EXAMPLES</th>
<th>LIGANDS</th>
<th>DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadherins</td>
<td>E-Cadherin</td>
<td>Homophilic</td>
<td>Epithelia</td>
</tr>
<tr>
<td></td>
<td>P-Cadherin</td>
<td></td>
<td>Placenta, skin, epithelia</td>
</tr>
<tr>
<td></td>
<td>N-Cadherin</td>
<td></td>
<td>Neural &amp; mesenchymal tissue</td>
</tr>
<tr>
<td></td>
<td>Desmosomal Cadherins</td>
<td></td>
<td>Skin, epithelia</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>ICAM-1</td>
<td>β2 integrins</td>
<td>Endothelium, fibroblasts</td>
</tr>
<tr>
<td></td>
<td>V-CAM</td>
<td>VLA-4 integrin</td>
<td>Endothelium</td>
</tr>
<tr>
<td></td>
<td>N-CAM</td>
<td>homophilic</td>
<td>Neural &amp; mesenchymal tissue</td>
</tr>
<tr>
<td>Integrins</td>
<td>β1 integrins (VLA-1-8)</td>
<td>Extracellular matrix</td>
<td>Widespread - epithelia, mesenchymal</td>
</tr>
<tr>
<td></td>
<td>β2 integrins (LFA-1)</td>
<td>ICAM-1</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>Selectins</td>
<td>L-Selectin</td>
<td>MadCAM, CD34</td>
<td>Leukocytes</td>
</tr>
<tr>
<td></td>
<td>E-Selectin</td>
<td>Sialyl Lewis α</td>
<td>Activated endothelium</td>
</tr>
<tr>
<td></td>
<td>P-Selectin</td>
<td>PSGL</td>
<td>Platelets, endothelium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sialyl Lewis x (all)</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>Multiple isoforms due to alternative splicing</td>
<td>Hyaluronic acid, col, ln, homotypic.</td>
<td>Leukocytes (90kDa variant) Epithelial &amp; mesenchymal cells (160kDa variant)</td>
</tr>
</tbody>
</table>

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).
Chapter 1: Introduction

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-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, 1999).

![Fig. 1.10: Structure of E-Cadherin and Interaction with Catenins and Actin Cytoskeleton](image-url)

The text contains a cell death programme, indicating a link between Cadherin-mediated adhesive interactions and the polarized phenotype in Caenorhabditis elegans. The text relevant for our understanding of the extracellular domain structure and function in particular the effects of mutants on cell adhesion and migration was provided by Takeichi et al, 1995. The text relevant for our understanding of the extracellular domain structure and function in particular the effects of mutants on cell adhesion and migration was provided by Takeichi et al, 1995.
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 p120CAS. 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;...
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). p120CAS 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
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 $\alpha_4\beta_1$ and $\alpha_5\beta_1$ receptors (Guan & Hynes, 1990), whereas in other cases the integrins bind to the same region of the protein, e.g. the binding of $\alpha_5\beta_1$ and $\alpha_3\beta_1$ 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 $\alpha$ and $\beta$ subunits possess large extracellular domains - 675-700 amino acids in $\beta$ chains, 950-1100 amino acids in $\alpha$ chains. Each $\beta$ subunit contains 56 cysteine residues, which suggests a highly folded conformation, and in the N-terminal domain there is extensive internal disulfide bonding. In $\alpha$ 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 $\alpha$ 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 $\beta_4$ subunit which is 1000 amino acids long, the $\beta$ chain
### Table 1.6: The Integrin Family of Receptors and their Ligands

<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>LIGAND</th>
<th>DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1 VLA-1</td>
<td>α1β1</td>
<td>Ln, Col</td>
</tr>
<tr>
<td>VLA-2</td>
<td>α2β1</td>
<td>Ln, Col</td>
</tr>
<tr>
<td>VLA-3</td>
<td>α3β1</td>
<td>Ln, Col, Fn, Epiligrin</td>
</tr>
<tr>
<td>α3ββ1</td>
<td>Fn (V25), VCAM-1</td>
<td>B &amp; T cells, mac. neural crest cells.</td>
</tr>
<tr>
<td>VLA-4</td>
<td>α4β1</td>
<td></td>
</tr>
<tr>
<td>VLA-5</td>
<td>α5β1</td>
<td>Fn (RGD)</td>
</tr>
<tr>
<td>VLA-6</td>
<td>α6Aβ1</td>
<td>Ln</td>
</tr>
<tr>
<td>α6ββ1</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>VLA-7</td>
<td>α7Aβ1</td>
<td>Ln</td>
</tr>
<tr>
<td>α7ββ1</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>VLA-8</td>
<td>α8β1</td>
<td>Fn, Vn</td>
</tr>
<tr>
<td>αVβ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2 LFA-1</td>
<td>αL β2</td>
<td>ICAM-1, ICAM-2, ICAM-3</td>
</tr>
<tr>
<td>CR3, Mac-1</td>
<td>αM β2</td>
<td>C3bi, factor X, Fbn</td>
</tr>
<tr>
<td>p150,95</td>
<td>αX β2</td>
<td></td>
</tr>
<tr>
<td>β3 gpIIb/IIIa</td>
<td>αIIb β3</td>
<td>Fbn, Fn, vW factor, Vn, Thrsp</td>
</tr>
<tr>
<td>VNR</td>
<td>αV β3</td>
<td>Fbn, Fn, vW factor, Vn, Thrsp, osteopontin</td>
</tr>
<tr>
<td>β4</td>
<td>α6A β4</td>
<td>Laminin 5</td>
</tr>
<tr>
<td>α6B β4</td>
<td>Laminin 5</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>β5</td>
<td>αV β5</td>
<td>Vn</td>
</tr>
<tr>
<td>β6</td>
<td>αV β6</td>
<td>Fn</td>
</tr>
<tr>
<td>β7</td>
<td>α4 β7, αE β7</td>
<td>Fn, VCAM-1, MadCAM-1, E-Cadherin</td>
</tr>
<tr>
<td>β8</td>
<td>αE β8</td>
<td>?</td>
</tr>
<tr>
<td>α9 β8</td>
<td></td>
<td>?</td>
</tr>
</tbody>
</table>

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 
\( \beta_4 \) subunit is entirely novel having a larger cytoplasmic domain compared to other \( \beta \) chains, and linking to intermediate filaments rather than to the actin cytoskeleton. In contrast to the sequence similarities seen among the \( \beta \) chains, the \( \alpha \) 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. \( \alpha 6\beta 4 \) 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).
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
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
\( \alpha 5 \beta 1 \) has been shown to regulate both tumour cell invasion and proliferation. Clones of Chinese hamster ovary (CHO) cells overexpressing \( \alpha 5 \beta 1 \) grow less rapidly in nude mice compared to clones lacking the fibronectin receptor (Schreiner et al, 1989). Furthermore, transfection of CHO cells with \( \alpha 5 \beta 1 \) 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 \( \alpha 5 \beta 1 \) 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 \( \alpha v \beta 3 \) 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 \( \alpha 2 \beta 1 \) 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 \( \alpha v \beta 3 \) integrin and both the vertical growth phase of melanoma and metastasis (Nesbit & Herlyn, 1994). Further evidence for the role of integrins in
malignancy is demonstrated by the specific inhibition of migration and invasion of glioma cells with blocking antibodies to $\alpha 3\beta 1$ 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 $\alpha 2$ integrin, and this effect is abrogated by antisense $\alpha 2$ 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 - $\alpha 4\beta 1$ and $\alpha 5\beta 1$ - co-operate in the control of three MMPs, MMP-1, MMP-3 and MMP-9: cross-linking of $\alpha 5\beta 1$, 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 $\alpha 4\beta 1$ 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
induction of MMP-1 in response to growth in a collagen matrix has been shown to be dependent on α2β1 integrin, and MMP-1 synthesis is inhibited when the cells are transfected with antisense α2 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 α3β1 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 α5β1 integrin and αvβ3 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 β1 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 et 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
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 α6β4 integrin, CTB columns exhibit down-regulation of these cell adhesion molecules and switch on expression of α1β1 and α5β1 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
Chapter 1: Introduction

Floating villus Anchoring villus

Basement membrane
Cytotrophoblast
Syncitiotrophoblast

CTB

Decidual cell

Cytotrophoblast columns (CTB)

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.

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 (MelCAM/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 \(\alpha_2\beta_1\) is frequently up-regulated in malignant melanoma, which reflects in-vitro studies, where \(\alpha_2\beta_1\) expression on melanoma cell lines is associated with enhanced invasion (Klein et al, 1991a; Klein et al, 1991b).

Furthermore, \(\alpha_2\beta_1\) 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 \(\alpha_3\beta_1\) 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 \(\alpha_\nu\beta_3\) integrin and both the vertical growth phase of melanoma and metastasis (Albedo et al, 1990; Nip et al, 1992; Nesbit & Herlyn, 1994), and the presence of \(\alpha_\nu\beta_3\) on melanoma cells correlates with their tumourigenic potential in athymic mice (Cheresh, 1991). Cell culture experiments have shown that interaction of \(\alpha_\nu\beta_3\) 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).
Chapter 1: Introduction

E-Cadherin

Normal melanocyte

Naevus cells

\[ \text{E-Cadherin} \]

\[ \text{Mel Cam} \]

Melanoma cells

\[ \text{E-Cadherin} \]

\[ \text{Mel Cam} \]

Advanced Melanoma cells

(verticall growth phase or metastatic)

\[ \text{MMP-1} \]

\[ \text{MMP-2} \]

\[ \text{MT1-MMP} \]

Vascular endothelium

\[ \text{V-Cam} \]

\[ \alpha 3 \beta 1 \]

\[ \alpha 2 \beta 1 \]

\[ \alpha 4 \beta 1 \]

\[ \alpha 2 \beta 1 \]

\[ \alpha 4 \beta 1 \]

\[ \alpha v \beta 3 \]

\[ \alpha v \beta 3 \]

\[ \text{MT1-MMP} \]

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 αvβ3 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 α4β1 is significantly increased in metastatic melanomas (Schadendorf et al, 1993). As well as binding to fibronectin, the α4β1 integrin is a receptor for VCAM-1 (Vascular Cell Adhesion Molecule), which is expressed by endothelial cells. The α4β1/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.
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

<table>
<thead>
<tr>
<th>TECHNIQUE</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Gel Zymography</td>
<td>• Active &amp; latent enzymes detected • TIMP-complexed enzyme detected • No antibodies required • Sensitive (4 ng/ml)</td>
<td>• Reproducibility requires extreme care • Not specific due to overlapping enzyme activity • Absolute quantitation difficult • Labour intensive</td>
</tr>
<tr>
<td>Radiolabelled substrate assay</td>
<td>• Sensitive (4 ng/ml)</td>
<td>• Poor reproducibility • Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes</td>
</tr>
<tr>
<td>Chromophore assay</td>
<td>• Some methods are highly sensitive</td>
<td>• Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes</td>
</tr>
<tr>
<td>In-situ zymography</td>
<td>• Sensitive • Localisation of enzyme activity</td>
<td>• Difficult to ensure reproducibility • Non-specific due to overlapping enzyme substrate specificities</td>
</tr>
<tr>
<td>ELISA</td>
<td>• Sensitive • Rapid, multiple sample analysis • Absolute quantitation possible</td>
<td>• Does not detect active and latent enzyme in one test • Does not detect enzyme-inhibitor complexes • Dependent on antibody specificity</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>TYPE</th>
<th>GRADE</th>
<th>LN+</th>
<th>LN-</th>
<th>NK</th>
<th>ER+</th>
<th>ER-</th>
<th>NK</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular</td>
<td>I</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>IDC</td>
<td>I</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>IDC</td>
<td>II</td>
<td>13</td>
<td>11</td>
<td>2</td>
<td>22</td>
<td>3</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>IDC</td>
<td>III</td>
<td>21</td>
<td>20</td>
<td>4</td>
<td>15</td>
<td>26</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>ILC</td>
<td>II</td>
<td>12</td>
<td>11</td>
<td>3</td>
<td>20</td>
<td>3</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>ILC</td>
<td>III</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>54</td>
<td>50</td>
<td>10</td>
<td>71</td>
<td>33</td>
<td>10</td>
<td>114</td>
</tr>
</tbody>
</table>

Table 2.2: Clinicopathological features of carcinomas studied
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 Concanavalin 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).
**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,
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

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

#### 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 75 cm³ sterile tissue culture flasks in DMEM without phenol red with 10% FBS and 2 mM L-Glutamine at 37°C in a 5% CO₂ humidified atmosphere. At ~80% confluence, the culture media was aspirated, centrifuged at 10,000 r.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 0.1 μg/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
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
(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 Bartlett's 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,
Chapter 2: MMP and CAM in Breast Tissue

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
Chapter 2: MMP and CAM in Breast Tissue

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 prehybridisation 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>
<thead>
<tr>
<th>VARIABLE</th>
<th>HT 1080</th>
<th>TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe Concentration (ng/ml)</td>
<td>100, 200, 400, 800</td>
<td>100, 200, 400, 800</td>
</tr>
<tr>
<td>Stringency (% Formamide)</td>
<td>20, 30, 50</td>
<td>20, 30, 50</td>
</tr>
<tr>
<td>Protease K Concentration (µg/ml)</td>
<td>None, 2</td>
<td>2, 5, 10</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>ANTIBODY</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 (ss)</td>
<td>1:2500</td>
</tr>
<tr>
<td>MMP-9 (ss)</td>
<td>1:5000</td>
</tr>
<tr>
<td>TIMP-1 (ss)</td>
<td>1:7500</td>
</tr>
<tr>
<td>TIMP-2 (ss)</td>
<td>1:5000</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>1:500</td>
</tr>
</tbody>
</table>

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).

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.

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

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

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 only 10 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
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 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).

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

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

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

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).
Chapter 2: MMP and CAM in Breast Tissue

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).
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 macrophages (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

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

ss : Antibodies from Dr. Stetler-Stevenson, N.I.H., Bethesda
os : Antibodies from Oncogene Science
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-myoeplithelial junction. Staining was not
Fig 2.9: Expression of Cell Adhesion Molecules in Normal Breast

Staining for α2- and α3-integrin is present at the cell-basement membrane junction (arrows top left) and at cell-cell borders. No convincing membrane reactivity is observed for α5-integrin. The arrows indicate non-specific staining of proteinaceous material in gland lumens. Staining for α6-integrin is seen at cell-cell borders with accentuation of reactivity at the cell-basement membrane interface. β1-integrin has a similar distribution to that of α2- and α3-integrins, whilst reactivity for β4-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 α2- and α3-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 (α3-integrin, right panel). Tumour cells do not stain for α5-integrin, but reactivity is observed in stromal fibroblasts around tumour groups. Many tumours lack α6-integrin, staining being observed only in small blood vessels (arrow). Reactivity for β1 is similar to that seen for α2- and α3-integrins, with staining of tumour cell membranes. In those tumours displaying reactivity for β4-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 \( \alpha_2 \) integrin was inversely related to stromal reactivity for \( \alpha_5 \) integrin (p=0.04).

| Table 2.8b Summary of Extent and Pattern of Staining for Integrins in Carcinomas* |
|---------------------------------|---------|---------|---------|---------|---------|
| Staining Pattern               | \( \alpha_2 \) integrin | \( \alpha_3 \) integrin | \( \alpha_6 \) integrin | \( \beta_1 \) integrin | \( \beta_4 \) integrin |
|                                | % (no)  | % (no)  | % (no)  | % (no)  | % (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)|

* \( \alpha_5 \) integrin limited to the stromal compartment (see text).

Both \( \alpha_6 \) and \( \beta_4 \) integrins were less frequently expressed than the other subunits with only 33% staining for \( \alpha_6 \) integrin and 29% of tumours staining for \( \beta_4 \) 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 \( \beta_4 \) integrin lacked \( \alpha_6 \) integrin, and 6 cases were positive for \( \alpha_6 \) integrin but did not express the \( \beta_4 \) 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 \( \alpha_6 \); p= 0.02 and 0.01 for membrane and cytoplasmic
Chapter 2: MMP and CAM in Breast Tissue

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).
Chapter 2: MMP and CAM in Breast Tissue

Fig. 12a: The relationship between membrane and cytoplasmic α6 integrin and tumour grade. A significant association is demonstrated between membrane α6 (p=0.01) and cytoplasmic α6 (p=0.001) and high tumour grade.

Fig. 12b: The relationship between membrane and cytoplasmic β4 integrin and tumour grade. A significant association is demonstrated between presence of membrane β4 (p=0.02) and cytoplasmic β4 (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*

<table>
<thead>
<tr>
<th></th>
<th>Membrane % (no)</th>
<th></th>
<th>Cytoplasmic % (no)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neg.</td>
<td>&lt;20%</td>
<td>20-50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Tubular</td>
<td>1 (1)</td>
<td>0</td>
<td>2 (2)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>IDC</td>
<td>8 (9)</td>
<td>7 (8)</td>
<td>18 (21)</td>
<td>36 (41)</td>
</tr>
<tr>
<td>ILC</td>
<td>24 (27)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade I**</td>
<td>1 (1)</td>
<td>3 (3)</td>
<td>3 (3)</td>
<td>11 (9)</td>
</tr>
<tr>
<td>Grade II</td>
<td>5 (4)</td>
<td>3 (3)</td>
<td>9 (8)</td>
<td>13 (11)</td>
</tr>
<tr>
<td>Grade III</td>
<td>6 (5)</td>
<td>2 (2)</td>
<td>14 (12)</td>
<td>30 (26)</td>
</tr>
<tr>
<td>LN +ve</td>
<td>9 (8)</td>
<td>6 (5)</td>
<td>16 (14)</td>
<td>16 (14)</td>
</tr>
<tr>
<td>LN -ve</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>9 (8)</td>
<td>33 (29)</td>
</tr>
</tbody>
</table>

*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 $\alpha 6$ 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, $\beta 4$ 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 \(\alpha_5\) 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 \((r=-0.187; \ p=0.03, \ \text{and} \ r=-0.180; \ p=0.02 \ \text{for membrane and cytoplasmic staining, respectively})\), and the presence of membrane E-Cadherin was inversely related to the presence of stromal TIMP-1 reactivity \((r=-0.375; \ p=0.0001 \ \text{for TIMP-1(ss)} \ \text{and} \ r=-0.277; \ p=0.0001 \ \text{for TIMP-1(os)})\).

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 \((r= 0.897; \ \text{fig. 2.15})\).
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

<table>
<thead>
<tr>
<th></th>
<th>Gel A</th>
<th>Gel B</th>
<th>Gel C</th>
<th>Gel D</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng pro-MMP-2</td>
<td>250</td>
<td>255</td>
<td>285</td>
<td>265</td>
<td>265</td>
<td>15.5</td>
</tr>
<tr>
<td>10 ng pro-MMP-2</td>
<td>1600</td>
<td>1920</td>
<td>1290</td>
<td>1890</td>
<td>1675</td>
<td>294.4</td>
</tr>
<tr>
<td>50 ng pro-MMP-2</td>
<td>5700</td>
<td>6350</td>
<td>5890</td>
<td>5270</td>
<td>6052</td>
<td>309.0</td>
</tr>
</tbody>
</table>

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 Wilcoxon 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.](image)

![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).](image)
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 12 IDC, 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>
<thead>
<tr>
<th>No</th>
<th>RW No</th>
<th>TYPE</th>
<th>GRDE</th>
<th>MMP-9</th>
<th>MMP-9</th>
<th>MT1-MMP</th>
<th>MT1-MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>628</td>
<td>IDC</td>
<td>II</td>
<td>Neg</td>
<td>Neg</td>
<td>TM 4; S++</td>
<td>T 1; S+++</td>
</tr>
<tr>
<td>2</td>
<td>653</td>
<td>IDC</td>
<td>III</td>
<td>Tcyt 1; S -ve</td>
<td>Neg</td>
<td>TM 2; S ++</td>
<td>Occ T +; S +++</td>
</tr>
<tr>
<td>3</td>
<td>738</td>
<td>TUB</td>
<td>I</td>
<td>Neg</td>
<td>Neg</td>
<td>TM 1; S +</td>
<td>T -ve; S +</td>
</tr>
<tr>
<td>4</td>
<td>812</td>
<td>IDC</td>
<td>III</td>
<td>Neg</td>
<td>Neg</td>
<td>TM 1; S +++</td>
<td>T -ve; S ++</td>
</tr>
<tr>
<td>5</td>
<td>824</td>
<td>IDC</td>
<td>II</td>
<td>Neg</td>
<td>TM 2; S +++</td>
<td>Occ T +; S ++</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>831</td>
<td>IDC</td>
<td>III</td>
<td>Tcyt 2; S +/-</td>
<td>Neg T; S +/-</td>
<td>TM 1; S +++</td>
<td>T -ve; S +++</td>
</tr>
<tr>
<td>7</td>
<td>840</td>
<td>IDC</td>
<td>III</td>
<td>Tcyt 2; S +++</td>
<td>Neg T; S ++</td>
<td>TM 2; S ++</td>
<td>T 1; S ++</td>
</tr>
<tr>
<td>8</td>
<td>845</td>
<td>IDC</td>
<td>I</td>
<td>Tcyt 1; S +</td>
<td>Neg T; S +</td>
<td>TM 2; S +</td>
<td>Occ T +; S +</td>
</tr>
<tr>
<td>9</td>
<td>867</td>
<td>IDC</td>
<td>III</td>
<td>Tcyt 2; S -</td>
<td>Occ T +</td>
<td>T -ve; S +++</td>
<td>T -ve; S +++</td>
</tr>
<tr>
<td>10</td>
<td>903</td>
<td>IDC</td>
<td>II</td>
<td>Tcyt 2; S +/-</td>
<td>Occ T +; S -</td>
<td>TM -ve; S +</td>
<td>T -ve; S +</td>
</tr>
<tr>
<td>11</td>
<td>941</td>
<td>IDC</td>
<td>III</td>
<td>Neg</td>
<td>Neg</td>
<td>TM -ve; S ++</td>
<td>T -ve; S ++</td>
</tr>
<tr>
<td>12</td>
<td>961</td>
<td>IDC</td>
<td>III</td>
<td>Neg</td>
<td>TM -ve; S +++</td>
<td>T -ve; S +++</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>861</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 2; S +</td>
<td>T 1; S +</td>
<td>TM -ve; S +</td>
<td>T -ve; S +</td>
</tr>
<tr>
<td>14</td>
<td>892</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 3; S +</td>
<td>T 3; S +/-</td>
<td>TM 3; S +++</td>
<td>T 2; S +++</td>
</tr>
<tr>
<td>15</td>
<td>894</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 2; S +</td>
<td>T 1; S +/-</td>
<td>TM 3; S +++</td>
<td>T 1; S ++</td>
</tr>
<tr>
<td>16</td>
<td>1022</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 4; S +/-</td>
<td>T 2; S -</td>
<td>TM -ve; S +</td>
<td>T -ve; S +/-</td>
</tr>
<tr>
<td>17</td>
<td>1046</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 2; S -</td>
<td>Occ T +; S -</td>
<td>TM -ve; S +/-</td>
<td>T -ve; S -</td>
</tr>
<tr>
<td>18</td>
<td>1181</td>
<td>ILC</td>
<td>III</td>
<td>Tcyt 3; S -</td>
<td>T 1; S -</td>
<td>TM 1; S +</td>
<td>T -ve; S +/-</td>
</tr>
<tr>
<td>19</td>
<td>1302</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 4; S +</td>
<td>T 2; S +/-</td>
<td>TM -ve; S +</td>
<td>T -ve; S +/-</td>
</tr>
<tr>
<td>20</td>
<td>1589</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 3; S +</td>
<td>T 1; S +</td>
<td>TM 1; S ++</td>
<td>T -ve; S +</td>
</tr>
<tr>
<td>21</td>
<td>1614</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 3; S +/-</td>
<td>Occ T +; S -</td>
<td>TM 1; S +++</td>
<td>T -ve; S ++</td>
</tr>
<tr>
<td>22</td>
<td>1637</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt Neg; S ++</td>
<td>T -ve; S ++</td>
<td>T 1; S ++</td>
<td>T -ve; S ++</td>
</tr>
<tr>
<td>23</td>
<td>1654</td>
<td>ILC</td>
<td>II</td>
<td>Tcyt 4; S ++</td>
<td>T 2; S +</td>
<td>TM -ve; S +++</td>
<td>T -ve; S +++</td>
</tr>
</tbody>
</table>

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 than observed for the MMP-9 protein.

• Reduced expression of $\alpha_2$, $\alpha_3$ and $\beta_1$ integrins was seen in carcinomas, the extent of which was associated with tumour grade (p=0.0001 for $\alpha_2$ and $\beta_1$, 0.05 for $\alpha_3$). Whereas all tumours retained some level of expression of these integrins, $\alpha_6$ and $\beta_4$ 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 $\alpha_5$ 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 $\alpha_5$ integrin was positively correlated with stromal MMP-9, and membrane $\alpha_6$ and cytoplasmic $\beta_4$ 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 notably 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
Chapter 2: MMP and CAM in Breast Tissue

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 explanation. 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,
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
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
Chapter 2: MMP and CAM in Breast Tissue

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 α1 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 α5 integrin in normal breast. Again, expression of this subunit has not been shown by other groups, and there was no evidence of reactivity for α5 integrin in this study. This discrepancy between studies is difficult to explain: each study has used different (though all monoclonal) antibodies to α5 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 α5 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 α2, α3 and β1 integrin subunits was observed, with greater loss of reactivity in poorly differentiated tumours, a relationship that was particularly strong for α2 and β1 integrins. A similar relationship between α2β1 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 α2 or α3 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 \( \alpha_2\beta_1 \) in the induction of breast epithelial differentiation. Transfection of a poorly differentiated mouse mammary tumour cell line with \( \alpha_2 \) integrin was shown to promote glandular morphogenesis (Zutter et al, 1995), whilst expression of anti-sense \( \alpha_2 \) mRNA into a well-differentiated \( \alpha_2 \)-expressing breast cancer cell line inhibited gland formation in three-dimensional collagen matrices (Keely et al, 1995). Reduced \( \alpha_3 \) expression in parallel with \( \alpha_2 \) and \( \beta_1 \) has been described by Pignatelli et al (1992), but has not been confirmed by other groups. However, the role of \( \alpha_3\beta_1 \) in breast appears to be distinct from that of \( \alpha_2\beta_1 \), since antibodies to \( \alpha_2 \) integrin block branching morphogenesis in three-dimensional gels but this is not seen with blocking antibodies to \( \alpha_3 \) integrin (Berdichevsky et al, 1992).

In this study whilst \( \alpha_5 \) 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 \( \alpha_5 \) reactivity is commented on in one study (Pignatelli et al, 1992). The \( \alpha_5 \) integrin has been implicated in the regulation of cell growth and tumourigenicity; overexpression of \( \alpha_5 \) 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 \( \alpha_5\beta_1 \)-mediated suppression of MAP kinase activity (Gong et al, 1998). In fibroblasts in-vivo, however, signalling by \( \alpha_5\beta_1 \) 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 \( \alpha_5 \) integrin neo-expression in tumour-associated fibroblasts, as identified in this study. The limitation of fibroblast-associated \( \alpha_5 \) 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 α6 integrin and β4 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 β4 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, α6β4 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 α6β4 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 α6β4, its expression was associated with the more aggressive, high grade tumours (p=0.01 for α6 integrin and p=0.02 for β4 integrin). Friedrichs et al (1995) found expression of α6 was associated with the presence of lymph node metastases and a reduced patient survival compared to α6-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 α6β4 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 α6β4 integrin and laminin, suggesting that α6β4 plays an important role in tumour progression particularly when it interacts with its ligand. In apparent contrast to this tumour-promoting role for α6β4, using an approach of RT-PCR differential display, Sager et al (1993) found α6
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 $\alpha_6\beta_4$ integrin expression in breast carcinomas is a poor prognostic marker (Tagliabue et al, 1998).

Whereas in the normal breast, $\alpha_6\beta_4$ 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 $\alpha_6\beta_4$ 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 $\alpha_6\beta_4$ 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 $\alpha_6\beta_4$ 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 $\beta_1$ and $\beta_4$-integrins that modulates tissue organisation and function. The present study has demonstrated an inverse relationship between membrane levels of $\beta_1$ and $\beta_4$ 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; Vleminkz 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 \( \alpha \)-catenin, to either \( \beta \)-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; Hulskens et al,
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 α4β1 and α5β1 integrin receptors (Huhtala et al, 1995). MMP synthesis is induced in wound-associated keratinocytes in an α5β1-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 α6 reactivity and the presence of membrane MMP-2 (ss), and with cytoplasmic, but not membrane, β4 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 β4 as well as the α6 integrin. A further relationship was identified between stromal reactivity for α5 integrin and stromal MMP-9 expression. Activation of a MAP-kinase signalling pathway following α5β1-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
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 α2β1 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 α3β1
(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 α2, α3 and β1 integrin is related to loss of differentiation whereas presence of α6 and β4 integrin is associated with high tumour grade, and an inverse relationship is seen between β1 and β4 integrins. A positive correlation exists between membrane expression of α6 integrin and both membrane and cytoplasmic MMP-2, but this relationship is not seen for β4 integrin, and its significance is not clear. These findings support a role for integrins in modulating differentiation. The presence of α6β4 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.
Chapter 3: In vitro studies of CAM and MMPs

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 α6β4 integrin in MDA-MB 231 cells following treatment with TGF-β1 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 Lamsweerde 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...
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 cm² 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.
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.
Chapter 3: In vitro studies of CAM and MMPs

Table 3.1: Oligonucleotide Primer and Probe Sequences

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMERS (5' - 3')</th>
<th>SIZE</th>
<th>PROBE (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>F: ATT GAT GCG GTA TAC GAG GC</td>
<td>350 bp</td>
<td>CTC CAG AAT TTG TCT</td>
</tr>
<tr>
<td></td>
<td>R: GGC ACC CTG GAA GAA GTA GC</td>
<td></td>
<td>CCA GC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>F: TTC TAC GGG CCA CTA CTG TGC</td>
<td>250 bp</td>
<td>GTT GCA GGC ATC GTC</td>
</tr>
<tr>
<td></td>
<td>R: CGC CCA GAG AAG AAG AAA AG</td>
<td></td>
<td>CAC CGG ACTCAA AGG</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>F: TCG CCA ATG GAA AGA CCT AC</td>
<td>315 bp</td>
<td>CAT CCA GAA GAG AGC</td>
</tr>
<tr>
<td></td>
<td>R: TGA TGA TCA CTT CCG TCT CC</td>
<td></td>
<td>AGC ATC AAT CTT GTC</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>F: TGG GGA CAC CAG AAG TCA AC</td>
<td>350 bp</td>
<td>GTA GTG ATG TGC AAG</td>
</tr>
<tr>
<td></td>
<td>R: CAG GGG ATG GAT AAA CAG GG</td>
<td></td>
<td>AGT CC</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>F: AAC GAC ATT TAT GGC AAC CC</td>
<td>250 bp</td>
<td>TCT ATA TCC TTC TCA</td>
</tr>
<tr>
<td></td>
<td>R: ACC TGT GGT TCA GGC TCT TC</td>
<td></td>
<td>GGC CC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AGA ACA TCA TCC CTG CCT AC</td>
<td>350 bp</td>
<td>GTT GAA GTC AGA GGA</td>
</tr>
<tr>
<td></td>
<td>R: GCC AAA TTC GTT TGC ATA CC</td>
<td></td>
<td>GAC C</td>
</tr>
<tr>
<td>β4 INTEGRIN</td>
<td>F: AGG AGA ACC TGA ACG AGG TC</td>
<td>125 bp</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>R: ATC AGC ACT GTG TCC ACA ATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 sulfoxide (DMSO), avidin, β-2-mercaptoethanol, p-nitrophenyl phosphate (PNPP), p-aminophenylmercuric acid (APMA) were from Sigma (Poole, UK). Magnesium sulphate anhydrate (MgSO$_4\cdot$7H$_2$O), disodium hydrogen orthophosphate (Na$_2$HPO$_4\cdot$2H$_2$O) were from Fisons (Loughborough, UK). Potassium chloride (KCl), magnesium chloride (MgCl$_2$), sodium hydroxide (NaOH) were from Fisher Scientific UK (Loughborough, UK). Sodium dihydrogen orthophosphate (NaH$_2$PO$_4\cdot$2H$_2$O) 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.
Chapter 3: In vitro studies of CAM and MMPs

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 x10⁵
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% CO₂/95% 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 0.25mm² 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:

\[
\frac{\text{no. cells lower membrane} + \text{no. cells in lower well}}{\text{total no. cells (upper membrane + lower membrane + lower well)}} \times 100\% 
\]

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 1 µg, 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 10⁵ 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, pelleted 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:
Chapter 3: In vitro studies of CAM and MMPs

**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 20\(\mu\)g/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 (50\(\mu\)g/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 \(\mu\)l of each sample, diluted 1:10 in sterile
water, was added to the appropriate well followed by 90 \(\mu\)l 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 100\(\mu\)l 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 100\(\mu\)l
digoxigenin-labelled oligonucleotide probe diluted to a final concentration of 0.2
pmol/100\(\mu\)l Rapid Hybridisation Buffer for 1.5 hours at 42\({^\circ}\)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\({^\circ}\)C. The
OD was read at 405 nm with a differential of 630 nm on a multiwell plate reader (Denley
Instruments Ltd., Billingshurst, UK).
Chapter 3: In vitro studies of CAM and MMPs

**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.
Chapter 3: In vitro studies of CAM and MMPs

- **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 x 10⁵ and 1 x 10⁴, 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>
<thead>
<tr>
<th>ANTIBODY</th>
<th>DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>All integrin antibodies</td>
<td>1: 200</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>1: 50</td>
</tr>
<tr>
<td>Vinculin</td>
<td>1: 20</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1: 200</td>
</tr>
<tr>
<td>EMA</td>
<td>1: 500</td>
</tr>
<tr>
<td>CK14</td>
<td>1: 5</td>
</tr>
<tr>
<td>CK18</td>
<td>neat</td>
</tr>
<tr>
<td>ER</td>
<td>1: 100</td>
</tr>
<tr>
<td>EGFR</td>
<td>1: 100</td>
</tr>
<tr>
<td>Plectin/HD1</td>
<td>neat; 1:10, 1:50, 1:100</td>
</tr>
</tbody>
</table>
3.3.6 Western Blotting

Protein was extracted from cell lines grown under different conditions and Western blot analysis carried out for β4 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,
Chapter 3: In vitro studies of CAM and MMPs

Fig. 3.2: Effect of Fibroblast Conditioned Medium (CM) on Invasion.

$\text{x axis indicates proportion of CM to DMEM added to lower chamber of invasion assay}$

Fig. 3.3: Effect of Matrigel Barrier on Invasion.

$\text{x axis indicates concentration of Matrigel in } \mu\text{g/ml}$
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.
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
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.

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
<th>HT 1080</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus RT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive RT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**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
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
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>
<thead>
<tr>
<th>Antibody</th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 468</th>
<th>MDA-MB 231</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>$+$</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>EMA</td>
<td>$+$</td>
<td>$+$</td>
<td>+/-</td>
<td>$-$</td>
</tr>
<tr>
<td>CK14</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>+/-</td>
</tr>
<tr>
<td>CK18</td>
<td>$+$</td>
<td>$+$</td>
<td>$+$</td>
<td>+/-</td>
</tr>
<tr>
<td>Vimentin</td>
<td>$-$</td>
<td>$-$</td>
<td>$+$</td>
<td>$+$</td>
</tr>
<tr>
<td>EGFR</td>
<td>$-$</td>
<td>$-$</td>
<td>$+$</td>
<td>$+$</td>
</tr>
</tbody>
</table>

$+$: 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 = 25μm)
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 = 25 µm)
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.](image)

- 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
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.

![Graph](image)

**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
Chapter 3: In-vitro studies of CAM and MMPs

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
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.
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.
Chapter 3: In-vitro studies of CAM and MMPs

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 α2, α3, and β1 integrins, which have a punctate distribution at the cell periphery, resembling focal contact formation. There is up-regulation of α6 and β4 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)
Chapter 3: In-vitro studies of CAM and MMPs

3.4.6 Changes in Level of $\beta 4$ Integrin Protein in TGF-$\beta 1$-Treated MDA-MB 231 Cells

To assess whether TGF-$\beta 1$ treatment of MDA-MB 231 cells leads to increased levels of $\beta 4$ 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-$\beta 1$. 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 $\beta 4$ integrin. Figure 3.17 demonstrates stronger bands for $\beta 4$ integrin in the TGF-$\beta 1$ treated cells compared to controls.

![Western Blot](image)

Fig. 3.17: Western Blot Analysis of $\beta 4$ Integrin Protein in Control and TGF-$\beta 1$-Treated MDA-MB 231 Cells

3.4.7 Relationship Between $\alpha 6$$\beta 4$ 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 $\alpha 6$$\beta 4$ integrin in mediating this invasion was investigated using a blocking-antibody approach. Figure 3.18a demonstrates that the mean invasive index of MDA-MB 231 cells is significantly reduced in the presence of either anti-$\alpha 6$-integrin antibodies or anti-$\beta 4$-integrin antibodies ($p<0.01$), but not in the presence of mouse IgG control antibodies. In contrast the invasive behaviour of MCF-7 cells, which do not express $\alpha 6$$\beta 4$ integrin, showed no significant change in the presence of both integrin antibodies and control antibodies.
To assess whether the enhanced expression of α6β4 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) 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.
Chapter 3: In-vitro studies of CAM and MMPs

• 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 β1 and β4 integrin was concentrated (fig. 3.19 & 3.20). In TGF-β1-treated cells, punctate staining was identified at the periphery of cells, in a pattern closely reflecting that of β1 integrin, and suggesting localisation to focal contacts (fig. 3.19 & 3.20).

• Cytoskeletal solubilisation experiments
To investigate the association of β1 and β4 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 β1 or β4 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-β1-treated cells with HSB demonstrated loss of staining for β1 integrin, but staining for β4 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).
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 extract the actin cytoskeleton, the focal reactivity for $\beta_1$ and $\beta_4$ 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 $\beta_1$ and $\beta_4$ Integrins in Control MDA-MB 231 Cells

(scale bars = 25$\mu$m)
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 β₁ and β₄ integrin, however, β₁ integrin is lost following HSB extraction of the actin cytoskeleton, in contrast to β₄ integrin which is largely retained. Punctate staining for vinculin is observed in a pattern mirroring β₁ 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 β₁ and β₄ Integrins in TGF-β₁-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: Transmission 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 thickened membrane plaque. Similar structures are identified in myoepithelial cells. These resemble Type II hemidesmosomes. Scale bar=200 nm.](image-url)
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 231 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++ |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| MDA-MB 231      | MMP-2           | MT1-MMP         | TIMP-2           | MMP-9           | TIMP-1          |
| CONTROL         | N/E             | 0.711           | 0.768            | 0.617           | 0.819           |
| MDA-MB 231      | N/E             | 0.950*          | 1.111*           | 0.834*          | 1.114**         |
| + TGFβ          |                 |                 |                 |                 |                 |
| MCF-7           | MMP-2           | MT1-MMP         | TIMP-2           | MMP-9           | TIMP-1          |
| CONTROL         | N/E             | N/E             | 0.891            | N/E             | 0.858           |
| MCF-7           | N/E             | N/E             | 0.941            | N/E             | 0.857           |
| + TGFβ          |                 |                 |                 |                 |                 |

++Values represent the mean gene:GAPDH ratio of four OD readings, corrected for background/–RT readings.
*=p<0.05; **=p<0.001
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 α6β4 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 α6β4 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 α6β4 integrin. In control MDA-MB 231 cells, staining for α6β4 integrin is lost when the actin cytoskeleton is solubilised, however, the majority of α6β4 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 α6β4-dependent, and an increase in MMP and TIMP expression in an α6β4-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 α6β4 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 α6β4, 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
Chapter 3: In-vitro studies of CAM and MMPs

231 cells is due to hypermethylation of the promoter 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 API transcription 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
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 environmental 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,
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 α3β1 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 α6β4 comes from Mercurios’ group, who found that localisation of α6β4 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-β1 -treated cells were plated on poly-d-lysine, however, TGF-β1 is known to influence ECM protein synthesis, therefore one could postulate that TGF-β1 could promote translocation of α6β4 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-β1, there was up-regulation and redistribution of α6β4 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
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).

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

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCE</th>
<th>SIZE</th>
<th>REFERENCE</th>
</tr>
</thead>
</table>
| β 4  | F: AGGAGAACCTGAACGAGGTC  
      | R: ATCAGCACTGTGTCCACAATG | 124  | N/A        |
| CK14 | F:CCCTACTTTCAAGACCATTGAGG  
      | R:GTCACGCACTGCTCGTTCAGAA  | 319  | N/A        |
| CALLA| F:TTGTAAGCAGCCTCAGCC  
      | R:TTGTCCACCTTTTCTCGG     | 460  | Gomm et al, 1995 |
| EMA  | F:GAGGATCCGCTCACCCTCTCAAA  
      | 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 1 mm 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 IgG1 conjugated with anti-β4 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

<table>
<thead>
<tr>
<th>EPITHELIAL</th>
<th>MYOEPITHELIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA-conjugated IgG1 beads</td>
<td>CALLA-conjugated IgG1 beads</td>
</tr>
<tr>
<td>BerEP4 labelled beads</td>
<td>β4-integrin-conjugated IgG1 beads</td>
</tr>
</tbody>
</table>

- **labelling of beads:** Aliquots of the sheep anti-mouse IgG1 coated beads (1 x 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-β4 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 $2 \times 10^6$ cells, were incubated sequentially for 30 mins at 4°C with $1 \times 10^6$ labelled beads as follows:
  - first round incubation with anti-β4 integrin labelled beads
  - second round incubation with anti-β4 integrin labelled beads
  - incubation with $1 \times 10^6$ 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:
Chapter 4: Study of cell interactions

Reduction Mammoplasty

Digestion
200 iu/ml collagenase
125 iu/ml hyaluronidase

Mixed population organoids, fibroblasts, stroma

Loose pellet

Sedimentation

Supernatant

Organoid rich

Culture in enriched medium

Fibroblast rich

Culture in basic medium

Trypsinise and Filter

Myoepithelial cell rich suspension

β4 labelled beads

Myoepithelial cells

2nd round extraction

β4 labelled beads

For positive BerEP4 selection

Supernatant

BerEP4 labelled beads

Fibroblasts

Epithelial cells

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.
Table 4.3: Details of Antibodies used to Characterise Bead-Separated Cell Populations

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>TARGET CELL</th>
<th>DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Cadherin</td>
<td>Epithelial &amp; Myoepithelial</td>
<td>1:50</td>
</tr>
<tr>
<td>HMFG-1</td>
<td>Epithelial</td>
<td>1:100</td>
</tr>
<tr>
<td>β4-integrin</td>
<td>Myoepithelial</td>
<td>1:200</td>
</tr>
<tr>
<td>CALLA</td>
<td>Myoepithelial</td>
<td>1:100</td>
</tr>
<tr>
<td>CK14</td>
<td>Myoepithelial &amp; subpopulation of Epithelial cells</td>
<td>1:5</td>
</tr>
<tr>
<td>CK18</td>
<td>Epithelial</td>
<td>neat</td>
</tr>
<tr>
<td>Smooth Muscle Actin</td>
<td>Myoepithelial &amp; subpopulation of stromal cells</td>
<td>1:100</td>
</tr>
</tbody>
</table>

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°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

<table>
<thead>
<tr>
<th>TARGET POPULATION</th>
<th>EFFECTOR POPULATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts F518</td>
<td>Tumour cell line</td>
</tr>
<tr>
<td>Primary breast fibroblasts</td>
<td>Tumour cell line</td>
</tr>
<tr>
<td>Tumour cell line</td>
<td>Fibroblasts F518</td>
</tr>
<tr>
<td>Tumour cell line</td>
<td>Primary breast fibroblasts</td>
</tr>
<tr>
<td>Tumour cell line</td>
<td>Myoepithelial cells</td>
</tr>
<tr>
<td>F518 &amp; Tumour cell line</td>
<td>Myoepithelial cells</td>
</tr>
<tr>
<td>Primary fibroblasts &amp; Tumour cell line</td>
<td>Myoepithelial cells</td>
</tr>
</tbody>
</table>
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.

![Invasion Assay Modified for use with Co-cultures](image)

**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 $1 \times 10^5$ 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.
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).
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 $\beta_4$-integrin-selected cells grow as cohesive well-demarcated sheets. Magnetic beads are internalised by the cells and strong staining for $\beta_4$ 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 β3-Integrin Selected Cells

(scale bars = 25 μm)
Fig. 4.7: Characteristics of Negatively Selected Cells
The cells yielded following positive selection with β4-labelled beads and BerEP4-labelled beads show a typical fibroblastic morphology. In keeping with this, they lack β4 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 = 25\,\mu m)
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.
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).

![Graph showing MMP-2 and GAPDH expression in different cell lines and culture conditions.](image)

**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.
Table 4.5: Effect of Tumour Cell - Fibroblast Co-culture on Tumour Cell MMP and TIMP Expression ++

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
<td>0.535</td>
</tr>
<tr>
<td>+ F518</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
<td>0.746 *</td>
</tr>
<tr>
<td>MMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
<td>0.556</td>
</tr>
<tr>
<td>+ 1° Fib</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
<td>0.794 **</td>
</tr>
<tr>
<td>MT1-MMP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>0.678</td>
<td>0.650</td>
</tr>
<tr>
<td>+ F518</td>
<td>N/E</td>
<td>N/E</td>
<td>1.020 **</td>
<td>0.967 **</td>
</tr>
<tr>
<td>MT1-MMP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>0.712</td>
<td>0.666</td>
</tr>
<tr>
<td>+ 1° Fib</td>
<td>N/E</td>
<td>N/E</td>
<td>1.063 **</td>
<td>1.002 **</td>
</tr>
<tr>
<td>TIMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.769</td>
<td>0.772</td>
<td>0.701</td>
<td>0.751</td>
</tr>
<tr>
<td>+ F518</td>
<td>0.732</td>
<td>0.734</td>
<td>0.955 **</td>
<td>0.759</td>
</tr>
<tr>
<td>TIMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.708</td>
<td>0.701</td>
<td>0.789</td>
<td>0.810</td>
</tr>
<tr>
<td>+ 1° Fib</td>
<td>0.7556</td>
<td>0.710</td>
<td>0.797</td>
<td>0.799</td>
</tr>
<tr>
<td>MMP-9:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>0.713</td>
<td>0.728</td>
</tr>
<tr>
<td>+ F518</td>
<td>N/E</td>
<td>N/E</td>
<td>1.039 **</td>
<td>1.027 **</td>
</tr>
<tr>
<td>MMP-9:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N/E</td>
<td>N/E</td>
<td>0.666</td>
<td>0.626</td>
</tr>
<tr>
<td>+ 1° Fib</td>
<td>N/E</td>
<td>N/E</td>
<td>1.000 **</td>
<td>0.986 **</td>
</tr>
<tr>
<td>TIMP-1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.677</td>
<td>0.638</td>
<td>0.802</td>
<td>0.744</td>
</tr>
<tr>
<td>+ F518</td>
<td>0.699</td>
<td>0.649</td>
<td>0.806</td>
<td>0.764</td>
</tr>
<tr>
<td>TIMP-1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.758</td>
<td>0.762</td>
<td>0.784</td>
<td>0.733</td>
</tr>
<tr>
<td>+ 1° Fib</td>
<td>0.753</td>
<td>0.751</td>
<td>0.806*</td>
<td>0.746</td>
</tr>
</tbody>
</table>

++ 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.

![Image of a gel electrophoresis with bands for MT1-MMP and GAPDH](image-url)

**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.
Chapter 4: Study of cell interactions

- 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>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2: F518</td>
<td>0.677</td>
<td>0.788 **</td>
<td>0.743 *</td>
<td>1.159 **</td>
<td>1.055 **</td>
</tr>
<tr>
<td>1º Fib</td>
<td>0.691</td>
<td>0.798 *</td>
<td>0.773 *</td>
<td>1.126 *</td>
<td>1.117 *</td>
</tr>
<tr>
<td>MT1-MMP: F518</td>
<td>0.782</td>
<td>0.843</td>
<td>0.832</td>
<td>1.259 *</td>
<td>1.128 *</td>
</tr>
<tr>
<td>1º Fib</td>
<td>0.709</td>
<td>0.772 *</td>
<td>0.789 **</td>
<td>1.222 **</td>
<td>1.091 **</td>
</tr>
<tr>
<td>TIMP-2: F518</td>
<td>0.679</td>
<td>0.697</td>
<td>0.714 *</td>
<td>0.939 **</td>
<td>0.962 **</td>
</tr>
<tr>
<td>1º Fib</td>
<td>0.639</td>
<td>0.664</td>
<td>0.665</td>
<td>0.981 **</td>
<td>0.943 **</td>
</tr>
<tr>
<td>MMP-9: F518</td>
<td>0.549</td>
<td>0.665 **</td>
<td>0.654 **</td>
<td>0.832 *</td>
<td>0.853 **</td>
</tr>
<tr>
<td>1º Fib</td>
<td>0.643</td>
<td>0.668</td>
<td>0.687 *</td>
<td>0.811 *</td>
<td>0.811 *</td>
</tr>
<tr>
<td>TIMP-1: F518</td>
<td>0.756</td>
<td>0.792 *</td>
<td>0.842 **</td>
<td>0.989 **</td>
<td>0.892 **</td>
</tr>
<tr>
<td>1º Fib</td>
<td>0.737</td>
<td>0.809 **</td>
<td>0.849 **</td>
<td>1.000 **</td>
<td>0.893 **</td>
</tr>
</tbody>
</table>

++ 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.

![Zymogram Image]

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*

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>690</td>
<td>870</td>
<td>875</td>
<td>1620</td>
<td>1580</td>
</tr>
<tr>
<td>pro-MMP-2</td>
<td>315</td>
<td>430</td>
<td>525</td>
<td>980</td>
<td>950</td>
</tr>
<tr>
<td>active MMP-2</td>
<td>145</td>
<td>220</td>
<td>297</td>
<td>1450</td>
<td>1625</td>
</tr>
<tr>
<td>F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>580</td>
<td>720</td>
<td>780</td>
<td>1480</td>
<td>1650</td>
</tr>
<tr>
<td>pro-MMP-2</td>
<td>250</td>
<td>390</td>
<td>480</td>
<td>1660</td>
<td>1590</td>
</tr>
<tr>
<td>active MMP-2</td>
<td>120</td>
<td>190</td>
<td>210</td>
<td>1300</td>
<td>995</td>
</tr>
</tbody>
</table>

*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.
Chapter 4: Study of cell interactions

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.
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.

![Graph showing effect of myoepithelial cells on tumour cell invasion](image)

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

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F518</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>N/E</td>
<td>0.523 **</td>
</tr>
<tr>
<td>1° Fib</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>N/E</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>N/E</td>
<td>0.439 **</td>
</tr>
<tr>
<td>MT1-MMP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F518</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>1.041</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>0.526 **</td>
<td>0.499 **</td>
</tr>
<tr>
<td>1° Fib</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>1.074</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>0.588 **</td>
<td>0.516 **</td>
</tr>
<tr>
<td>TIMP-2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F518</td>
<td>Control + Myo</td>
<td>0.761</td>
<td>0.750</td>
<td>0.705</td>
</tr>
<tr>
<td></td>
<td>0.759</td>
<td></td>
<td>0.688</td>
<td>0.772</td>
</tr>
<tr>
<td>1° Fib</td>
<td>Control + Myo</td>
<td>0.742</td>
<td>0.751</td>
<td>0.776</td>
</tr>
<tr>
<td></td>
<td>0.735</td>
<td></td>
<td>0.760</td>
<td>0.774</td>
</tr>
<tr>
<td>MMP-9:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F518</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>1.014</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>0.594 **</td>
<td>0.516 **</td>
</tr>
<tr>
<td>1° Fib</td>
<td>Control + Myo</td>
<td>N/E</td>
<td>N/E</td>
<td>0.966</td>
</tr>
<tr>
<td></td>
<td>N/E</td>
<td></td>
<td>0.633 *</td>
<td>0.458 **</td>
</tr>
<tr>
<td>TIMP-1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F518</td>
<td>Control + Myo</td>
<td>0.692</td>
<td>0.667</td>
<td>0.856</td>
</tr>
<tr>
<td></td>
<td>0.686</td>
<td></td>
<td>0.842</td>
<td>0.915</td>
</tr>
<tr>
<td>1° Fib</td>
<td>Control + Myo</td>
<td>0.795</td>
<td>0.712</td>
<td>0.780</td>
</tr>
<tr>
<td></td>
<td>0.776</td>
<td></td>
<td>0.785</td>
<td>0.778</td>
</tr>
</tbody>
</table>

++ 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.
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

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB 231</th>
<th>MDA-MB 468</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-2:</strong> F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.717</td>
<td>0.741</td>
<td>1.032</td>
<td>1.070</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.634 *</td>
<td>0.644 **</td>
<td>0.743 **</td>
<td>0.704 **</td>
</tr>
<tr>
<td><strong>MMP-2:</strong> 1° Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.716</td>
<td>0.736</td>
<td>1.063</td>
<td>1.063</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.628 **</td>
<td>0.642 *</td>
<td>0.727 **</td>
<td>0.727 **</td>
</tr>
<tr>
<td><strong>MT1-MMP:</strong> F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.751</td>
<td>0.747</td>
<td>1.152</td>
<td>0.915</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.601 **</td>
<td>0.616 **</td>
<td>0.631 **</td>
<td>0.673 *</td>
</tr>
<tr>
<td><strong>MT1-MMP:</strong> 1° Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.746</td>
<td>0.776</td>
<td>1.146</td>
<td>1.146</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.545 **</td>
<td>0.556 **</td>
<td>0.577 **</td>
<td>0.664 **</td>
</tr>
<tr>
<td><strong>TIMP-2:</strong> F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.708</td>
<td>0.703</td>
<td>1.030</td>
<td>0.989</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.656 *</td>
<td>0.660 **</td>
<td>0.709 **</td>
<td>0.684 *</td>
</tr>
<tr>
<td><strong>TIMP-2:</strong> 1° Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.655</td>
<td>0.627</td>
<td>0.850</td>
<td>0.858</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.615</td>
<td>0.586</td>
<td>0.597 **</td>
<td>0.597 **</td>
</tr>
<tr>
<td><strong>MMP-9:</strong> F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.632</td>
<td>0.649</td>
<td>0.891</td>
<td>0.912</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.574 *</td>
<td>0.595 *</td>
<td>0.672 **</td>
<td>0.712 **</td>
</tr>
<tr>
<td><strong>MMP-9:</strong> 1° Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.637</td>
<td>0.703</td>
<td>0.721</td>
<td>0.721</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.565 **</td>
<td>0.606 **</td>
<td>0.643 **</td>
<td>0.643 **</td>
</tr>
<tr>
<td><strong>TIMP-1:</strong> F518</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.785</td>
<td>0.738</td>
<td>1.001</td>
<td>0.979</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.607 *</td>
<td>0.629 *</td>
<td>0.643 *</td>
<td>0.661 *</td>
</tr>
<tr>
<td><strong>TIMP-1:</strong> 1° Fib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.769</td>
<td>0.746</td>
<td>0.969</td>
<td>0.924</td>
</tr>
<tr>
<td>+ Myo</td>
<td>0.647</td>
<td>0.616 *</td>
<td>0.615 *</td>
<td>0.615 *</td>
</tr>
</tbody>
</table>

++ 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).

![Substrate Gel Zymogram on Conditioned Medium from Control (C=Tumour-Primary Fibroblast) and Myoepithelial (M) Co-cultures.](image)

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 was 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 equivalent 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
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 assess 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 myoepithelial 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 β4 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 β4 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
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 α2, α3, α6, β1 and β4 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
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 promoter 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 α6β4 integrin. My previous work in this laboratory has demonstrated up-regulation and redistribution of this integrin heterodimer in the presence of TGF-β1. The role of α6β4 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 α6β4-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 α6β4 integrin, thus indicating a cascade of related but independent events in response to this growth factor.

In order to address the mechanism by which α6β4 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 α6β4 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 α6β4 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 (Kedeslin 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.
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 Na₂HPO₄, 140 mM NaH₂PO₄, 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
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking Buffer</td>
<td>2.5 mM Tris-HCl (pH 7.6), 7.5 mM NaCl, 5% dry defatted milk</td>
</tr>
<tr>
<td>Washing Buffer</td>
<td>2.5 mM Tris-HCl (pH 7.6), 7.5 mM NaCl, 0.1% ml Polyoxyethylene sorbiton monolaureate (Tween 20)</td>
</tr>
<tr>
<td>Diaminobenzidine</td>
<td>0.1% 3,4,3,4 Tetra-amino Biphenyl Hydrochloride (DAB) in PBS, 3% hydrogen peroxide</td>
</tr>
<tr>
<td>Citric Acid Buffer</td>
<td>10 mM citric acid, pH 6.0</td>
</tr>
<tr>
<td>2x Non-Reducing Sample Buffer</td>
<td>0.5 M Tris-HCl pH 6.8, 10% glycerol 10% SDS, 0.2% bromophenol blue</td>
</tr>
<tr>
<td>Tank Buffer</td>
<td>0.25 M Tris, 1.92 M glycine, 1% SDS, pH 8.3</td>
</tr>
<tr>
<td>Incubation Buffer</td>
<td>0.5 M Tris, 0.1 M CaCl₂, 0.05% Brij, pH 7.6</td>
</tr>
<tr>
<td>Staining Solution</td>
<td>0.05% Coomassie Blue G-250, 30% methanol, 10% acetic acid</td>
</tr>
<tr>
<td>Destaining Solution</td>
<td>30% methanol, 10% acetic acid</td>
</tr>
<tr>
<td>20 X Standard Saline Citrate (SSC)</td>
<td>3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0</td>
</tr>
<tr>
<td>ISH Blocking Solution</td>
<td>TBS containing 0.1% Triton X-100, 3% w/v BSA</td>
</tr>
<tr>
<td>ISH Substrate Buffer</td>
<td>0.1 M Tris-HCl 0.05 M MgCl₂, 0.1 M NaCl, pH 9.5</td>
</tr>
<tr>
<td><strong>Paraformaldehyde</strong></td>
<td>4% paraformaldehyde in 10 X DEPC/PBS, diluted 1:10 in DEPC H₂O to give 0.4% solution</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>10 X PE</strong></td>
<td>0.5 M Tris-HCl, 1% sodium pyrophosphate, 2% polyvinylpyrrolidine, 2% Ficoll, 50 mM EDTa, pH 7.5</td>
</tr>
<tr>
<td><strong>10 X TE</strong></td>
<td>0.1 M Tris-HCl, 0.01 M EDTA, pH 8.0</td>
</tr>
<tr>
<td><strong>ISH Blocking Solution</strong></td>
<td>TBS, 0.1% BSA, 0.01% Triton X-100</td>
</tr>
<tr>
<td><strong>Buffer 3</strong></td>
<td>0.1 M Tris-HCl, 0.05 M MgCl₂, 0.1 M NaCl, pH 9.5</td>
</tr>
<tr>
<td><strong>Diluent Buffer</strong></td>
<td>30 % 20 X SSC in DEPC H₂O, 0.2 mg salmon sperm DNA</td>
</tr>
<tr>
<td><strong>BCIP</strong></td>
<td>0.5% 5-bromo-4-chloro-3-indolylphosphate w/v in dimethyl formamide</td>
</tr>
<tr>
<td><strong>NBT</strong></td>
<td>0.4 mM nitroblue tetrazolium in 70% dimethyl formamide</td>
</tr>
<tr>
<td><strong>10 x AJ PCR buffer</strong></td>
<td>45 mM Tris-HCl pH 8.8, 11 mM (NH₄)₂SO₄, 4.5 mM MgCl₂, 200 μM each dNTPs, 110 μg/ml BSA, 6.7 mM β-mercaptoethanol, 4.4 μM EDTA pH 8.0, 501.8 μl PCR H₂O</td>
</tr>
<tr>
<td><strong>Cova buffer 1</strong></td>
<td>0.02% w/v Tween 20, 1 x PBS (filtered)</td>
</tr>
<tr>
<td><strong>ELOSA wash buffer 2</strong></td>
<td>11.69% w/v NaCl, 1% w/v MgSO₄.7H₂O, 0.5% w/v Tween 20, 1 x PBS (filtered)</td>
</tr>
</tbody>
</table>
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% Acrylamide/Bis-acrylanide, 100 µl 10% w/v SDS, 5 µl TEMED, 50 µl 10% APS, 4.0 mls ddH2O
3.75% Stacking Gel: 2.5 mls Stacking buffer, 1.3 mls 30% Acrylamide/Bis-acrylanide, 100 µl 10% w/v SDS, 10 µl TEMED, 50 µl 10% APS, 6.1 mls ddH2O

Appendix 3: Substrate Gel Zymogram
10% Gelatin Substrate Gel: 10 mg collagen (Type III, Sigma) in 4.0 mls ddH2O, 2.5 mls Resolving buffer, 3.3 mls 30% Acrylamide/Bis-acrylanide, 100 µl 10% w/v SDS, 5 µl TEMED, 50 µl 10% APS
Appendix 4: Zymography on Primary Breast Carcinomas

<table>
<thead>
<tr>
<th>Case No.</th>
<th>RW No</th>
<th>IHC</th>
<th>Pro-MMP-2*</th>
<th>Active MMP-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>628</td>
<td>high</td>
<td>570</td>
<td>498</td>
</tr>
<tr>
<td>2</td>
<td>616</td>
<td>low</td>
<td>3210</td>
<td>926</td>
</tr>
<tr>
<td>3</td>
<td>824</td>
<td>high</td>
<td>358</td>
<td>485</td>
</tr>
<tr>
<td>4</td>
<td>767</td>
<td>high</td>
<td>220</td>
<td>390</td>
</tr>
<tr>
<td>5</td>
<td>844</td>
<td>low</td>
<td>1265</td>
<td>560</td>
</tr>
<tr>
<td>6</td>
<td>861</td>
<td>low</td>
<td>1325</td>
<td>522</td>
</tr>
<tr>
<td>7</td>
<td>867</td>
<td>low</td>
<td>1572</td>
<td>483</td>
</tr>
<tr>
<td>8</td>
<td>845</td>
<td>high</td>
<td>110</td>
<td>525</td>
</tr>
<tr>
<td>9</td>
<td>565</td>
<td>low</td>
<td>368</td>
<td>297</td>
</tr>
<tr>
<td>10</td>
<td>824</td>
<td>high</td>
<td>425</td>
<td>621</td>
</tr>
<tr>
<td>11</td>
<td>601</td>
<td>low</td>
<td>1925</td>
<td>326</td>
</tr>
<tr>
<td>12</td>
<td>840</td>
<td>high</td>
<td>215</td>
<td>328</td>
</tr>
<tr>
<td>13</td>
<td>648</td>
<td>low</td>
<td>2875</td>
<td>898</td>
</tr>
<tr>
<td>14</td>
<td>618</td>
<td>high</td>
<td>210</td>
<td>1220</td>
</tr>
<tr>
<td>15</td>
<td>653</td>
<td>high</td>
<td>92</td>
<td>425</td>
</tr>
<tr>
<td>16</td>
<td>734</td>
<td>low</td>
<td>6987</td>
<td>492</td>
</tr>
<tr>
<td>17</td>
<td>953</td>
<td>high</td>
<td>5620</td>
<td>512</td>
</tr>
<tr>
<td>18</td>
<td>961</td>
<td>low</td>
<td>4752</td>
<td>490</td>
</tr>
<tr>
<td>19</td>
<td>941</td>
<td>low</td>
<td>3800</td>
<td>185</td>
</tr>
<tr>
<td>20</td>
<td>948</td>
<td>low</td>
<td>6820</td>
<td>297</td>
</tr>
<tr>
<td>21</td>
<td>738</td>
<td>high</td>
<td>259</td>
<td>980</td>
</tr>
<tr>
<td>22</td>
<td>903</td>
<td>low</td>
<td>1500</td>
<td>382</td>
</tr>
</tbody>
</table>

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


Apt, 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.


References


References


References


References


References


References


References


References

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

Distribution of Myoepithelial Cells and Basement-Membrane Proteins in the Normal Breast and in

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.


866-874.

Expression in Methacarn and Formalin-Fixed Material from in-situ Ductal Carcinoma of the Breast.
*Journal of Pathology*, 171, 257-262.


Cadherin Adhesion Molecules in Metastatic Ovarian Tumor-Cells. *Japanese Journal of Cancer
Research*, 80, 459-463.

Mainardi, C.L. (1990). Human Neutrophil Collagenase - a Distinct Gene-Product with homology to


Activity of Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) for a wide range of cells - a possible

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
labelled RNA. *J. Histochem. Cytochem.*, 26, 677-679.

*Proceedings of the National Academy of Sciences of the United States of America*, 86, 2632-2636.


References


References


References


correlations with their functions as Receptors and Cell-Adhesion Molecules. American Journal of Pathology, 139, 787-799.


References


References


References


References


References


References


References


References


References


References


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 cancer cell line alters invasive potential

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.

J.L. Jones, & R.A. Walker

Cell-cell and cell-stromal interactions in breast cancer invasion and metastasis.

R.A. Walker, J.L. Jones, S. Chappell, T. Walsh & J.A. Shaw

Molecular pathology of breast cancer and its application to clinical management.

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 \(\alpha 6\beta 4\) 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.

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.