MODULATION OF THE MYOFIBROBLAST PHENOTYPE IS VIA INTERACTION OF EXTRACELLULAR MATRIX PROTEINS WITH INTEGRIN RECEPTORS

by

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This report is submitted in fulfilment of the requirements for the degree of PhD in the University of London

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I declare that all the experiments in this thesis were carried out by myself, unless otherwise stated:

The work described in Chapter Five, entitled **Results 3: Oral Squamous Cell Carcinoma Derived TGF-β Promotes MF differentiation and SF dependant invasion**, was done in collaboration with colleagues in the Richard Dimbleby/CRUK labs at St. Thomas’ Hospital, London.

The results obtained for the immunohistochemistry and invasion assays were performed at St. Thomas’ and were not done by myself.
This work is dedicated to my loving parents Brian and Sarah, and sister Beth.
Summary: Abstract

Myofibroblasts are differentiated fibroblast cells and are implicated in a wide range of normal and pathological conditions. They are characterised by the production of the cytoskeletal protein α-smooth muscle actin (α-sma), contained within stress fibres and the production of abundant amounts of extracellular matrix (ECM) proteins. They play an important role in normal wound contraction and scar production as well as in embryogenesis, fibrosis, hypertrophic scarring and tumourigenesis. Differentiation of fibroblast to myofibroblast can be brought about by stimulation of the cells with the multifunctional cytokine transforming growth factor -β1 (TGF-β1), which has also been implicated in ECM deposition. This suggests a situation where myofibroblast differentiation, TGF-β1, and the ECM are intimately connected. Interaction of myofibroblasts with their surrounding matrix involves cell surface integrin receptors.

The aims of the study were:
(i) to identify the integrin involvement in fibroblast to myofibroblast differentiation.
(ii) to establish whether such participation in the differentiation process had implications in cell contraction, cell migration and MMP-2 production.
(iii) to determine any phenotypic differences between oral and dermal fibroblasts and their implication in scarless wound healing
(iv) to discover the implication of myofibroblast differentiation in tumour progression by investigating whether oral squamous cell carcinoma cells are able to generate myofibroblasts and the effect that these cells then have on tumour invasion and progression.

A panel of integrins on human oral and dermal fibroblasts and myofibroblasts were screened by flow cytometry and Western blotting. Initially 3 integrin subunits (αv, α5 and β1) were targeted for blocking studies. By adding blocking antibodies simultaneously to TGF-β1 it was discovered that the α-sma upregulation seen upon differentiation was prevented. This prevention of differentiation also inhibited the TGF-β1-induced collagen gel contraction.

The integrins subunits were also found to play a part in cell migration, but were unrelated to Matrix Metalloproteinase (MMP) -2 production in both fibroblasts and TGF-β1 induced myofibroblasts.

Oral squamous cell carcinoma cell lines were able to generate a myofibroblast phenotype in human oral fibroblasts. These myofibroblasts in turn increased the carcinoma cell invasion.
Papers/Communications:

Papers:

NB * Joint first authors


Published abstracts:


Oral Presentations:

March 2004, IADR, Honolulu, Hawaii, USA

K.A. Lygoe, P.M. Speight and M.P. Lewis. *α5 Integrin: its role in Myofibroblast Differentiation, Contraction and Migration.*

June 2003, IADR, Göteborg, Sweden

KA Lygoe, PM Speight and MP Lewis. *Regulation of Myofibroblast Differentiation & Wound Contraction by αv & β1 Integrins.* Entry for the Senior Colgate Prize
June 2003, IADR, Göteborg, Sweden


Sept 2002, PEF of IADR, Cardiff, UK

KA Lygoe, PM Speight and MP Lewis. Integrin involvement in myofibroblast differentiation of human gingival and human skin fibroblasts.

Poster Presentation:

July 2002, XVIIIth meeting of the Federation of European Connective Tissue Societies (FECTS), Brighton, UK

KA Lygoe, PM Speight and MP Lewis. αv and α5 integrin involvement in α-smooth muscle actin expression and cell migration in human gingival and human skin fibroblasts.
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Thank you to Dr Chris Irwin (Queen’s University, Belfast) for providing me with patient matched samples of oral and dermal fibroblasts used throughout the whole of this study. Thank you to Dr Jill Norman (University College, London) for helping me out in the lab, and Dr John Marshall (Cancer Research UK, London) for supplying me with ‘bucket loads’ of antibody! I think he thought I was drinking it at one point!

Thank you also to my family and friends, who have provided me with an occasional, but welcome distraction from myofibroblasts!! To name but a few (in no particular order!!!): Mum, Dad and Beth, Rob Benson, Suzy and Lee Smith, Claire Peppiatt, Natalie Fillingham, Michele Carron, Alice Webb, Louise Brown, Scott Hasler, Justin Barnes, Lisa Peppiatt, Pat and Penny Rigby and Karl Wallace (for whom I wouldn’t be where I am now if his ambitiousness for science wasn’t catching!!!).

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Contents

Declaration ........................................................................................................................2
Dedication ...........................................................................................................................3
Summary: Abstract ...........................................................................................................4
Papers/Communications: ...............................................................................................5
  Published abstracts: ......................................................................................................5
Acknowledgements ..........................................................................................................7
Contents ..............................................................................................................................8
List of Abbreviations ......................................................................................................13
List of Figures ..................................................................................................................16
List of Tables ...................................................................................................................18

Chapter One: Introduction

1.1 Wound Healing .....................................................................................................19
1.2 Myofibroblasts ......................................................................................................26
  1.2.1 Introduction ....................................................................................................26
  1.2.2 Myofibroblasts in Normal Tissues ..............................................................30
  1.2.3 Myofibroblasts in Wound Healing ..............................................................31
    1.2.3.1 Adult Wound Healing ...............................................................................31
    1.2.3.2 Fetal and Oral Wound Healing ..................................................................32
  1.2.4 Myofibroblasts in Pathological Conditions .................................................34
    1.2.4.1 Keloids/Hypertrophic Scarring ..................................................................34
    1.2.4.2 Fibrosis .......................................................................................................34
    1.2.4.3 Tumourigenesis ..........................................................................................35
  1.2.5 Factors Influencing Myofibroblast Differentiation ....................................37
    1.2.5.1 Transforming Growth Factor-β (TGF-β) .................................................37
    1.2.5.2 Extracellular Matrix ...................................................................................38
1.3 Transforming Growth Factor-β ............................................................................41
  1.3.1 Introduction ....................................................................................................41
  1.3.2 Latency, and Activation of TGF-β ..............................................................41
  1.3.3 TGF-β Receptor Activation .........................................................................44
  1.3.4 TGF-β Intracellular Signalling .....................................................................44
  1.3.5 TGF-β and Wound Repair ............................................................................47
1.4 Integrins .................................................................................................................49
  1.4.1 Introduction to Integrins ...............................................................................49
  1.4.2 Integrin Structure ...........................................................................................49
  1.4.3 Ligand Binding to Integrins ..........................................................................53
    1.4.3.1 Introduction ............................................................................................53
    1.4.3.2 Fibronectin ..............................................................................................55
    1.4.3.3 Vitronectin .............................................................................................58
    1.4.3.4 Collagens ................................................................................................58
  1.4.4 Integrin Clustering .........................................................................................60
Chapter Two: Materials and Methods

2.1 Materials ................................................................. 75
  2.1.1 Chemical Reagents and Kits .................................. 75
  2.1.2 Antibodies .......................................................... 76

2.2 Cell Culture .............................................................. 77
  2.2.1 Routine Cell Culture ........................................... 77
  2.2.2 Routine Passage .................................................. 77
  2.2.3 Determining Cell Density .................................... 78
  2.2.4 Freezing Down Cell Stocks ................................. 79
  2.2.5 Thawing Out Cell Stocks ...................................... 79

2.3 Flow Cytometry ....................................................... 80
  2.3.1 Introduction ...................................................... 80
  2.3.2 Preparation of Cells for Analysis ......................... 80
  2.3.3 Flow Cytometry Analysis ................................. 81

2.4 Western Blotting ...................................................... 82
  2.4.1 Introduction ...................................................... 82
  2.4.2 Preparation of Cell Lysates ................................. 82
  2.4.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins .......................... 83
  2.4.4 Membrane Transfer (Western Blotting) .................. 84
  2.4.5 Immunodetection of Antigen ............................. 86

2.5 Immunocytochemistry ............................................. 88
  2.5.1 Introduction ...................................................... 88
  2.5.2 Preparing and Coating Glass Coverslips ................ 88
  2.5.3 Preparing and Fixing the Cells ......................... 88
  2.5.4 Immunodetection of Antigen ............................. 89
  2.5.5 Visualising Antigen .......................................... 89

2.6 Collagen Gel Contraction Assay .............................. 90
  2.6.1 Introduction ...................................................... 90
  2.6.2 Contraction Assay ............................................ 90

2.7 Scratch Assay ......................................................... 92
Chapter Three: Results One

Integrin Involvement in Myofibroblast Differentiation

3.1 Introduction ...........................................................................................................97

3.2 Methods ................................................................................................................100

3.3 Results ..................................................................................................................102
  3.3.1 Expression of α-smooth muscle actin (α-sma) by Human Oral and Dermal Fibroblasts ..................................................................................................................102
  3.3.2 Integrin Expression on Control and TGF-β1 Treated HOF and HDF ....107
  3.3.3 Functional Blockade of αv Integrin can Block TGF-β1 Induced Myofibroblast Differentiation ....................................................................115
  3.3.4 Functional Blockade of β1 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation ....................................................................118
  3.3.5 Functional Blockade of α5 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation ....................................................................119
  3.3.6 Functional Blockade of the αvβ3 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation ....................................................................121
  3.3.7 Functional Blockade of αvβ5 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation ....................................................................123
  3.3.8 α-smooth muscle actin (α-sma) Expression Correlates with Collagen Gel Contraction ........................................................................125
  3.3.9 α-smooth muscle actin (α-sma) Expression in Oral Compared to Dermal Fibroblasts ........................................................................132
  3.3.10 Integrin Binding to ECM Ligands can cause Myofibroblast Differentiation via Integrin Signalling Mechanisms ..................134

3.4 Results Summary .................................................................................................137

Chapter Four: Results Two

Functional Consequences of Integrin Blockade of Myofibroblast Differentiation

4.1 Introduction .........................................................................................................138

4.2 Methods ................................................................................................................140

4.3 Results ..................................................................................................................141
  4.3.1 Migration of Fibroblasts and Myofibroblasts in Response to Wounding ..............................................................................141
4.3.2 Functional Blockade of αv Integrin can Block Fibroblast Migration ... 144
4.3.3 Functional Blockade of β1 Integrin can Block Fibroblast Migration ... 147
4.3.4 Functional Blockade of α5 Integrin has no Effect on Fibroblast Migration ......................................................................................................150
4.3.5 Differentiation to Myofibroblast has no Effect on the Production of Matrix Metalloproteinases ............................................................. 153
4.3.6 Functional Blockade of αv Integrin has no Effect on the Production of Matrix Metalloproteinases ............................................................. 155
4.3.7 Functional Blockade of β1 Integrin has no Effect on the Production of Matrix Metalloproteinases ............................................................. 158
4.3.8 Functional Blockade of α5 Integrin has no Effect on the Production of Matrix Metalloproteinases ............................................................. 161

4.4 Results Summary ...............................................................................................164

Chapter Five: Results Three

Tumour Derived TGF-β Modulates Myofibroblast Differentiation and Promotes Scatter Factor Dependant Invasion of Oral Squamous Carcinoma Cells

5.1 Introduction ..........................................................................................................165
5.2 Methods ...............................................................................................................167
  5.2.1 Immunohistochemistry ...............................................................................167
  5.2.2 Cell Culture ..................................................................................................167
  5.2.3 Preparation and Use of Medium Conditioned by Oral Squamous Carcinoma Cells (OSCC) .......................................................... 168
  5.2.4 Preparation and Use of Medium Conditioned by Fibroblasts and Myofibroblasts .......................................................... 168
  5.2.5 Analysis of Conditioned Medium for TGF-β1 and HGF/SF Content ... 169
  5.2.6 Invasion Assays ...........................................................................................169

5.3 Results .................................................................................................................170
  5.3.1 Myofibroblasts are seen in the Stroma of Oral Squamous Cell Carcinomas in vivo.........................................................................................170
  5.3.2 OSCC Cells Induce Myofibroblast Differentiation Through Secretion of TGF-β1 .........................................................................................172
  5.3.3 Myofibroblast Conditioned Medium Promotes Invasion of OSCC ...... 177
  5.3.4 Myofibroblasts Upregulate Secretion of Hepatocyte Growth Factor (HGF/SF) .........................................................................................180
  5.3.5 Inactivation of HGF/SF in Myofibroblast Conditioned Medium Inhibits Invasion of OSCC Cells.........................................................................182

5.4 Results Summary .................................................................................................184

Chapter Six: Discussion

6.1 Introduction ........................................................................................................185
6.2 The Role of Integrin receptors in Myofibroblast Differentiation .............. 186
6.3 The Role of Myofibroblasts and Cell Adhesion in a Wound Contraction Model ............................................................................................................. 191
6.4 The Role of Fibroblasts and Myofibroblasts in Cell Migration and Protease Production ..................................................................................................... 193
6.5 The Role of Myofibroblasts in Oral Squamous Cell Carcinoma Invasion ......................................................................................................................... 196
6.6 Conclusion ........................................................................................................... 199
6.7 Future Studies ...................................................................................................... 199

Appendix
1. Media and Solutions ............................................................................................. 200
2. Antibody Concentrations Used Throughout the Study ..................................... 203
3. Data From Results Chapters ................................................................................ 204

References .................................................................................................................... 206
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>α-minimal essential medium</td>
</tr>
<tr>
<td>α-sma</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitres, volume</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADS</td>
<td>Antibody diluting solution</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius, temperature</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Col-1</td>
<td>Collagen, type 1</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common mediator Smad</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenyindole</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence associated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activation protein</td>
</tr>
<tr>
<td>FCM</td>
<td>Fibroblast conditioned medium</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGM</td>
<td>Fibroblast growth medium</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>FL1-H</td>
<td>Fluorescence seen through a green filter</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FPCL</td>
<td>Fibroblast populated collagen lattice</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GMF</td>
<td>Geometric mean fluorescence</td>
</tr>
<tr>
<td>GS</td>
<td>Glycine-serine</td>
</tr>
<tr>
<td>h</td>
<td>Hour, time</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HDF</td>
<td>Human dermal fibroblast</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Hepatocyte Growth Factor/Scatter Factor</td>
</tr>
<tr>
<td>HOF</td>
<td>Human oral fibroblast</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>iC3B</td>
<td>Complement component</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>I-Smad</td>
<td>Inhibitory Smad</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>kd</td>
<td>Kilodaltons, weight</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LLC</td>
<td>Large latent complex</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGF-β-binding protein</td>
</tr>
<tr>
<td>MadCAM</td>
<td>Mucosal addressin cell adhesion molecule</td>
</tr>
<tr>
<td>MCM</td>
<td>Myofibroblast conditioned medium</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>min</td>
<td>Minute, time</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre, volume</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre, length</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram, weight</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PA</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS-Tween 20</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>pFN</td>
<td>Plasma fibronectin</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartate (Arg-Asp-Gly)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute, speed</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-regulated Smad</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad Anchor for Receptor Activation</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SCCM</td>
<td>Squamous cell carcinoma conditioned medium</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecysulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Second, time</td>
</tr>
<tr>
<td>SLC</td>
<td>Small latent complex</td>
</tr>
<tr>
<td>Smad</td>
<td>A merger of Sma from <em>Caenorhabditis elegans</em> and Mad from <em>Drosophila mothers against decapentaplegic</em></td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SF/HGF</td>
<td>Scatter factor/hepatocyte growth factor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>V</td>
<td>Voltage, power</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VN</td>
<td>Vitronectin</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1 Phases of Wound repair
Figure 1.2 The role myofibroblasts play in wound healing
Figure 1.3 Stages of differentiation of fibroblast to myofibroblast
Figure 1.4 Schematic diagram representing the large latent TGF-β complex
Figure 1.5 Schematic diagram representing downstream signalling cascade of the TGF-β1/Smad pathway
Figure 1.6 Diagram of the structure of an integrin receptor
Figure 1.7 Integrin subunit combinations
Figure 1.8 Structure of the fibronectin dimer
Figure 1.9 Cytoskeletal and signalling complexes in focal adhesions
Figure 1.10 Interactions among proteins involved in the integrin signalling pathways

Figure 2.1 Haemocytometer slide
Figure 2.2 Component of SDS-PAGE
Figure 2.3 Western Blot Transfer Equipment
Figure 2.4 Mini Trans-Blot Cell Description and Assembly of Parts
Figure 2.5 Principles of ECL Western Blotting - the reaction and principle behind enhanced chemiluminescence
Figure 2.6 Scratch assay configuration

Figure 3.1 Myofibroblast differentiation in response to TGF-β1 is both dose and Time dependant
Figure 3.2 TGF-β1 induces α-sma expression in HOF and HDF
Figure 3.3 TGF-β1 induces α-sma expression in HOF and HDF
Figure 3.4 Morphology of HOF and HDF changes upon treatment with TGF-β1
Figure 3.5 Integrin profile of HOF
Figure 3.6 Integrin profile of HDF
Figure 3.7 αv and β1 integrin expression in HOF and HDF
Figure 3.8 α5 integrin expression in HOF and HDF
Figure 3.9 Integrin expression on HOF and HDF
Figure 3.10 Effect of blocking αv integrin on TGF-β1 induced expression of α-sma
Figure 3.11 Effect of blocking β1 integrin on TGF-β1 induced expression of α-sma
Figure 3.12 Effect of blocking α5 integrin on TGF-β1 induced expression of α-sma
Figure 3.13 Effect of blocking αvβ3 integrin on TGF-β1 induced expression of α-sma
Figure 3.14 Effect of blocking αvβ5 integrin on TGF-β1 induced expression of α-sma
Figure 3.15 3D gel contraction correlates with α-sma expression
Figure 3.16 Effect of integrin blocking antibodies on TGF-β1 induced collagen gel contraction in HOF
Figure 3.17 Effect of integrin blocking antibodies on TGF-β1 induced collagen gel contraction in HDF
Figure 3.18 Phase contrast microscopy illustrating morphology of HOF cultured within collagen gel lattices
| Figure 3.19 | Phase contrast microscopy illustrating morphology of HDF cultured within collagen gel lattices |
| Figure 3.20 | $\alpha$-sma expression and gel contraction |
| Figure 3.21 | Expression of $\alpha$-sma by HOF and HDF grown on different matrices |
| Figure 3.22 | Effect of inhibition of FAK on TGF-β1 induced expression of $\alpha$-sma |
| Figure 4.1 | Cell migration/wound closure is decreased upon treatment with TGF-β1 in HOF |
| Figure 4.2 | Cell migration/wound closure is decreased upon treatment with TGF-β1 in HDF |
| Figure 4.3 | Blocking $\alpha v$ integrin prevents cell migration of HOF |
| Figure 4.4 | Blocking $\alpha v$ integrin prevents cell migration of HDF |
| Figure 4.5 | Blocking $\beta 1$ integrin prevents cell migration of HOF |
| Figure 4.6 | Blocking $\beta 1$ integrin prevents cell migration of HDF |
| Figure 4.7 | Blocking $\alpha 5$ integrin has no effect on cell migration of HOF |
| Figure 4.8 | Blocking $\alpha 5$ integrin has no effect on cell migration of HDF |
| Figure 4.9 | Treatment with TGF-β1 had no effect on MMP-2 production by HOF and HDF |
| Figure 4.10 | Treatment with $\alpha v$ blocking antibody had no effect on MMP-2 production by HOF |
| Figure 4.11 | Treatment with $\alpha v$ blocking antibody had no effect on MMP-2 production by HDF |
| Figure 4.12 | Treatment with $\beta 1$ blocking antibody had no effect on MMP-2 production by HOF |
| Figure 4.13 | Treatment with $\beta 1$ blocking antibody had no effect on MMP-2 production by HDF |
| Figure 4.14 | Treatment with $\alpha 5$ blocking antibody had no effect on MMP-2 production by HOF |
| Figure 4.15 | Treatment with $\alpha 5$ blocking antibody had no effect on MMP-2 production by HDF |
| Figure 5.1 | Immunohistochemistry showing $\alpha$-sma expression in myofibroblasts in oral squamous cell carcinoma |
| Figure 5.2 | Squamous carcinoma conditioned medium induces HOF $\alpha$-sma expression |
| Figure 5.3 | Fibroblast $\alpha$-sma expression is TGF-β1 dependent |
| Figure 5.4 | Squamous carcinoma conditioned medium induces fibroblast $\alpha$-sma expression, which is TGF-β1 dependent |
| Figure 5.5 | OSCC cell lines secrete TGF-β1 |
| Figure 5.6 | Myofibroblast conditioned medium (MCM) promotes invasion of OSCC cells |
| Figure 5.7 | Myofibroblasts secrete scatter factor/hepatocyte growth factor (SF/HGF) |
| Figure 5.8 | Inactivation of HGF/SF in MCM inhibits invasion of OSCC cells |
| Figure 6.1 | Proposed method of myofibroblast activation by treatment with TGF-β1 |
List of Tables

Table 1.1 Cytokines and growth factors involved in the different stages of wound healing
Table 1.2 Myofibroblasts: normal distribution and function
Table 1.3 ECM components and function
Table 1.4 The vertebrate Smad protein family
Table 1.5 Integrin ligand specificities
Chapter One

Introduction

1.1 Wound Healing

Introduction

Wound healing is a physiological event which occurs when there is tissue injury and enables the restoration of the structure and function of the damaged tissue as efficiently and effectively as possible. Wound healing is not a simple step-by-step sequence of events; it is a number of complex interactions involving many cell types, soluble mediators and matrix molecules. Wound healing consists of three overlapping phases – inflammation, tissue formation and tissue remodelling (Falanga V, 2001; Singer and Clark, 1999). See Figure 1.1.

Figure 1.1 Phases of wound repair (Clark, 1996).
Chapter One: Introduction

Inflammation

The skin serves as the body’s protective barrier, so any break in it must be rapidly and efficiently repaired (Martin, 1997). A temporary repair is achieved by the formation of a fibrin-rich clot. The formation of the clot by blood coagulation and platelet aggregation not only serves to protect the denuded area, but also provides a provisional matrix for cell migration (Aukhil, 2000; Clark, 1996; Martin, 1997; Singer and Clark, 1999). The clot consists of platelets embedded in a meshwork of fibrin fibres which have been derived by the thrombin catalysed cleavage of fibrinogen from the clotting cascade, as well as small amounts of plasma fibronectin, vitronectin and thrombospondin (Clark, 1996; Martin, 1997). The wound bed is rich in cytokines and growth factors such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) produced by degranulating platelets, activated coagulation pathways and injured cells. It is these soluble factors present in the clot which, attract inflammatory cells into the wounded area (Clark, 1996) and may provide the start signals for wound repair (Aukhil, 2000). After clot formation, infiltration of neutrophils and monocytes by numerous chemoattractive signals from the circulating blood usually predominates. Neutrophils cleanse the wound of foreign particles, bacteria and debris (Aukhil, 2000). Neutrophils normally begin to arrive at the wound site within minutes and unless the wound is greatly infected they usually disappear within a few days (Martin, 1997). Monocytes infiltrating the wound area become activated macrophages (Singer and Clark, 1999). Macrophages serve to phagocytose any remaining organism as well as cellular and molecular debris (Aukhil, 2000; Martin, 1997). They also release a host of cytokines and growth factors (Table 1.1) (Clark, 1996; Singer and Clark, 1999)), continuing and amplifying the earlier signals released by degranulating platelets, activated coagulation pathways and injured cells (Aukhil, 2000; Martin, 1997; Singer and Clark, 1999). The growth factors derived from macrophages are necessary for the initiation of new tissue formation in the healing of a wound (Singer and Clark, 1999).
Chapter One: Introduction

Reepithelialisation

Reepithelialisation is a process where the epithelial cells migrate over the wound edge and begins within hours of injury (Ravanti and Kahari, 2000). The epithelial cells that form the cut edge start to migrate, using the provisional fibrin matrix for attachment. In order to detach and migrate quickly across the defect, the epithelial cells dissolve their hemidesmosomal connections with the basement membrane (Clark, 1996). Migrating epithelial cells are extremely phagocytic, which allows them to penetrate any tissue debris or clot. They express integrin receptors on their cell surface which allow them to interact with a wide variety of extracellular matrix (ECM) molecules present in the wound space (Aukhil, 2000; Felsenfeld et al., 1996; Lai et al., 2000; Singer and Clark, 1999). They also produce a number of matrix degrading proteins – the matrix metalloproteinases (MMPs), such as MMP-1 (interstitial collagenase), MMP-9 (type IV collagenase) and MMP-10 (stromelysin), which are likely to be required to permeate the provisional matrix (Hakkinen et al., 2000). One to two days post injury the epithelial cells start to proliferate. The stimuli for epithelial cell proliferation has not been determined, but several possibilities exist, including local release of growth factors and the absence of neighbouring cells. It is unknown whether the stimuli by chemotactic guidance, loss of neighbouring cells, or active contact guidance, or a combination of all three; however, cell migration is independent from cell proliferation (Clark, 1996). Once contact with the opposing epithelial cells is established and reepithelialisation is complete, components of the basal lamina are synthesised and deposited and the epithelial cells revert back to their original phenotype (Aukhil, 2000).

Granulation Tissue Formation

Granulation tissue forms below the epithelium and usually starts around day 4 post wounding (Aukhil, 2000; Hakkinen et al., 2000; Hinz et al., 2001a; Linares, 1996; Lorena et al., 2002; Singer and Clark, 1999). It is the framework for the repair process and provides support for the migrating epithelial cells (Linares, 1996). Granulation tissue consists mainly of fibroblasts, new capillaries and macrophages embedded within a loose matrix of fibronectin, collagen and hyaluronic acid.
(Mutsaers et al., 1997). The granulation tissue is a complex reservoir of growth factors and cytokines (Table 1.1) which possess chemoattractive, mitogenic and phenotypic expression properties. Fibroblasts, macrophages and blood vessels are activated and move into the wound space at the same time. The macrophages provide a source of cytokines and growth factors required for fibroblast migration and proliferation, matrix production (fibroplasia) and blood vessel formation (angiogenesis) (Hakkinen et al., 2000; Singer and Clark, 1999). Fibroblasts can use the fibronectin and vitronectin within the matrix for movement by binding directly to these proteins via expression of various integrin matrix receptors (Clark, 1996; Irwin et al., 1994; Stephens et al., 1997). It is important to understand that fibroblasts, like keratinocytes have to rearrange the expression of such receptors in order to proliferate, migrate and attach to different matrix molecules (Aukhil, 2000). The integrin receptor superfamily will be discussed in more detail later in the chapter (Section 1.4 - ‘Integrins’). After migration into the wound site, the fibroblasts begin synthesizing new ECM to replace the injured tissue with a connective tissue scar. TGF-β contributes to the regulation of this fibrotic process by recruiting the fibroblasts and stimulating their synthesis of a collagen rich matrix (Aukhil, 2000; Singer and Clark, 1999).

**Angiogenesis**

The term angiogenesis refers to the formation of new capillaries and blood vessels. Fibroblast growth factor (FGF)-2, released by damaged endothelial cells and macrophages in the wound site, and VEGF released by keratinocytes and macrophages at the wound edge are responsible for the promotion of angiogenesis (Martin, 1997). It is clear that angiogenesis is a complex process relying on specific surrounding matrix, therefore, as in the case of fibroblasts and keratinocytes, the endothelial cells are required to upregulate specific integrin receptors (αvβ3) on their cell surface to be able to respond to any angiogenic signals (Aukhil, 2000). Endothelial cells release plasminogen activator and procollagenase, which liberate plasmin and collagenase respectively, in response to FGF (Linares, 1996). These enzymes degrade the basement membrane and release the endothelial cells. They are then able to migrate into the granulation tissue, form tubes surrounded by their own
provisional matrix and a basement membrane, and new blood flow results (Aukhil, 2000; Clark, 1996).

Table 1.1 Cytokines and growth factors involved in the different stages of wound healing (Martin, 1997; Singer and Clark, 1999).

<table>
<thead>
<tr>
<th>Cytokine/Growth Factor</th>
<th>Source</th>
<th>Target Cells and Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>Platelets</td>
<td>Keratinocyte motility and proliferation</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Macrophages, keratinocytes</td>
<td>Keratinocyte motility and proliferation</td>
</tr>
<tr>
<td>bFGF</td>
<td>Macrophages, endothelial cells</td>
<td>Angiogenesis and fibroblast proliferation</td>
</tr>
<tr>
<td>KGF</td>
<td>Fibroblasts</td>
<td>Keratinocyte motility and proliferation</td>
</tr>
<tr>
<td>TGF-β1/β2</td>
<td>Platelets, macrophages</td>
<td>Keratinocyte migration, chemotaxis of macrophages and fibroblasts, ECM synthesis and remodelling</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Macrophages</td>
<td>Anti-scarring properties</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelets, macrophages, keratinocytes</td>
<td>Fibroblast proliferation and chemotaxis, macrophage chemotaxis and activation</td>
</tr>
<tr>
<td>VEGF</td>
<td>Keratinocytes, macrophages</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>IL-1 &amp; TNF-α</td>
<td>Neutrophils</td>
<td>Activators of growth factor expression in macrophages, keratinocytes and fibroblasts</td>
</tr>
<tr>
<td>Insulin-like growth factor 1</td>
<td>Fibroblasts, keratinocytes</td>
<td>Re-epithelialisation and granulation tissue formation</td>
</tr>
</tbody>
</table>

Wound Contraction and Tissue Remodelling

One of the functions of the granulation tissue is to bring the wound margins closer together to allow efficient, effective wound closure. To enable this to happen, contraction of the wound begins around 7-14 days post wounding (Clark, 1996). Fibroblasts present within the granulation tissue develop several ultrastructural features associated with muscle cells, including force generation and are termed myofibroblasts. A myofibroblast is distinguished by the expression of α-smooth muscle actin (α-sma) incorporated into stress fibres, vinculin-containing fibronexus adhesion complexes and fibronectin fibrils. The multifunctional cytokine TGF-β1 is a direct inducer of the myofibroblast phenotype (Clark, 1996; Grinnell, 1994; Hakkinen et al., 2000; Hinz et al., 2001a; Hu et al., 2003; Singer and Clark, 1999;
Tomasek et al., 2002). Contraction is brought about by attachment of the myofibroblasts to the collagen and fibronectin rich matrix via interaction with integrin receptors (Hakkinen et al., 2000; Singer and Clark, 1999). At 15 days post wounding around 70% of fibroblasts in the granulation tissue have differentiated to the myofibroblast phenotype (Hakkinen et al., 2000). A detailed discussion of this cell type is given in Section 1.2 - ‘Myofibroblasts’, and a summary of the role of myofibroblasts in wound healing can be seen in the following diagram (Figure 1.2), taken from Tomasek et al. (2002).

Post contraction, cellular apoptosis occurs, resulting in an acellular collagen-rich scar. Endothelial cells appear to be the first cells to apoptose, followed by the myofibroblasts (Clark, 1996). New fibroblasts with properties typical to normal connective tissue fibroblasts appear. The final process in wound healing is the tissue remodelling and scar resolution phase (Mutsaers et al., 1997). This takes place over many months and is dependant on continual collagen synthesis and degradation, brought about by the action of specific MMPs and tissue inhibitors of MMPs (TIMPs) (Lewis et al., 2001) expressed by macrophages, epithelial cells, endothelial cells and fibroblasts.
Figure 1.2 The role myofibroblasts play in wound healing (Tomasek et al., 2002).

Upon wounding, local growth factors within the clot stimulate fibroblasts from the surrounding intact stroma to invade this provisional matrix (a). Migrating fibroblasts exert forces on the collagen matrix, which allows cellular reorganization along the lines of stress, and results in the formation of proto-myofibroblasts (b). Stimulation by cytokines (e.g. TGF-β) causes the differentiation to myofibroblast and the production of matrix molecules such as ED-A fibronectin and collagen by the myofibroblasts (c). Wound contraction and remodelling occurs over many weeks and months and, in the healing of a normal wound, the myofibroblasts disappear by apoptosis and leave an acellular scar (d), however, in many pathological conditions the myofibroblasts remain present and continue to remodel the ECM, resulting in connective tissue contracture (e).
1.2 Myofibroblasts

1.2.1 Introduction

The term myofibroblast was first described by Gabbiani and co-workers in the early 1970's (Gabbiani et al., 1972; Majno et al., 1971), and was used to describe the presence of smooth-muscle like cells in the granulation tissue of a healing wound, and their role in wound contraction. They demonstrated that strips of granulation tissue respond to pharmacological agents in a similar manner to smooth muscle, and the resemblance to smooth muscle was confirmed by chemical and immunological methods (Gabbiani et al., 1972). Myofibroblasts have, in recent years, been implicated in a number of different situations associated with force generation and ECM production, which are summarised in Table 1.2.

The simplest definition of myofibroblasts is that they are differentiated fibroblast cells which exhibit characteristics of smooth muscle cells, and have developed a contractile phenotype, although they have also been referred to as activated smooth muscle cells, or stellate cells (Gabbiani, 1998; Grinnell, 1994; Powell et al., 1999a; Tomasek et al., 2002; Vaughan et al., 2000). They possess several distinguishing morphological characteristics, which are used to characterise them. They display cytoplasmic bundles of microfilaments, or stress fibres that contain bundles of actin filaments with associated contractile proteins, such as α-sm, non-muscle myosin, vimentin and desmin. They are connected to each other by gap junctions on the cell surface (Gabbiani et al., 1978) and connected to the ECM by focal contacts or adhesions (also termed the fibronexus). Focal contacts are adhesion complexes, which use transmembrane integrin receptors to link the intracellular stress fibres to extracellular fibronectin fibrils, see Figure 1.3 (Dugina et al., 1998; Dugina et al., 2001; Powell et al., 1999a; Powell et al., 1999b; Serini et al., 1998; Serini and Gabbiani, 1999; Tomasek et al., 2002; Vaughan et al., 2000).
Table 1.2 Presence of myofibroblasts in different normal and pathological situations.

<table>
<thead>
<tr>
<th>Situation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulation tissue</td>
<td>(Aukhil, 2000; Clark, 1996; Cornelissen et al., 2000; Desmouliere et al., 1993; Desmouliere, 1995; Desmouliere and Gabbiani, 1994; Desmouliere and Gabbiani, 1996; Gabbiani, 1998; Grinnell, 1994; Hakkinen et al., 2000; Hinz et al., 2001a; Lorena et al., 2002; Moulin et al., 1998; Schurch et al., 1998; Serini and Gabbiani, 1999; Vaughan et al., 2000; Yokozeki et al., 1997)</td>
</tr>
<tr>
<td>Normal tissue under some degree of tension, e.g. the lung</td>
<td>(Clark, 1996; Gabbiani, 1998; Grinnell, 1994; Tomasek et al., 2002)</td>
</tr>
<tr>
<td>Hypertrophic scarring &amp; Dupuytren's contracture</td>
<td>(Badid et al., 2000; Desmouliere et al., 1997; Kloen, 1999; Neely et al., 1999; Ronnov-Jessen and Petersen, 1993)</td>
</tr>
<tr>
<td>Fibrosis of the kidney</td>
<td>(Badid et al., 2001; el Nahas et al., 1997; Lewis and Norman, 1998; Muchaneta-Kubara and el Nahas, 1997; Norman and Fine, 1999; Schelling et al., 2002)</td>
</tr>
<tr>
<td>Fibrosis of the lung</td>
<td>(Hashimoto et al., 2001; Levine et al., 2000; Scaffidi et al., 2001; Sheppard, 2001; Sime and O'Reilly, 2001)</td>
</tr>
<tr>
<td>Fibrosis of the liver</td>
<td>(Andrade et al., 1999; Kato et al., 2001)</td>
</tr>
<tr>
<td>Scleroderma lesions</td>
<td>(Denton and Abraham, 2001; Holmes et al., 2003; Leask et al., 2002; Ronnov-Jessen and Petersen, 1993)</td>
</tr>
<tr>
<td>Bleomycin-injured lung</td>
<td>(Munger et al., 1999; Shukla et al., 1999)</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>(Tuxhorn et al., 2001; Tuxhorn et al., 2002)</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>(Ronnov-Jessen and Petersen, 1993; Sieuwerts et al., 1998)</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>(Tomasek et al., 2002)</td>
</tr>
<tr>
<td>Ovary carcinoma</td>
<td>(Tomasek et al., 2002)</td>
</tr>
</tbody>
</table>
There are some locations in vivo, such as the lung alveolar septa and early granulation tissue, where the fibroblasts exhibit all of the other characteristics of myofibroblasts, but do not express α-sma (Hinz et al., 2001b; Tomasek et al., 2002). These cells have recently been termed ‘proto-myofibroblasts’ and are thought to be a pre-requisite to the fully differentiated myofibroblast phenotype. These partially differentiated fibroblasts develop under mechanical stress. They form actin-containing stress fibres, focal adhesion sites and express fibronectin at the cell surface. These cells can generate low level contractile force. Increased mechanical tension, production of ED-A fibronectin and action of growth factors such as TGF-β1 promote the modulation from proto-myofibroblast to fully differentiated myofibroblast (Bochaton-Piallat et al., 2000). These cells are able to generate a greater amount of contractile force, characterised by the expression of α-sma in their stress fibres (Tomasek et al., 2002; Vaughan et al., 2000). The differentiation from fibroblast to proto-myofibroblast to myofibroblast can be summarised by Figure 1.3 (taken from Tomasek et al., 2002). The difference between fibroblast and proto-myofibroblast is clearly seen in vivo, but this is less obvious in vitro, as due to the presence of serum, almost all fibroblasts develop features of the proto-myofibroblast (Tomasek et al., 2002; Vaughan et al., 2000).
Figure 1.3 Stages of differentiation of fibroblast to myofibroblast (Tomasek et al., 2002).

Fibroblasts can form partially differentiated fibroblasts called ‘proto-myofibroblasts’ when they are under some degree of mechanical tension. They form actin-containing stress fibres, focal adhesion sites and express ED-A fibronectin at the cell surface. These cells are able to generate low level contractile force. Differentiation to myofibroblast is characterised by the expression of α-sma in more extensively developed stress fibres.
Myofibroblasts are able to produce abundant amounts of ECM proteins and have been shown to synthesize urokinase-type plasminogen activator (uPA), fibroblast activation protein (FAP), MMPs, TIMPs, TGF-β and other growth factors that promote angiogenesis and aid wound healing. Differentiation from fibroblast to myofibroblast can be induced by a number of different growth factors and cytokines such as TGF-β1, -β2, granulocyte macrophage-colony stimulating factor (GM-CSF), PDGF, FGFs and connective tissue growth factor (CTGF) both in vivo and in vitro (Desmouliere et al., 1993; Desmouliere and Gabbiani, 1994; Gabbiani, 1998; Leask et al., 2002; Serini and Gabbiani, 1999). It is the inductive mechanisms by which these substances cause the fibroblast to myofibroblast differentiation which are poorly understood.

1.2.1 Myofibroblasts in Normal Tissues

Myofibroblasts have been found in normal tissues which undergo a high degree of remodelling. Principle areas where they have been described are the intestinal mucosa, pulmonary alveolar septa, breast stroma, uterus, liver, ovary, prostate, bone marrow and oral mucosa. A summary of location and function can be seen in Table 1.3. (Date et al., 2000; Desmouliere and Gabbiani, 1996; Schmitt-Graff et al., 1994; Schurch et al., 1998).
Table 1.3 Myofibroblasts: normal distribution and function (Powell et al., 1999a)

<table>
<thead>
<tr>
<th>Tissue or Organ</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach and intestine</td>
<td>Regulation of motility, &quot;pacemaker&quot; activity</td>
</tr>
<tr>
<td>Interstitial cell of Cajal</td>
<td>Epithelial growth and differentiation; contraction of gastric glands and intestinal villi; regulation of intestinal absorption and secretion</td>
</tr>
<tr>
<td>Subepithelial myofibroblast</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Alveolus formation</td>
</tr>
<tr>
<td>Interstitial contractile cell</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>Epithelial growth and differentiation; possibly contraction and expulsion of milk</td>
</tr>
<tr>
<td>Stromal myofibroblast</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>Regeneration of endometrium after menses</td>
</tr>
<tr>
<td>Endometrial myofibroblasts</td>
<td>Structure of placental stem villus</td>
</tr>
<tr>
<td>Placental myofibroblasts</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Endothelial and sinusoid structure and function; regulation of blood flow; vitamin A storage</td>
</tr>
<tr>
<td>Perisinusoidal stellate cell (Ito cell)</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>Meiosis and ovulation</td>
</tr>
<tr>
<td>Theca cells</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Nurture stem cells and promote hematopoiesis</td>
</tr>
<tr>
<td>Stromal cells</td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>Growth and differentiation of prostate gland</td>
</tr>
<tr>
<td>Stromal cells</td>
<td></td>
</tr>
<tr>
<td>Mouth</td>
<td>Attachment of teeth</td>
</tr>
<tr>
<td>Periodontal ligament</td>
<td>Structure of gum</td>
</tr>
<tr>
<td>Gingival myofibroblasts</td>
<td>Structure of palate</td>
</tr>
<tr>
<td>Palatal mucosa</td>
<td></td>
</tr>
</tbody>
</table>

1.2.2 Myofibroblasts in Wound Healing

1.2.2.1 Adult Wound Healing

The following is a brief summary of the role of myofibroblasts in adult wound healing, myofibroblast involvement in wound healing has also been discussed in other sections, as referred to throughout. See also Section 1.1 - ‘Wound Healing’, for a complete overview of the wound healing process.

Fibroblasts and myofibroblasts play a vital role in wound repair, presumably as an extension to their role in normal growth and differentiation and in maintenance of normal tissue integrity. In wound healing they are involved in the formation and repair of the ECM, release many cytokines, growth factors, chemokines and
inflammatory mediators, and the contractile nature of myofibroblasts facilitates wound contraction - they are capable of generating strong contractile forces to bring the wound edges together. (Gabbiani, 2003; Gailit et al., 1996; Grinnell, 1994; Grinnell, 2000; Grinnell and Ho, 2002; Powell et al., 1999a; Tomasek et al., 2002). Wound contraction involves a complex series of events, encompassing cells, ECM and cytokines. Around day 7-14 post wounding, fibroblasts present in the wound differentiate into myofibroblasts, and this differentiation coincides with the beginning of wound contraction (Singer and Clark, 1999). Contraction requires the adhesion of cells to each other (via gap junctions) and to the surrounding ECM (via focal contacts and integrin receptors). After wound contraction has finished, wound and scar remodelling take place. The myofibroblasts disappear via apoptosis and leave a scar which becomes less cellular and new connective tissue fibroblasts emerge (Desmouliere et al., 1993; Hakkinen et al., 2000; Hinz et al., 2001b; Levinson et al., 2001; Schmitt-Graff et al., 1994). Indeed, it is because the myofibroblasts persist that gives rise to pathological conditions (See Section 1.2.3 - 'Myofibroblasts in Pathological Conditions').

1.2.2.2 Fetal and Oral Wound Healing

In contrast to adult wound healing, early-gestation fetal wounds are well known to heal rapidly and without scar formation (Bullard et al., 2003; Moulin and Plamondon, 2002; Nodder and Martin, 1997; Roh et al., 2001), as characterised by the lack of fibroplasia, inflammation, and collagen deposition. This observation was first noted over 20 years ago; however the specific mechanisms for regulation of the scarless phenomenon still remain poorly understood. There are a number of both intrinsic and extrinsic differences between the adult and fetus, which may influence wound healing. One major difference is that the fetal wound is surrounded by a warm, sterile, fluid-filled environment rich in growth factors and ECM components (McCallion and Ferguson, 1996). The matrix macromolecule hyaluronan (HA) has been well documented in playing a role in the scarless phenomenon of fetal wound healing and it has been reported to inhibit scar formation when applied topically to adult dermal healing wounds (Ellis and Schor, 1996). Unlike adult fibroblasts, fetal fibroblasts do not decrease the amount of HA they produce with increasing cell
fetal fibroblasts also have two- to four-fold more HA receptors than adult fibroblasts (Bullard et al., 2003; Ellis and Schor, 1996). Myofibroblasts are absent in early-gestation, scarless wound healing, but have been observed in late-gestation fetal wounds and the onset of myofibroblast production has been seen to coincide with the beginning of scarring (Bullard et al., 2003). Fetal and adult wounds also differ with respect to the presence and participation of various cytokines and growth factors, particularly EGF, PDGF and TGF-β (Bullard et al., 2003; Ellis and Schor, 1996; McCallion and Ferguson, 1996). TGF-β has been the most widely studied and is believed to play a major role in scar production (Shah et al., 1995), yet still its role in fetal repair remains unclear. TGF-β is known to cause production of ECM components by fibroblasts, chemoattraction of monocytes and leucocytes, induction of angiogenesis, induction of wound contraction and regulates the production of other inflammatory cytokines and growth factors (Dillon et al., 1994). There are 3 isoforms of TGF-β: β1, β2, and β3. Low levels of TGF-β1 and high levels of TGF-β3 (Bullard et al., 2003) have been associated with scarless healing. A combination of all of these factors could play a part in the phenomenon of scarless wound healing.

There has been a general observation that adult oral wounds have the ability to heal more quickly and with less scarring than extraoral wounds such as in the case of skin (Hakkinen et al., 2000; Irwin et al., 1994; Lee and Eun, 1999; Schor et al., 1996; Stephens et al., 2001b). The reasons for this privileged healing of the oral mucosa are unknown, but may be due to factors present within the saliva and the specific microflora of the oral cavity (Hakkinen et al., 2000). Additionally it may be that phenotypic differences between the cells themselves exist, indeed several investigations have compared adult oral and dermal fibroblasts with fetal fibroblasts. These studies have revealed that oral fibroblasts share some phenotypic properties to fetal fibroblasts, such as increased growth and migration, production and response to cytokines and production of migration stimulating factor (Hakkinen et al., 2000; Schor et al., 1996). It is possible that the unique phenotype of oral fibroblasts contributes to the privileged healing seen within the adult oral cavity.
1.2.3 Myofibroblasts in Pathological Conditions

1.2.3.1 Keloids/Hypertrophic Scarring

Keloids and hypertrophic scars are benign fibrous growths formed by the excessive deposition of connective tissue, in particular collagen (English and Shenefelt, 1999; Neely et al., 1999). Contrary to normal scars, keloids and hypertrophic scars result typically in disfigurement, contractures, itchiness and pain (Tuan and Nichter, 1998). Clinically, hypertrophic scars form within the boundary of the original wound, whereas keloids extend beyond the original wound boundary and tend to remain elevated (Bayat et al., 2003; Neely et al., 1999). Histologically, fibronectin deposition is as much as 4 times greater within keloid scars compared to normal dermal scars; similarly, collagen synthesis is around 20 times greater in keloid scars and 3 times greater in hypertrophic scars (English and Shenefelt, 1999). Myofibroblasts have been implicated in this type of excessive scarring due to their ability to produce abundant amounts of ECM proteins and contractile capability, indeed hypertrophic scars have been shown to contain abundant amounts of myofibroblasts (Tuan and Nichter, 1998). TGF-β and its actions in fibroblast proliferation, differentiation to myofibroblast, migration and ECM deposition has been implicated in keloids and hypertrophic scars (Schurch et al., 1998; Tuan and Nichter, 1998).

1.2.3.2 Fibrosis

Repeated tissue injury, followed by cycles of inflammation and repair can lead to excessive scarring, or fibrosis. Fibrosis is characterised by the accumulation of excessive numbers of fibroblasts and myofibroblasts, the deposition of abundant amounts of ECM proteins and the distortion of normal tissue architecture (Sime and O'Reilly, 2001). Myofibroblasts have been implicated in fibrosis of many organs such as the kidney (Badid et al., 2001; el Nahas et al., 1997; Lewis and Norman, 1998; Muchaneta-Kubara and el Nahas, 1997; Norman et al., 2000; Schelling et al., 2002), lung (Hashimoto et al., 2001; Levine et al., 2000; Scaffidi et al., 2001; Sheppard, 2001; Sime and O'Reilly, 2001), liver (Andrade et al., 1999; Kato et al.,...
2001) and scleroderma lesions (Denton and Abraham, 2001; Folger et al., 2001; Leask et al., 2002; Ronnov-Jessen and Petersen, 1993). The differentiation to myofibroblasts is an early event in the development of fibrosis (Badid et al., 2001; Denton et al., 2003; Grupp et al., 2001; Schelling et al., 2002) and these cells are presumed to play a pivotal role in the accumulation and deposition of ECM proteins. Therefore understanding the mechanisms of their differentiation could have a significant impact in controlling the progression of such pathological states.

### 1.2.3.3 Tumourigenesis

Historically, most studies of neoplastic transformation and progression have focussed on the tumour cell. However, in addition to transformed cells, tumours are also partly composed of stromal tissue comprising fibroblasts, ECM, newly formed blood vessels and immune components. Although stroma was initially thought to passively support tumour development, there is increasing evidence that it actually contributes to malignant progression (Derynck et al., 2001; Liotta and Kohn, 2001; Pupa et al., 2002).

A common finding in many types of solid tumour is that stromal fibroblasts become 'activated' and express a number of contractile proteins, particularly \( \alpha \text{-sma} \) (Huber et al., 2003; Tlsty and Hein, 2001). These cells have been referred to as peritumour fibroblasts, carcinoma-associated fibroblasts and activated stroma, but are now more commonly called myofibroblasts. The process of activation of fibroblasts is associated with increased proliferation, increased deposition of collagen and spliced-variant forms of fibronectin, assembly of vinculin-containing fibronexus adhesion complexes and acquisition of smooth muscle cell characteristics, e.g. \( \alpha \text{-sma} \). The structural changes, such as assembly of fibronexi, and accumulation of cytoskeletal \( \alpha \text{-sma} \), modulate myofibroblast contractility and reduce their migratory potential (Serini and Gabbiani, 1999). Conversely, myofibroblasts upregulate secretion of numerous growth factors, chemokines and cytokines, as well as ECM proteins and proteases (Powell et al., 1999a; Powell et al., 1999b).
Chapter One: Introduction

A number of cytokines including PDGF, IIκ4, insulin-like growth factor II and TGF-β1 may be involved in the differentiation of fibroblasts to myofibroblasts, and these can be derived from a number of different cell types (Kunz-Schughart et al., 2001; Powell et al., 1999a; Powell et al., 1999b; Stamenkovic, 2000). Among these cytokines TGF-β1 is considered to have a central role in inducing the myofibroblastic phenotype, because it is capable of upregulating fibroblast α-sma and collagen both in vitro and in vivo (Tuxhorn et al., 2001). In many types of cancers TGF-β1 is over-expressed by carcinoma cells (Ronnov-Jessen and Petersen, 1996), and it has been proposed previously that expression of this cytokine by breast and prostate carcinoma cells induces reactive stroma (Ronnov-Jessen and Petersen, 1996; Tuxhorn et al., 2002). TGF-β1 has many effects, in addition to inhibiting epithelial cell proliferation it also promotes secretion of matrix proteins and proteases. Its powerful anti-proliferative effect has led to it being thought of as a tumour suppressor in carcinomas. However, it is now apparent that TGF-β1 may be pro-oncogenic, driving malignant progression, invasion and metastasis (Wakefield and Roberts, 2002). This is partly explained by observations of carcinomas, including oral squamous cell carcinoma (OSCC), that become refractory to the anti-proliferative effect of TGF-β1. However, another mechanism by which TGF-β1 could promote tumour development is by inducing the differentiation of stromal fibroblasts, producing an activated myofibroblast-rich stromal microenvironment. For example, it has been shown that TGF-β1 produced by breast cancer cells activates normal breast stromal fibroblasts and promotes them to produce uPA, a serine protease important in cancer cell invasion and metastasis (Sieuwerts et al., 1998). Such changes have a potential role in tumourigenesis since if tumour stroma becomes activated and immobilized in the vicinity of tumour cells, paracrine interactions may be established between the separate cellular compartments, some of which could encourage tumour development.

To date, there has been little work investigating potential interactions between squamous cell carcinomas and the surrounding stroma. Maas-Szabowski et al., (2000) showed that IL-1 produced by epidermal keratinocytes induced expression of keratinocyte growth factor by dermal fibroblasts, which in turn stimulated keratinocyte proliferation. It has also been suggested that PDGF-activated stromal
cells may maintain elevated keratinocyte proliferation via a paracrine mechanism (Skobe and Fusenig, 1998). It has been demonstrated (Ramos et al., 1997) that peritumour fibroblast-conditioned medium promoted OSCC migration on tenascin, and that this effect could be partially inhibited by blocking EGF, TGF-β1 or hepatocyte growth factor/scatter factor (HGF/SF). Additionally, paracrine interactions have been demonstrated between squamous carcinoma cells and other cell types found in stroma. Liss et al.,(2001) found tumour-derived TGF-β1 and monocyte chemotactic protein-1 attracted and activated monocytes. They suggested (tumour necrosis factor) that macrophages secreted TNFα and IL-1, which in turn stimulated tumour cells to produce IL-8 and VEGF, the latter cytokine then inducing angiogenesis.

1.2.4 Factors Influencing Myofibroblast Differentiation

Little is known about the actual mechanisms involved in myofibroblast differentiation, although a number of mediators of differentiation have been identified, such as TGF-β, ECM proteins, GM-CSF, PDGF and FGFs. The role of TGF-β and ECM proteins are discussed in more detail below.

1.2.4.1 Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β1 has been the most widely studied and is one of the most well characterised stimulators of wound healing, whose actions encompass cell migration, proliferation and differentiation of a wide variety of cells. It has been shown to be a potent inducer of the myofibroblast phenotype (measured by α-sma expression) both in vitro and in vivo. (Desmouliere et al., 1993; Desmouliere, 1995; Vaughan et al., 2000; Yokozeki et al., 1997). However, the intracellular signalling pathways leading to myofibroblast differentiation and mechanism of induction have not yet been determined (Desmouliere et al., 1993; Hashimoto et al., 2001). The source of TGF-β in damaged tissue is from macrophages, platelets, epithelial cells, or from the myofibroblast itself. Of the three isoforms of TGF-β (β1, β2 and β3), TGF-β1 and -β2 induce myofibroblast differentiation both in vivo and in vitro. TGF-
β3 however, acts as a negative regulator of the myofibroblast phenotype \textit{in vivo}, but induces differentiation \textit{in vitro} (Serini and Gabbiani, 1999). As well as stimulating α-sma expression, TGF-β1 is known to upregulate fibrillar collagen and fibronectin expression (Serini et al., 1998). Integrin mediated adhesion to ECM is known to regulate the transmission of activated growth factor receptor signaling pathways and that the interaction between integrin signaling pathways is required for proper stimulation of cell growth, differentiation and gene expression (Clark et al., 1998; Clark and Brugge, 1995; Serini et al., 1998). TGF-β1 receptors signal from the cell membrane to the nucleus via a Smad family of signal transducers (see Section 1.3.4 - ‘TGF-β Intracellular Signalling’). TGF-β1 is also discussed in more detail in Section 1.3 - ‘Transforming Growth Factor β’.

1.2.4.2 Extracellular Matrix

The ECM is a complex network of polysaccharides and proteins secreted locally by cells, in which they are embedded (Alberts et al., 1994). It serves as a structural element and comprises a substantial part of any tissue. It forms a network which, as well as imposing structure, provides a reservoir for information transfer (Mutsaers et al., 1997). The ECM is comprised of a large number of components with varying structural and cell regulatory functions depending on the tissue. A summary of the components of the ECM can be seen by the following table (Table 1.4; adapted from Mutsaers et al., 1997).
Table 1.4 ECM components and function

<table>
<thead>
<tr>
<th>ECM Component</th>
<th>Cellular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagens</td>
<td>Tissue architecture, tensile strength, cell-matrix interactions, matrix-matrix interactions</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Tissue architecture, cell-matrix interactions, matrix-matrix interactions, cell proliferation, cell migration</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>cell-matrix interactions, matrix-matrix interactions, haemostasis,</td>
</tr>
<tr>
<td>Elastin</td>
<td>Tissue architecture and elasticity</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>Cell-matrix interactions, matrix-matrix interactions, cell proliferation, binding and storage of growth factors</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Cell-matrix interactions, matrix-matrix interactions, cell proliferation, cell migration</td>
</tr>
<tr>
<td>Laminin</td>
<td>Basement membrane component, cell migration, cell-matrix interactions</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Cell proliferation, cell migration, haemostasis</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Modulates cell-matrix interactions, anti-adhesive, anti-proliferative</td>
</tr>
<tr>
<td>SPARC (secreted protein acidic and rich in cysteine)</td>
<td>Modulates cell-matrix interactions, anti-adhesive, anti-proliferative</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Modulates cell-matrix interactions</td>
</tr>
<tr>
<td>Adhesion Molecules</td>
<td>Cell surface proteins mediating cell adhesion to matrix or adjacent cells, Mediators of transmembrane signals</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>Mediates platelet adhesion, carrier for procoagulant VIII</td>
</tr>
</tbody>
</table>

The specific ECM molecules fibronectin, vitronectin and collagen will be discussed in more detail in Section 1.4.3 - 'Ligand Binding to Integrins'. The ECM plays many roles in the wound repair process, including that proteins interact directly with the cell surface receptors to initiate signal transduction pathways and to modulate the pathways that have been triggered by growth factors and cell differentiation (Streuli, 1999).

Myofibroblasts are able to attach to the ECM at discrete adhesive sites called fibronexi. (For more detail see Section 1.4 - 'Integrins' and more specifically Section 1.4.4 - 'Integrin Clustering', also Figure 1.3 and Section 1.2.1 - 'Introduction'). Adhesion to the ECM via this mechanism can trigger intracellular signalling pathways, thereby controlling cell shape, migration, proliferation and
differentiation (Serini and Gabbiani, 1999). Recently it has been identified that the
differentiation of fibroblasts to myofibroblasts requires the presence of matrix
molecules, in particular, the ED-A domain of fibronectin. It has been shown that in
both skin granulation tissue (Serini et al., 1998), and a hepatic wound model
(Jarnagin et al., 1994), that the presence of the fibronectin ED-A is required to
stimulate the TGF-β induced differentiation to myofibroblast. The precise
mechanism of involvement of the fibronectin is yet to be discovered.
1.3 Transforming Growth Factor β

1.3.1 Introduction

The TGF-β superfamily consists of a large number of structurally related molecules each capable of regulating a large variety of cellular responses, including cell proliferation, differentiation, migration, ECM synthesis, adhesion and apoptosis, in many different cell types (Eickelberg, 2001; Kloos et al., 2002; Ling and Robinson, 2002; Massague, 1998; Moustakas et al., 2001; Nakao et al., 1997; ten Dijke et al., 2000; Verrecchia and Mauviel, 2002). TGF-β family members include TGF-βs, bone morphogenetic proteins (BMPs), activins, nodals, anti-Müllerain hormone along with many other related factors found in vertebrates, insects and nematodes (Massague, 1998; Moustakas et al., 2001; Verrecchia and Mauviel, 2002). Such members of the TGF-β family have crucial roles in embryogenesis, modulation of the immune system, growth, development and wound repair of most tissues (Hakkinen et al., 2000; Heldin et al., 1997; O'Kane and Ferguson, 1997; ten Dijke et al., 2000).

1.3.2 Latency, and Activation of TGF-β

TGF-βs are mainly produced in a latent form, requiring activation before they can bind to their receptor and initiate an intracellular signalling cascade. Latent, inactive forms of TGF-β in the ECM may provide the surrounding cells with a readily available source of the growth factor. Latent TGF-β’s are secreted by cells as large pro-peptide molecules consisting of 3 distinct regions; an amino terminal signalling sequence, a pro-domain: LAP (latency-associated peptide), and a C-terminal mature TGF-β molecule (Koli et al., 2001; Massague, 1998; O'Kane and Ferguson, 1997). LAP and TGF-β are non-covalently associated, this dimer is known as the small latent complex (SLC). LAP can furthermore disulfide-link to members of another family of proteins called the latent TGF-β-binding proteins (LTBP’s), the
combination of the SLC and LTBP is known as the large latent complex (LLC), and it is in this form that the TGF-β is predominantly secreted (Massague, 1998; Munger et al., 1998; Munger et al., 1999; Oklu and Hesketh, 2000) See Figure 1.4. LTBP can bind to the ECM thereby incorporating TGF-β. Human β1 and β3 LAP, and LTBP-1 contain the integrin recognition sequence RGD, suggesting that the LLC may be a possible ligand for integrins (Munger et al., 1998; Munger et al., 1999).

Once secreted by the cell, TGF-β1 can exist as the LLC, or as the SLC (which is retained in fibrin clots after release from surrounding platelets). The size of the LLC is 200kD, with the mature TGF-β molecule being a mere 25kD, consisting of two monomers of 12.5kD each (O’Kane and Ferguson, 1997). LAP and LTBP must both be removed before TGF-β becomes active. This is a complex process, that can be brought about by the addition of acid, alkali, proteases, or glycosylases in vitro, causing the release of the TGF-β molecule (Massague, 1998; Verrecchia and Mauviel, 2002). Fewer methods are known in vivo, and the exact mechanism of activation has not yet been fully established.
Figure 1.4 Schematic diagram representing the large latent TGF-β complex. The small latent complex consists of the TGF-β and LAP dimers. The LTBP associates with ECM molecules by its N-terminus. Modified from Saharinen et al., (1999).
1.3.3 TGF-β Receptor Activation

Once activated extracellularly, TGF-β family members initiate their intracellular response by binding to receptors on the cell surface. These TGF-β receptors have intrinsic serine/threonine kinase activity (Heldin et al., 1997; Huse et al., 2001; Kloos et al., 2002; Massague, 1998; Verrecchia and Mauviel, 2002). There are two TGF-β receptors - type I and type II, they are structurally similar, with extracellular cysteine-rich domains and intracellular kinase domains. The activated TGF-β binds to and phosphorylates the type II receptor at its serine residues - the type II receptor has a region in the juxtamembrane domain rich in glycine and serine amino acid residues (GS domain). This phosphorylation activates the type I receptor to complex with the type II receptor, as the type I receptor cannot directly bind the active TGF-β molecule in the complex. It is the type I receptor that then determines the downstream signalling pathways within the cell (See Figure 1.5) (Heldin et al., 1997; Koli et al., 2001; Massague, 1998; ten Dijke et al., 2000).

1.3.4 TGF-β Intracellular Signalling

Once activated, type I receptors initiate intracellular signalling pathways which carry signals to the nucleus by phosphorylation of specific Smad proteins (Heldin et al., 1997; Kloos et al., 2002; ten Dijke et al., 2000; Verrecchia and Mauviel, 2002). Genetic screens in *Drosophila* and *Caenorhabditis elegans* produced the first Smad-related genes. ‘Smad’ is derived from a fusion of the two gene names *Drosophila mothers against decapentaplegic* (Mad) and *Caenorhabditis elegans*, small (Sma) (Heino et al., 1989; Heldin et al., 1997; Kloos et al., 2002; Massague, 1998; ten Dijke et al., 2000; Verrecchia and Mauviel, 2002). There are three sub-families of Smad proteins, based on both structural and functional differences, they are receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads) and inhibitory Smads (I-Smads) (ten Dijke et al., 2002). The Smad family can be summarised in the following table (Kloos et al., 2002): (Table 1.5)
Table 1.5 The vertebrate Smad protein family

<table>
<thead>
<tr>
<th>R-Smads</th>
<th>Co-Smads</th>
<th>I-Smads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad1</td>
<td>Smad4</td>
<td>Smad6</td>
</tr>
<tr>
<td>Smad2</td>
<td>Smad4β/10</td>
<td>Smad7</td>
</tr>
<tr>
<td>Smad3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smad5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smad8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Activation of the type I receptor recruits and phosphorylates R-Smad (on two serine residues at it's C termini), by association with a membrane-bound protein called SARA (Smad Anchor for Receptor Activation). Activated R-Smad then forms a complex with a Co-Smad, which translocates into the nucleus to activate transcriptional responses. Once in the nucleus, the complex initiates such responses by binding to a specific promoter region of target genes (Ashcroft and Roberts, 2000; Heldin et al., 1997; Kloos et al., 2002; Massague, 1998; ten Dijke et al., 2000). Inhibitory Smads, as their name suggests, prevent the phosphorylation of R-Smads by type I receptor by forming stable associations with the receptor.
Figure 1.5 Schematic diagram representing downstream signalling cascade of the TGF-β1/Smad pathway. Modified from (Heldin et al., 1997; Kloos et al., 2002; Massague, 1998; ten Dijke et al., 2000; Verrecchia and Mauviel, 2002).
1.3.5 TGF-β and Wound Repair

TGF-β is known to have many distinct roles in the wound repair process as previously mentioned. This section aims simply to summarise the effects that TGF-β has in wound repair in one place.

Of the many different cytokines involved in wound repair, TGF-β has undoubtedly the broadest effects. It is known to exert its effects from inflammation at the initiation of healing, right through to the final scar remodelling phase. TGF-β is released from degranulating platelets, as well as being secreted by all major cell types involved, including macrophages, lymphocytes, fibroblasts, epithelial cells, endothelial cells and smooth muscle cells (Crowe et al., 2000; O'Kane and Ferguson, 1997; Roberts and Sporn, 1996), it can also be produced in an autocrine fashion by the cell from which it is affecting, thus amplifying its own response.

Initially, TGF-β is released in its large latent complex form from the α granules of platelets when they degranulate. Once activated, TGF-β acts as a potent stimulator of chemotaxis, attracting cells to the wound site, and then stimulating the formation of granulation tissue. As well as an initial chemoattractant and activator for inflammatory cells to the wound site, TGF-β can also play a role in suppressing and reversing such inflammatory responses, along with other cytokines and inflammatory suppressors such as IL-4. TGF-β1 has been shown to stimulate the initial keratinocyte migration in re-epithelialization, and is involved in neovascularization. However, although these actions of TGF-β1 are of utmost importance to the wound repair process, it is the effects of TGF-β during the wound contraction and ECM production and remodelling phase that is of significance to this work. TGF-β1 has been shown to be a potent inducer of the myofibroblast phenotype and it is these cells which are involved in wound contraction, synthesis and secretion of ECM molecules, and has also been implicated in upregulation of integrin receptors (Cai et al., 2000). TGF-β has been shown to play a pivotal role in the initiation and degree of conditions such as fibrosis, and attempts to inhibit or antagonize TGF-β activity have led to increasingly positive results in terms of downregulating or reversing such
pathological conditions (Hakkinen et al., 2000; Heldin et al., 1997; O'Kane and Ferguson, 1997; ten Dijke et al., 2000).
1.4 Integrins

1.4.1 Introduction to Integrins

Integrins are a large family of transmembrane, cation dependent, cell-cell and cell-ECM receptor proteins. They are the predominant protein family involved in binding and response of cells to their surrounding matrix - mediating bidirectional interactions between the ECM and the actin cytoskeleton. Integrins are involved in a wide variety of cellular functions, which regulate the migration, growth and survival of the cell. Integrins have been shown to play key roles in haemostasis, development, immune responses, tumourigenesis and leukocyte traffic. They have been implicated in many human diseases, are a target of effective drugs against thrombosis and inflammation, and can also be receptors for many viruses and bacteria (Calderwood et al., 2000; Cukierman et al., 2001; Ehrlich et al., 1998; Geiger et al., 2001; Hynes, 2002; McDonald, 2000; Yamada et al., 1996).

1.4.2 Integrin Structure

Functional integrins consist of two transmembrane glycoprotein subunits called alpha (α) and beta (β). The α subunits vary in size from 120–180 kD and the β subunits from 90–110 kD, they are non-covalently bound and serve as a link between the ECM and the cytoskeleton of the cell (Giancotti and Ruoslahti, 1999; Holly et al., 2000; Hynes, 1992; Hynes and Zhao, 2000; Miranti and Brugge, 2002). 18 different α subunits and 8 β subunits have so far been identified (Hynes, 2002), which assemble into 24 distinct integrins. Figure 1.6 illustrates the structure of an individual integrin receptor, and Figure 1.7 shows the known αβ integrin combinations. The transmembrane and cytoplasmic domains are not required to form the heterodimer as truncated integrins which lack transmembrane and cytoplasmic tails can still form functional αβ dimers (Dana et al., 1991). Not all αβ combinations exist, for example, the β4 subunit can only form a heterodimer with the α6 subunit, and the β5, β6 and β8 subunits can only form heterodimers with the
αν subunit. In contrast, the β1 subunit can form heterodimers with 12 different α subunits (http://integrins.hypermart.net). Each integrin combination has a distinct ligand binding specificity. Integrins have been subdivided into families depending on their β subunit. For example, the β1 subfamily is the most widely expressed in many different cell types and principally involved in cell-substrate interactions. The β2 subfamily is expressed on leukocytes only, and is involved in cell-cell interactions and the β3 subfamily can be found on platelets. Most integrins can combine with more than one ligand and one ligand is often recognized by more than one integrin (Cheng et al., 2000). However, different integrins that bind to the same ligand may activate separate intracellular signalling pathways and initiate diverse responses within the cell. See section 1.4.3 ‘Ligand Binding to Integrins’, for discussion with regards to specific integrin ligands. Table 1.6 summarises the integrins and their ligand specificity.

Each integrin subunit is composed of a large, ligand-binding, globular N-terminal extracellular domain (1000 residues for the α subunit and approximately 750 residues for the β subunit), a transmembrane domain and a short (50 residues or less) C-terminal cytoplasmic tail, which links to cytoplasmic actin filaments (Alberts et al., 1994; Loftus et al., 1994). The exception to this being the β4 tail, which has a cytoplasmic domain of over 1000 residues (Hogervorst et al., 1990) and is linked primarily to intermediate filaments instead of actin filaments. The structure between the α subunits is very similar – all contain 7 homologous repeats of 30-40 amino acids in their extracellular domain, spaced out between stretches of 20-30 amino acids. Three or four of these repeats contain sequences with cation binding properties. It is these sequences that are thought to be involved in ligand-binding, as interaction of integrins with their ligand is cation dependent (Ca^{2+}, Mn^{2+} or Mg^{2+}, depending on the subunit).
Integrins are composed of two transmembrane glycoprotein subunits called α and β. Each subunit consists of a large ligand-binding, globular N-terminal extracellular domain (1000 residues for the α subunit and approximately 750 residues for the β subunit), a transmembrane domain and a short (50 residues or less) C-terminal cytoplasmic tail, which links to cytoplasmic actin filaments (Exception = the β4 tail which has a cytoplasmic domain of over 1000 residues).
Figure 1.7 Integrin subunit combinations (redrawn from ref. (Hynes, 2002)).
18 different α subunits and 8 β subunits have so far been identified, which assemble into 24 distinct integrins.
1.4.3 Ligand Binding to Integrins

1.4.3.1 Introduction

Integrins have the ability to bind to many diverse ligands via their extracellular domain, and it is the specific α/β pairings which denote the ligand binding specificity. Ligands include (most importantly) components of the ECM (See Table 1.6) - which reflects the significant function of integrin cell adhesion to their surrounding matrix as well as, components of microorganisms (Loftus et al., 1994; Plow et al., 2000) - which utilize integrins to gain entry into the cell and counter-receptors present on other cells, such as ICAMs, VCAM, MadCAM, or E-cadherin (Arnaout et al., 2002; Clark and Brugge, 1995; Hynes, 1999) - which mediate cell-cell interactions.

Many integrins recognise the short tripeptide arginine-glycine-aspartate (RGD) sequence, which is commonly found in ECM components such as fibronectin, vitronectin, collagen, laminin, thrombospondin, fibrinogen and von Willebrand factor (Mohri et al., 1996; Porter and Hogg, 1998). Some integrins will recognise one or more of these ligands in terms of their RGD sequence, whereas others are more restricted in their ligand-binding specificity. For example, αvβ3 will recognise fibronectin, vitronectin, thrombospondin, von Willebrand factor via it’s RGD sequence, whereas αvβ5 will recognise only fibronectin, and αvβ1, only fibronectin and vitronectin, yet all can be inhibited by using the same RGD peptides. It is therefore possible that there are several regions within a ligand that contribute to the specificity of ligand-binding interactions. Not all integrin adhesion is RGD dependant - whereas the integrin α5β1 binds fibronectin through it’s RGD sequence, the α4β1 receptor binds to fibronectin via the leucine-aspartic acid-valine (LDV) sequence.

Experimentally, genetically altered mice have provided a valuable insight into defining the function of different integrins. As can be seen from Table 1.6, β1
integrin is the most promiscuous subunit, combining with 12 different α subunits and binding to many extracellular ligands. Deletion of the β1 integrin subunit gene has massive implications in normal growth and development. Stephens et al and Fassler & Meyer recorded early embryonic lethality (Fassler and Meyer, 1995; Stephens et al., 1995) upon deletion of the gene, and Bloch et al recorded that β1 integrin is essential for benign tumour cell growth and normal angiogenesis as deletion caused much smaller, or absent cell masses, and a diffusely deposited connective tissue (Bloch et al., 1997). Mutation in the αv subunit gene is also lethal, although some degree of development proceeds almost normally in the absence of αv, resulting in around 20% of animals being born alive. These animals show extensive angiogenesis and vasculogenesis and organogenesis, resulting in intracerebral haemorrhages and cleft palates and the ability to support sustained tumour growth prior to an early death (Bader et al., 1998). Inhibition of αvβ3 and αvβ5 integrin function has been reported to suppress neovascularization and tumour growth (Kumar et al., 2001). Indeed, mice lacking the β3 and β5 integrins show enhanced angiogenesis, not only supporting tumourigenesis, but resulting in enhanced tumour growth as well (Reynolds et al., 2002). Genetic studies illustrate how essential integrins are in normal growth and development, but could however, underestimate their individual importance due to possible compensation mechanisms among other integrins.
Table 1.6 Integrin ligand specificities.

<table>
<thead>
<tr>
<th>β subunit</th>
<th>α subunit</th>
<th>Known Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1</td>
<td>α1</td>
<td>Collagens, Laminins</td>
</tr>
<tr>
<td>β1</td>
<td>α2</td>
<td>Collagens, Laminins</td>
</tr>
<tr>
<td>β1</td>
<td>α3</td>
<td>Laminins, Fibronectin, Thrombospondin</td>
</tr>
<tr>
<td>β1</td>
<td>α4</td>
<td>Fibronectin, VCAM</td>
</tr>
<tr>
<td>β1</td>
<td>α5</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>β1</td>
<td>α6</td>
<td>Laminins</td>
</tr>
<tr>
<td>β1</td>
<td>α7</td>
<td>Laminins</td>
</tr>
<tr>
<td>β1</td>
<td>α8</td>
<td>Fibronectin, Tenascin</td>
</tr>
<tr>
<td>β1</td>
<td>α9</td>
<td>Tenascin</td>
</tr>
<tr>
<td>β1</td>
<td>α10</td>
<td>Collagens</td>
</tr>
<tr>
<td>β1</td>
<td>α11</td>
<td>Collagens</td>
</tr>
<tr>
<td>β1</td>
<td>αv</td>
<td>Fibronectin, Vitronectin</td>
</tr>
<tr>
<td>β2</td>
<td>αL</td>
<td>ICAMs</td>
</tr>
<tr>
<td>β2</td>
<td>αM</td>
<td>Fibrinogen, ICAMS, ic3B</td>
</tr>
<tr>
<td>β2</td>
<td>αX</td>
<td>Fibrinogen, ic3B</td>
</tr>
<tr>
<td>β2</td>
<td>αD</td>
<td>VCAM, ICAMs</td>
</tr>
<tr>
<td>β3</td>
<td>αIIb</td>
<td>Collagens, Fibronectin, Vitronectin, Fibrinogen, von Willebrand factor, Thrombospondin</td>
</tr>
<tr>
<td>β3</td>
<td>αv</td>
<td>Fibronectin, Vitronectin, Fibrinogen, von Willebrand factor, Thrombospondin</td>
</tr>
<tr>
<td>β4</td>
<td>α6</td>
<td>Laminins</td>
</tr>
<tr>
<td>β5</td>
<td>αv</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>β6</td>
<td>αv</td>
<td>Fibronectin, Tenascin</td>
</tr>
<tr>
<td>β7</td>
<td>α4</td>
<td>Fibronectin, VCAM, MAdCAM</td>
</tr>
<tr>
<td>β7</td>
<td>αE</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>β8</td>
<td>αv</td>
<td>Collagens, Laminins, Fibronectin</td>
</tr>
</tbody>
</table>

1.4.3.2 Fibronectin

Fibronectin is the most widely distributed, and best characterised non-collagenous ECM molecule to date. It is a 250 kD polypeptide that, in tissues, is present in a dimeric or cross-linked multimeric form. The subunits are joined by a pair of disulphide bonds near their carboxyl termini, and each subunit is folded into a series of functionally distinct homology domains separated by regions of polypeptide chain (Alberts et al., 1994; Magnusson and Mosher, 1998; Pankov and Yamada, 2002). Each domain contains binding sites for cell surface receptors and for other ECM components. One domain, which has been isolated for its ability to bind the
fibronectin molecule to integrin receptors on cells is the domain containing the short polypeptide sequence Arg-Asp-Gly (RGD) (Damsky et al., 1992; Gailit et al., 1997; Mohri, 1996; Xiong et al., 2002). Integrin receptors known to bind fibronectin include α5β1 and αvβ3. See Table 1.6.

The three types of homology domains, based on their amino acid composition are termed types I, II and III (See Figure 1.8). The patterns of alternative splicing of fibronectin complicate matters further, since they can either insert, or delete cell-type-specific adhesion sites. For example, the V region of fibronectin can undergo alternative splicing that can produce human fibronectin molecules with 5 different sequence patterns within this region (termed CS1 - CS5). Furthermore, there are two other sites of alternative splicing in fibronectin that result from insertion or deletion of entire type III repeating units, termed ED-A and ED-B. These spliced sites have recently been shown to be involved in wound repair and embryonic cell migration and morphogenesis (Serini et al., 1998; Yamada and Clark, 1996), and more specifically, the ED-A fibronectin isoform has been implicated in the TGF-β1 induced differentiation of fibroblast to myofibroblast (Gabbiani, 2003; Powell et al., 1999a; Serini et al., 1998).

Fibronectin is essential in promoting cell adhesion and affects cell morphology, migration and differentiation, as well as cytoskeletal organisation in many cellular processes throughout the body (Hynes, 1993). Fibronectin has multiple functions within wound healing situations. It is incorporated into the blood clot with fibrin and this fibrin-fibronectin network provides a scaffold for various cells to migrate on. Fibronectin fragments are chemotactic to monocytes, fibroblasts and endothelial cells, it enhances phagocytosis of the wound debris by monocytes, fibroblasts and epidermal cells, and has been reported to activate monocytes and bind to growth factors (Shah, 1998).
Figure 1.8 Structure of the fibronectin dimer (Kindly redrawn and donated by Dr N Al-Hazmi, Eastman Dental Institute, UK).

Figure illustrates the fibronectin dimer molecule joined by a pair of disulphide bonds at the carboxyl termini. The A and B regions of the molecule are differentially spliced regions.
1.4.3.3 Vitronectin

Vitronectin is a multifunctional glycoprotein, which can be found in plasma, ECM and fibrin clots. It is a 75kD monomer which can dissociate to form two chains, one of 65kD and a second of 10kD linked by a disulphide bond (Mosher, 1993). Cell attachment to vitronectin is via an RGD binding domain and mutation of this sequence results in loss of cell attachment (Yamada and Clark, 1996). A wide variety of cells adhere to vitronectin, and attachment is through several different integrin receptors all of the αv family, for example αvβ1, αvβ3, and αvβ5. For a full list, see Table 1.6. Vitronectin has many diverse activities, it adsorbs strongly to tissue culture plastic and a variety of materials, even in the presence of high concentrations of other proteins - this mediates cell spreading through the integrin receptors. It can bind to heparin, through a heparin-binding domain, with high affinity and can neutralize heparin's anti-coagulant activity. Vitronectin can also bind plasminogen activator inhibitor (PAI-1), stabilizing its activity, or even activating mutated forms. These functions of vitronectin are important in many cellular processes, including tissue remodelling, implantation, cell migration and tumour cell invasion (Yamada and Clark, 1996).

1.4.3.4 Collagens

The collagens are the most important family of fibrous ECM proteins with a structural role as their primary function. They are secreted by connective tissue cells as well as by a variety of other cell types. There are 27 different known types of collagen, all are comprised of 3 polypeptide chains that form a unique protein structure called a triple helix. They can be classified into 6 groups, namely fibrillar, filamentous, network forming, fibril-associated with an interrupted triple helix, membrane-associated and multiplexin (Dugina, 2003). Collagen is a major component of connective tissue, skin and bone, comprising around 25% of total protein mass in mammals. The triple helix consists of three polypeptide (α) chains, each with a left-handed helical configuration, wound round each other to form a right-handed superhelix. Its' fundamental structural unit is long (300nm) and thin
Chapter One: Introduction

(1.5nm diameter) and consists of three coiled subunits: two α1 chains and one α2 chain. Each chain contains 1050 amino acids (Darnell et al., 1990), every third amino acid within is a glycine. This is to allow the different chains to be in close proximity in the structure as glycine is the simplest of the amino acids, having only a hydrogen atom as its side chain. Collagen is also rich in the amino acids proline and hydroxyproline (Darnell et al., 1990), which fold and stabilize the three chains. In fibrils, collagen molecules pack together side by side in parallel bundles with adjacent molecules being slightly displaced.

The main receptors for collagen attachment to the cell are the integrin receptors α1β1 and α2β1, for a full list, see Table 1.6. Cytokines such as, TGF-β1, TGF-β2 and IL-1 are known to have an influence on collagen synthesis. TGF-β1 and -β2 have been reported to have a great influence on collagen metabolism by activation of the biosynthesis of several proteins, such as collagen type I and inhibition of proteolytic activities (Eckes et al., 1996).

The balance between collagen synthesis and degradation has a major impact in all living tissues, and any disregulation can have massive implications. In terms of collagen in wound healing, collagen type III (a fibrillar collagen) and fibronectin are secreted by fibroblast cells in the initial phases of wound healing. In the remodeling and scar resolution phase, the collagen type III is gradually replaced with collagen type I, this isoform being the major structural protein of scar tissue (Eckes et al., 1996).

Examples of human genetic disease caused by mutations in collagen genes controlling cell-ECM interactions can be seen below:

- Collagen-I Osteogenesis imperfecta
- Collagen-II Chondrogenesis imperfecta
- Collagen-VI Bethlem myopathy
- Collagen-VII Dystrophic epidermolyis bullosa
1.4.4 Integrin Clustering

Integrins are different from other cell surface receptors in that they bind their ligands with a low affinity \(10^6-10^9\) litres/mole and are usually present at high concentrations on the cell surface (http://integrins.hypermart.net). Integrins can only bind to their ligands once a sufficient number are present at certain places called fibronexi (or focal contacts) and hemidesmosomes (Ballestrem et al., 2001; Geiger and Bershadsky, 2001; Giancotti and Ruoslahti, 1999). When integrins are widely distributed across the cell membrane, no/low adhesion will be present and migration will be extremely slow (Figure 1.9, point A), it is after certain stimuli that these integrins cluster together, for example in focal contacts, and these combined weak affinities for attachment gives rise to a position on the cell surface which has enough adhesive capacity to adhere to the ligand. This situation is extremely valuable to the cell as binding weakly to large numbers of matrix molecules allows the cell to explore its' environment without losing all attachment to it (Figure 1.9, point B). Stronger binding would presumably bind the cell to its surroundings more permanently and the cell would be unable to move (Figure 1.9, point C). This bell-shaped relationship between cell adhesion and migration was first described in 1997 (Palecek et al., 1997). See Figure 1.9.
Chapter One: Introduction

Optimal cell adhesion and migration is a function of matrix concentration, integrin density and integrin activation. When the integrin receptors are widely distributed and inactive, low adhesion and therefore slow migration result (point A), and when binding to the ECM is strong and integrins are in high concentration high adhesion results, rendering the cell unable to move (point C). An optimal rate of cell migration occurs with increasing adhesion (point B).

Figure 1.9 Relationship between cell migration and cell adhesion (Holly et al., 2000)
1.4.5 Integrin Signalling

Integrins were originally termed due to their integration of the extracellular matrix with the cell's cytoskeleton (Aplin et al., 1998; Hynes, 1992; Miranti and Brugge, 2002), however it is now known that these receptors not only act as structural receptors, but also as intracellular signal transducers. Integrins are able to signal through the cell membrane in either direction, and are therefore known as bi-directional signalling receptors. The two types of signal integrins can transmit are: (i) signals from the interior to the exterior of the cell in order to affect ligand binding by a process called integrin activation, or "inside-out" signalling. This involves the propagation of conformational changes of the cytoplasmic tails across the membrane towards the ligand-binding region, and (ii) whereby integrins bind their ligand, which activates intracellular pathways within the cytoplasm, termed "outside-in" signalling (Schoenwaelder and Burridge, 1999; Schwartz and Assoian, 2001). Some of the cellular responses after integrin binding include actin polymerization and cell spreading, induction of gene expression, suppression of apoptosis and induction of cellular differentiation. (Danen and Sonnenberg, 2003; Giancotti and Ruoslahti, 1999; Holly et al., 2000; Juliano and Haskill, 1993; Schwartz and Ginsberg, 2002).

Integrin-mediated cell adhesion can trigger $Ca^{2+}$ influx, activate and deactivate protein tyrosine kinases (e.g. focal adhesion kinase (FAK) and Src), serine/threonine protein kinases (e.g. MAP kinases, protein kinase C), regulate the activity of the Rho family of small GTPases, and activate inositol lipid metabolism (e.g. PIP$_2$ synthesis) (Table 1.9) (Aplin et al., 1998; Berrier et al., 2002; Bhowmick et al., 2001; Danen and Yamada, 2001; Giancotti and Ruoslahti, 1999; Schwartz and Ginsberg, 2002). Since the cytoplasmic tail is devoid of enzymatic activity and generally very short, integrins connect to these down stream signalling pathways via the recruitment of other signalling molecules and adapter proteins.
Table 1.7 Summary of the signalling molecules linked with integrin activation

<table>
<thead>
<tr>
<th>Signalling Molecule</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein tyrosine kinases</td>
<td>FAK</td>
</tr>
<tr>
<td>Src family of tyrosine kinases</td>
<td>Src, Ras, Raf, Mek, Erk, protein kinase C, protein kinase A, Syk/ZAP, ILK, Pyk2, MAP kinases,</td>
</tr>
<tr>
<td>Small GTPases</td>
<td>Rho, Rac, CDC42</td>
</tr>
<tr>
<td>Adaptor proteins</td>
<td>Nck, Grb-2</td>
</tr>
</tbody>
</table>

As mentioned earlier, ECM binding causes clustering of integrins and reorganisation of the actin cytoskeleton, which in turn stimulates further organisation of the integrins and their associated proteins into large cell focal adhesions (Danen and Sonnenberg, 2003; Geiger et al., 2001; Miranti and Brugge, 2002). These focal adhesions contain ECM proteins, integrins and cytoskeletal proteins, which assemble on each side of the membrane to provide a link between the ECM proteins and the cytoskeletal signalling complex and are thought to act as signal transduction units. These structures are known to contain many signalling proteins, such as FAK, Src, ILK and Erk within actin-associated proteins such as α-actinin, vinculin, tensin and paxillin, which promote the assembly of actin filaments. Reorganization of actin filaments into stress fibres in turn causes more integrin clustering, enhancing matrix binding in a positive feedback mechanism (Sastry and Burridge, 2000).

FAK has been implicated as a contributor to integrin signalling pathways for over 10 years (Hanks et al., 1992). FAK is a non-receptor associated protein tyrosine kinase, whose role is implicated fairly early on in the signalling cascade (See Figure 1.10). It is made up from a central catalytic domain contained within large N- and C-terminal non-catalytic domains. Phosphorylation of FAK in response to integrin activation results in the formation of phosphotyrosine docking sites for several classes of signalling molecules. The phosphorylation at Tyr397 creates a binding site for the SH2 domain of Src, phosphatidylinositol 3-kinase, phospholipase C and
adapter protein Grb7 (Hood and Cheresh, 2002; Miranti and Brugge, 2002; Parsons, 2003; Thannickal et al., 2003). The ability of FAK to activate and signal to multiple downstream pathways may be a factor in the ability of integrins to regulate multiple pathways and cellular outcomes.

As integrin-mediated signal transduction pathways are beginning to be defined, it has become apparent that many of the biochemical elements within these signalling pathways are shared with that of many growth factor receptor tyrosine kinase (RTK) signalling pathways, resulting in cell shape, proliferation, migration and differentiation being under joint control (Serini and Gabbiani, 1999). Also, ECM ligands can directly interact with RTKs and integrin dependant adhesion can depend on RTK activation (Thannickal et al., 2003; Trusolino et al., 1998)

With this in mind, it therefore becomes clear that there are many opportunities for integrin signals to modulate growth factor signals and \textit{vice versa} leading to a complex interaction of signalling messages rather than a simple chain of events (Schwartz and Ginsberg, 2002).

The following Figure, taken from a recently published review paper (Figure 1.10) illustrates the complexity surrounding integrin receptors and their integration of the extracellular matrix with the intracellular actin cytoskeleton and downstream signalling pathways.
Figure 1.10  Cytoskeletal and signalling complexes in focal adhesions (Miranti and Brugge, 2002).

The upper panel illustrates how integrin receptors integrate the ECM (green fibrils) with the intracellular cytoskeletal actin network (purple chains of actin) and its associated proteins. The lower panel illustrates the intracellular molecules that make up many diverse downstream integrin signalling cascades to the nucleus.
1.4.6 Regulation of Integrin Function

Integrin-mediated adhesion to it's ligand is a mechanism which is rapidly and precisely regulated, this process is central to integrin function and depends on the interaction between the intracellular tail of the integrin receptor with intracellular proteins (Liddington and Ginsberg, 2002). A distinct feature of integrins is their ability to modulate the level of adhesion rapidly and reversibly. Some integrin subunits are able to undergo alternate splicing of their cytoplasmic domains in a tissue-type specific regulated manner (for example α7, β1), which suggests that there are discrete intracellular functions for individual integrins (Aplin et al., 1998).

Cells can regulate their adhesive function by two methods, the first is by changing the overall strength of cell adhesion, known as integrin avidity, by the clustering of integrins into focal contacts on the cell surface. The second is by changing the strength of attraction of an integrin for its ligand, this is known as integrin affinity. Signalling at the cytoplasmic tail of the integrin alters the shape of the extracellular domain, changing the affinity of the integrin for its ligand and therefore the adhesive capacity of the cell (Carman and Springer, 2003; Hood and Cheresh, 2002; van Kooyk and Figdor, 2000).

Regulation of Integrin Affinity

There are several factors which can affect integrin affinity from outside the cell, both natural and synthetic, including the actions of cations, activating or function blocking antibodies and matrix ligands.

Cations such as Ca$^{2+}$, Mn$^{2+}$ or Mg$^{2+}$ are known to be crucial in integrin-ligand interactions. They have been shown to affect integrin activity by inducing conformational changes within the extracellular domain of the α subunit, resulting in the exposure of activation-dependant epitopes (Dransfield et al., 1992b; Dransfield et al., 1992a). It has been proposed that divalent cations may act as a bridge between ligand and receptor (Mould et al., 1995). For example, each cation (Ca$^{2+}$, Mn$^{2+}$ or Mg$^{2+}$) has been shown to exert distinct effects on the ligand-binding capacity of the
integrin α5β1 to the RGD binding domain of fibronectin. α5β1 integrin has been shown to contain 3 or more cation binding sites, a high affinity site for Mn^{2+}, a low affinity binding site for both Mg^{2+} and Ca^{2+} and a high affinity binding site for Ca^{2+} (Mould et al., 1995). At a low concentration, Ca^{2+} binds to the high affinity Ca^{2+} binding site, which promotes Mg^{2+} binding and induces increased cell adhesion. In contrast, at a high concentration, Ca^{2+} binds to the low affinity binding site and inhibits adhesion. Cations may be present within both natural, or synthetic integrin ligands. It has been suggested that the binding of the appropriate cation to the α subunit of integrins is necessary to induce and/or maintain specific conformations of the integrin, which facilitate it’s interaction with ligands.

Integrin-ligand binding can be activated, or inhibited by using specific antibodies. It has been suggested that binding of antibodies causes conformational changes within the integrin structure to inhibit or activate the receptor’s configuration (Wayner et al., 1988).

Integrin affinity can also be regulated from within the cell through intracellular signalling in a process termed ‘inside-out’ signalling (See section 1.4.5 - Integrin Signalling for more detail). Stimuli received from other cell surface receptors initiate signalling pathways that can interrupt, or impose on integrin cytoplasmic domains and alter the adhesiveness for the extracellular ligand. For example, protein kinase Cs have been demonstrated to be potent activators of the leukocyte integrin αLβ2, and PI3-kinase has also been found to be involved in αLβ2 activation by enhancing ICAM-1 binding (van Kooyk and Figdor, 2000).

**Regulation of Integrin Avidity**

On ligand binding, the cytoplasmic tails of integrins interact with initiators of intracellular signalling cascades, which lead to a wide variety of cellular responses. One of these signals, or it’s result, may eventually determine integrin distribution. For examples, activation of small GTP-binding proteins Rac and CDC42 and protein kinase C can lead to integrin clustering thereby increasing integrin avidity. Redistribution of integrin receptors has generally been observed after integrin-ligand
binding. However, this redistribution may also be found before, or in absence, of ligand binding. Changes in adhesion in response to certain physiological factors, may operate through changes in integrin avidity rather than affinity. Both mechanisms of regulation from inside the cell can be attributed to 'inside-out' signalling, although resulting in different biological consequences.
1.5 Regulation of the ECM by Proteases

1.5.1 Introduction

The constant production and degradation of the ECM is essential in both normal tissue remodelling and wound healing. A disturbance in the balance between the two can lead to pathological conditions such as chronic ulcers, rheumatoid arthritis, osteoarthritis, atherosclerosis, tumour invasion and tumour metastasis (the result of excessive ECM degradation) or fibrosis, keloid and hypertrophic scarring (the result of excessive ECM production and accumulation). This fine balance is maintained by the protease group of molecules, two of which are of great importance in these processes, namely the matrix metalloproteinases (MMPs) and their inhibitors, the tissue-derived inhibitors of MMPs (TIMPS), and secondly, a group of proteases called the serine proteases and their inhibitors.

1.5.2 Metalloproteases - MMPs and TIMPs

The MMPs are a well described family of structurally related zinc-dependant endopeptidases that are collectively capable of degrading all components of the ECM. They are precisely regulated at the level of transcription, by interaction with specific ECM components and by inhibition by endogenous inhibitors (Visse and Nagase, 2003). There are at least 20 members of the human MMP family and they are characterised into groups according to their structure and substrate specificity, known as the collagenases, gelatinases, stromelysins and membrane type MMPs (See Table 1.7). They are all proteinases which can degrade one or more components of the ECM, they contain a Zn$^{2+}$ ion and are inhibited by chelating agents, they are synthesized in a latent, proenzyme form and they are inhibited by TIMPs (Lewis et al., 2000; Lewis et al., 2001; Ma et al., 1999; Ravanti and Kahari, 2000; Thomas et al., 1999). It is important to note that most of the activities of MMPs and their substrates have been demonstrated in vitro only and not in vivo.
### Table 1.8 Human MMPs and their substrates (Lewis et al., 2001; Ravanti and Kahari, 2000)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td></td>
</tr>
<tr>
<td>Collagenase-1 (MMP-1)</td>
<td>Collagen I, II, III, VII, VIII, X, aggregan, serpins, α2M.</td>
</tr>
<tr>
<td>Collagenase-2 (MMP-8)</td>
<td>Collagen I, II, III, aggregan, serpins, α2M.</td>
</tr>
<tr>
<td>Collagenase-3 (MMP-13)</td>
<td>Collagen I, II, III, IV, IX, X, XIV, gelatine, fibronectin, laminin,</td>
</tr>
<tr>
<td></td>
<td>large tenasin C, aggregan, fibrillin, osteonectin, serpins.</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
</tr>
<tr>
<td>Stromelysin-1 (MMP-3)</td>
<td>Collagen IV, V, IX, X, fibronectin, elastin, gelatin, laminin, nidogen,</td>
</tr>
<tr>
<td></td>
<td>fibrillin, osteonectin, α1PI, myelin basic protein.</td>
</tr>
<tr>
<td>Stromelysin-like MMPs</td>
<td></td>
</tr>
<tr>
<td>Stromelysin-3 (MMP-11)</td>
<td>Serpins, α1PI.</td>
</tr>
<tr>
<td>Matrilysin (MMP-7)</td>
<td>Elastin, fibronectin, laminin, nidogen, collagen IV, tenasin, versican,</td>
</tr>
<tr>
<td></td>
<td>α1PI.</td>
</tr>
<tr>
<td>Metalloelastase (MMP-12)</td>
<td>Collagen IV, gelatin, fibronectin, laminin, vitronectin, elastin, fibrillin, α1PI, myelin basic protein, apolipoprotein A.</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
</tr>
<tr>
<td>Gelatinase A (MMP-2)</td>
<td>Gelatin, collagen I, IV, V, VII, X, fibronectin, tenasin, fibrillin,</td>
</tr>
<tr>
<td></td>
<td>osteonectin, α2M.</td>
</tr>
<tr>
<td>Gelatinase B (MMP-9)</td>
<td>Gelatin, collagen IV, V, VII, XI, XIV, elastin, fibrillin, osteonectin,</td>
</tr>
<tr>
<td></td>
<td>α2M.</td>
</tr>
<tr>
<td>Membrane-type MMPs</td>
<td></td>
</tr>
<tr>
<td>MT1-MMP (MMP-14)</td>
<td>Collagen I, II, gelatin, laminin, vitronectin, aggregan, tenasin, nidogen,</td>
</tr>
<tr>
<td></td>
<td>perlecan, fibrillin, α1PI, α2M, fibrin.</td>
</tr>
<tr>
<td>MT2-MMP (MMP-15)</td>
<td>Fibronectin, laminin, aggregan, tenasin, nidogen, perlecan.</td>
</tr>
<tr>
<td>MT3-MMP (MMP-16)</td>
<td>Collagen III, fibronectin, gelatin, casein. Cartilage,</td>
</tr>
<tr>
<td></td>
<td>proteoglycan, laminin, α1PI, α2M.</td>
</tr>
<tr>
<td>MT4-MMP (MMP-17)</td>
<td>Gelatin, TNF precursor.</td>
</tr>
<tr>
<td>MT5-MMP (MMP-24)</td>
<td>Not Determined.</td>
</tr>
<tr>
<td>MT6-MMP (MMP-25)</td>
<td>Not Determined.</td>
</tr>
<tr>
<td>Other MMPs</td>
<td></td>
</tr>
<tr>
<td>MMP-19</td>
<td>Gelatin.</td>
</tr>
<tr>
<td>Enamelysin (MMP-20)</td>
<td>Amelogenin.</td>
</tr>
<tr>
<td>MMP-23</td>
<td>Synthetic MMP substrate.</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Gelatin, α1PI, synthetic MMP substrates, TACE-substrate.</td>
</tr>
</tbody>
</table>

TIMPs are specific inhibitors of the MMPs that bind in a 1:1 ratio. They function by stabilizing the MMP proenzyme and inhibiting the active species. To date, there have been four TIMPs identified in vertebrates, named TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The TIMPs are 21-29kD in size and each member can inhibit all mammalian MMPs tested so far (Lewis et al., 2000; Visse and Nagase, 2003).

Proteolytic degradation of the ECM is required throughout wound repair, such as in degradation of the provisional matrix, angiogenesis, keratinocyte migration, and
remodelling of the granulation tissue and ECM. With the exception of MMP-7, MMP-8 and MMP-9, the expression of MMPs is not detected in vivo in normal skin, however, after injury, several MMPs are temporarily expressed throughout the wound healing process (Parks, 1999; Ravanti and Kahari, 2000).

1.5.3 Serine Proteases - the Plasminogen Activators

Serine proteases are so called due to the presence of a highly reactive serine residue in their active site. The major members of this family of proteases include the plasminogen activators (PAs), leukocyte elastase and cathepsin G, of which, the PAs are the best characterised of the three. PAs convert the inactive zymogen plasminogen to the active protease plasmin, which is then able to degrade several ECM components, including fibronectin, laminin, and proteoglycans. Plasmin cannot degrade native collagen, nor elastin, but can however degrade gelatin, the partially degraded, or denatured form of collagen (Mignatti et al., 1996). The term PA refers to two enzymes, which are very efficient activators of plasminogen, called urokinase-type PA (uPA) and tissue type PA (tPA)(Alberts et al., 1994).

The serine protease inhibitor family are specific, fast-acting inactivators, present in most body fluids and tissues. Members of the family include plasminogen activator inhibitor type 1 (PAI-1), PAI-2, protease nexin 1 and protein C inactivator (Werb, 1997). In wound healing, PAs have been implicated in inflammation, granulation tissue formation, matrix formation and reepithelialization. They can be produced by different cell types, such as keratinocytes, fibroblasts, endothelial cells, granulocytes and macrophages (Mignatti et al., 1996).
1.6 Summary

Myofibroblasts (characterised by the presence of the cytoskeletal protein $\alpha$-sma) are an important cell type implicated in growth and development, wound healing and disease. Cells with this phenotype are found at sites of injury (Grinnell, 1994; Hakkinen et al., 2000), in organ fibrosis, e.g. in the kidney (Grupp et al., 2001; Lewis and Norman, 1998) and in carcinomas (Sieuwerts et al., 1998; Tuxhorn et al., 2002). Many of their biological features and mechanisms of action still remain a mystery, although the factors which regulate their differentiation are becoming more and more clearly understood.

Adult dermal wound healing is a restorative process involving the formation of a collagen rich scar. In contrast, fetal wound healing, is a regenerative process characterised by the absence of any such scar (Schor et al., 1996). Oral wounds have been noted for their ability to exhibit this privileged ‘fetal-like’ method of healing (Stephens et al., 2001a). While this is no doubt related to the differing environments of the cell types, phenotypic differences may exist between the cells themselves. A number of growth factors, cytokines and extracellular matrix (ECM) molecules have been identified that can drive the transition of fibroblasts to myofibroblast, of these TGF-\(\beta\)1 has been most widely studied. $\alpha$-sma expression can be induced in vitro in fibroblasts from a variety of tissues by this factor, which is also well known for stimulating the accumulation of ECM. Furthermore, in vivo high levels of TGF-\(\beta\)1 are consistently associated with myofibroblast-containing lesions (Desmouliere et al., 1993; Lewis and Norman, 1998; Moulin et al., 1998; Serini and Gabbiani, 1999; Tuan and Nichter, 1998; Yokozeki et al., 1997). These data suggest a dynamic situation in which myofibroblast differentiation, ECM deposition and TGF-\(\beta\)1 are intimately connected. One mechanism by which changes in ECM and changes in cytoskeleton may be linked is via the integrin receptors.

The integrins are a family of heterodimeric transmembrane, cell surface ECM receptors, composed of non-covalently linked $\alpha$ and $\beta$ chains. In addition to their adhesive function, integrins mediate important signalling pathways regulating a diverse range of cell functions including motility, proliferation and apoptosis.
Specific integrin signals can affect the composition of connective tissue by modulating expression of ECM proteins (Wu et al., 1995a), matrix degrading enzymes and their inhibitors (Huhtala et al., 1995; Larjava et al., 1993; Niu et al., 1998; Tremble et al., 1995). Moreover integrins of the αv family can activate latent TGF-β1 by binding to the growth factor-bound LAP-β1; (Munger et al., 1999). It follows therefore we hypothesized that differential expression of integrins may be a feature of the transition from fibroblasts to myofibroblast. Indeed, recent in vitro data indicate that differential regulation of subsets of integrin receptors (particularly the αv integrins) occurs concomitantly with cellular differentiation in models of osteoclast (Rodan and Rodan, 1997), oligodendrocyte (Milner and Campbell, 2002), keratinocyte (Thomas et al., 2001a) and myoblast differentiation (Blaschuk et al., 1997).

The hypothesis that changes in integrin expression and integrin-mediated signaling induce the differentiation of fibroblasts to myofibroblast implies that there are differences in integrin expression and/or function of the two cell types. There are currently very little data on changes in integrin expression that occur when fibroblasts differentiate into myofibroblasts. Learning to interfere with and being able to control their differentiation ability has many implications in terms of trying to minimise scar formation and the prevention of many pathological conditions.
1.7 Aims of the Study

The aims of the study were:

(i) to identify any integrin involvement in fibroblast to myofibroblast differentiation, and identify any possible mechanisms involved;

(ii) to establish whether such participation in the differentiation process had functional implications on cell contraction, cell migration in response to wounding and MMP production;

(iii) to determine any phenotypic differences between oral and dermal fibroblasts and their implication in scarless wound healing;

(iv) to discover the implication of myofibroblast differentiation in tumour progression by investigating whether oral squamous cell carcinoma cells are able to generate myofibroblasts and the subsequent effect, if any, these cells have on tumour invasion and progression.
Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Chemical Reagents and Kits

- β-mercaptoethanol
- Acetic acid (Glacial)
- Acrylamide ProtoGel™
- APS
- Bromophenol blue
- BSA
- Citifluor
- Collagen type-1 (rat tail)
- Coomassie brilliant blue R250
- DAPI
- DMEM
- DMSO
- ECL® reagents
- FCS
- Fibronectin (human)
- Gelatin, Type A
- Glycerol
- Glycine
- Hydrochloric acid
- Hyperfilm-ECL®
- Isopropanol
- Matrigel® Matrix (growth factor reduced)
- Kaleidoscope prestained standards
- MEM (10X)
- Paraformaldehyde
- PBS (sterile, Ca²⁺/Mg²⁺ free)
- PBS (non-sterile tablets)
- Penicillin/streptomycin
- Poly-l-lysine
- Ponceau S
- PP2 & PP3

Sigma Chemical Co., Dorset, UK,
VWR International (BDH), Dorset, UK,
Flowgen, Leicestershire, UK,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Citifluor Ltd., London, UK,
BD Biosciences, Oxford, UK,
Sigma Chemical Co.,
Sigma Chemical Co.,
GIBCO/Invitrogen Corp., Paisley, UK,
Sigma Chemical Co.,
Amersham Biosciences, Bucks, UK,
PAA Laboratories, Somerset, UK,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
VWR International,
Amersham Biosciences,
VWR International,
BD Biosciences,
Bio-Rad, Herts, UK,
GIBCO/Invitrogen Corp.,
VWR International,
GIBCO/Invitrogen Corp.,
Oxoid, Hampshire, UK,
GIBCO/Invitrogen Corp.,
Sigma Chemical Co.,
VWR International,
Calbiochem, California, USA,
Propan-2-ol
Protease inhibitor cocktail
Quantikine® ELISA kits (HGF and TGF-β1)
SDS
Skimmed milk powder
SnakeSkin™ pleated dialysis tubing
Sodium bicarbonate (7.5%)
TBS
TEMED
rhTGF-β1
Trypsin-EDTA
Triton X-100
Trizma® Base
Trizma® Hydrochloride
Tween 20
Vitronectin (human)
X-Ray developer
X-ray fixer

VWR International,
Sigma Chemical Co.,
R&D Systems, Oxfordshire, UK,
VWR International,
Marvel, Cadbury, UK,
Pierce, Cheshire, UK,
GIBCO/Invitrogen Corp.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Sigma Chemical Co.,
Photosol, Essex, UK,
Photosol,

2.1.2 Antibodies

All antibodies are monoclonal mouse anti-human unless specified. Concentrations of use can be found in Appendix 2.

α1 integrin, clone FB12
α2 integrin, clone P1E6
α3 integrin, clone P1B5
α4 integrin, clone P1H4
α5 integrin, clone P1D6
α6 integrin, clone NK1-GoH3
α-sma, clone 1A4
αv integrin, clone P3G8
αv integrin, clone L230
αvβ3, clone LM609
αvβ5, clone P1F6
β1 integrin, clone JB1A
β1 integrin, clone A11B2
β-actin, clone AC-15
FITC. F(ab’); Rabbit anti-mouse
HGF/SF antibody, clone 24612
HRP. Affinity isolated Rabbit anti-mouse
MHC-class I, clone W632
TGF-β1 antibody, clone 9016

Chemicon International Inc.,
Harrow, UK,
Chemicon International Inc.,
Chemicon International Inc.,
Chemicon International Inc.,
Chemicon International Inc.,
Chemicon International Inc.,
Sigma Chemical Co.,
Chemicon International Inc.,
Dr J Marshall, CRUK, London, UK,
Chemicon International Inc.,
Chemicon International Inc.,
Chemicon International Inc.,
Dr J Marshall, CRUK, London, UK,
Sigma Chemical Co.,
Dako,
R&D systems,
Dako,
R&D Systems,
2.2 **Cell Culture**

All cells were grown in a humidified atmosphere at 37°C and 5% CO2. Solutions and media used can be found in the Appendix.

2.2.1 Routine Cell Culture

Three sets of paired human oral and dermal fibroblasts (HOF and HDF) were used in this study, and were a kind donation from Dr C Irwin (Queens’ University, Belfast, UK). They were obtained by explant culture from 4mm punch biopsy tissue samples taken from consenting, healthy female donors, aged between 20 and 25 years. Mucosal biopsies were taken from the buccal mucosa and skin biopsies from the forearm. Fibroblasts were grown in FGM (See Appendix) and the medium changed every 2-3 days. Cells were grown until they formed a monolayer sheet on the plastic (confluent), which took between 10 and 14 days. Cells up to passage 20 were used.

A panel of 3 oral squamous cell carcinoma (OSCC) cell lines were also used. The invasive VB6 cell line was generated previously by transfection and retroviral infection of integrin subunits to express high levels of the integrin αvβ6 (Thomas et al., 2001a). CA1 and 5PT cell lines were kind gifts from Professor I.C. Mackenzie (Cardiff Dental School, UK). Cells were grown in standard KGM (See Appendix) and the medium changed every 2-3 days.

2.2.2 Routine Passage

The growth medium was removed and the monolayer cell culture washed 3 times with Ca^{2+}/Mg^{2+}-free PBS. The cells were harvested using Trypsin-EDTA at 1ml per 80cm^2. The trypsin digests the ECM links and EDTA chelates Ca^{2+} on which cell-cell adhesion is dependent. The flask(s) were incubated until all of the cells had rounded up and detached from the plastic (approx. 5-10 mins). Trypsin action was terminated by the addition of 10ml FGM per 80cm^2 flask. The cell/medium mixture
was transferred to a 15ml centrifuge tube and centrifuged at 1000rpm, 4°C for 5 minutes to pellet the cells. The supernatant was discarded, the cells resuspended in 10ml FGM using a 10ml pipette, and re-centrifuged, to ensure there was no trace of Trypsin-EDTA. The supernatant was again discarded and the cells resuspended in 1ml of FGM and counted using a haemocytometer (see section 2.1.3 Determining Cell Density). Cells were seeded at 2x10^5 cells per 80cm^2 flask for continued growth.

**2.2.3 Determining Cell Density**

Cell density was determined using a haemocytometer viewed under a light microscope at 10X magnification. Cells were trypsinised and resuspended in 1ml growth medium. 10μl of the sample was collected and pipetted under a glass coverslip adhered to the top of the haemocytometer. The pipette was reloaded and the second chamber filled. At 10X magnification, the grid was located and the cells were counted within the four primary squares. The cells that are within or that touch the left or top boundary are counted, while those that touch or are outside the lower or right hand boundary are not counted. See Figure 2.1.

![Figure 2.1 Haemocytometer slide](image)

A = Top view of Improved Neubauer ruled haemocytometer, showing position of grid areas and placement of coverglass. B = Grid patterns showing cells distributed over a primary square and those that are counted.
The value was averaged for the four primary squares and by using the following calculation the cell density was calculated;

\[ c = \frac{n}{v} \]

Where \( c \) = cell concentration (cells/ml), \( n \) = number of cells counted, and \( v \) = volume counted (ml). For the Improved Neubauer slide, the depth of the chamber is 0.1mm, and the volume is \( 1 \times 10^{-4} \). Therefore, the formula becomes:

\[ c = n \times 10^{-4} \]

### 2.2.4 Freezing Down Cell Stocks

After trypsinisation and centrifugation, cells were counted and \( 1 \times 10^6 \) cells were resuspended in 1ml of freezing medium (See Appendix). This 1ml solution of cells/freezing medium was transferred to a cryovial, labelled and placed in a plastic container containing isopropanol, overnight at -70°C, before transfer to liquid nitrogen for storage.

### 2.2.5 Thawing Out Cell Stocks

Frozen vials of cells were recovered from the liquid nitrogen and thawed in a 37°C water bath. The contents of the cryovial was transferred to a 15ml centrifuge tube containing 10 ml FGM and centrifuged at 1000rpm, 4°C for 5 mins. The supernatant was discarded and the cells were resuspended in 20 ml FGM, and transferred into two 80cm² flasks so that each contained 10ml of cells/medium.
2.3 Flow Cytometry

2.3.1 Introduction

Flow Cytometry measures certain physical and chemical characteristics of cells as they move in a fluid stream past a fixed laser light beam. Light scattered by the cells at small angles (<2°) (forward scatter; FSC) is proportional to cell size, whereas light scattered at angles of 90° (side scatter; SSC) is proportional to the granularity and intracellular complexity of the cell. Flow Cytometry is also able to detect emitted fluorescence light, and the use of fluorescent dyes and fluorochrome-conjugated antibodies has also provided considerable information about relative levels of cell surface and intracellular antigens, as well as DNA.

2.3.2 Preparation of Cells for Analysis

HOF and HDF were trypsinised and counted (see section 2.1.2 and 2.1.3). Cells were aliquoted into Falcon plastic LP3 tubes at a density of $2 \times 10^5$ cells per tube, centrifuged at 1000rpm, 4°C for 3 mins, and the supernatant discarded. Primary antibodies to integrins were added in 250μl FACS buffer (See Appendix) to each tube. The cells were resuspended by vortexing and incubated on ice for one hour. 1000μl FACS buffer was added and the tubes centrifuged as before. The wash step was repeated twice more. The fluorescently labelled secondary antibody (FITC. F(ab')2 Rabbit anti-mouse) was added in 250μl of FACS buffer to the cells and incubated on ice for 30 minutes. The wash step was repeated three times and the cells resuspended in 500μl FACS buffer ready for analysis.
2.3.3 Flow Cytometry Analysis

A Becton Dickinson FACScan machine running Cell Quest™ software was used to analyse the samples. The machine was calibrated before each experiment. The negative control sample (control cells incubated with secondary antibody only) was analysed first, 10,000 cells were analysed and recorded. This reading gives a background level with which to compare the samples. Data was acquired as a dot plot with each dot representing a positively charged cell or cell fragment. The data was collected for each sample and analysed accordingly.
2.4 Western Blotting

2.4.1 Introduction

Western Blotting is a technique used to ascertain important characteristics of protein antigens within a cell sample, including the presence and the quantity of the antigen. Polyacrylamide gel electrophoresis separates individual proteins, by size, shape and charge. The separated proteins are transferred to a nitrocellulose membrane (blotting) for detection with specific antibodies.

2.4.2 Preparation of Cell Lysates

Post treatment, HOF and HDF were washed twice with Ca\(^{2+}/\)Mg\(^{2+}\)-free PBS and 100\(\mu\)l protein lysis solution (See Appendix) containing protease inhibitor cocktail was added to each well of a 6 well plate and then placed on ice for 30 min. The cells were then scraped into labelled 0.5ml microfuge tubes, heated to 95°C for 5 min to denature the protein, cooled in ice and centrifuged at 13,000rpm for 5 min. The cell lysates were transferred into clean, labelled microfuge tubes, leaving behind any cell debris. A protein assay was carried out to determine the concentration of each sample and the cells were stored at -20°C until required.

Determination of Protein Concentration

The Bio-Rad DC Protein Assay was used to determine the protein concentration within the cell samples. It is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well documented Lowry assay. It is based on the reaction of protein with an alkaline copper tartrate solution and a folin reagent. There are two steps which lead to the colour development, the reaction between protein and copper in an alkaline medium, and the subsequent reduction of folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan and to a lesser extent
cystine and histidine. Proteins affect a reduction of the folin reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750nm and minimum absorbance at 405nm.

A standard solution of 10mg/ml BSA in protein lysis solution was prepared and further diluted to form a series of standards. 5µl of duplicate samples of the standards and cell lysates were added to clean wells of a 96 well plate (duplicates were run). 20µl of reagent S (SDS) was added to 1ml of reagent A (an alkaline copper tartrate solution), and 25µl was added to each well containing the standards and samples. 200µl of reagent B (a dilute Folin reagent) was added to the wells and the plate left for 15 minutes. The absorbance was then read at 650nm on a plate reader. A standard curve was then plotted and relative concentrations of the cell lysates calculated using the equation \( y = mx + c \).

2.4.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

The glass plates and spacers were assembled in the clamp and clipped into the casting stand ensuring no leaks (See Figure 2.2). The 12.5% acrylamide separating gel (See Appendix) was made and poured into the plates to two-thirds full, propan-2-ol was overlaid and the gel left for 15 minutes to set. The 15µl samples were prepared by adding 3µl 5X sample buffer (See Appendix) to between 3 & 10µg protein (calculated from the protein assay) and if required were made up to 15µl with
distilled water (dH₂O). The propan-2-ol was decanted and the 4% acrylamide stacking gel (See Appendix) was made and added to the top of the separating gel. The comb was inserted into the stacking gel between the glass plates, ensuring there were no air bubbles at the bottom of the comb. The gel was left to set for 5-10 minutes, meanwhile 50ml of 10X running buffer (See Appendix) was diluted to 1X with dH₂O. Once the gel had set the clamps were transferred from the casting stand to the electrode assembly and clipped in (see Figure 2.2). 1X running buffer was poured into the upper chamber and the combs slowly removed, revealing the newly formed wells. Running buffer was also poured into the tank to cover the bottom of the electrode assembly and complete the circuit. The prepared cell samples and 10µl of Kaleidoscope pre-stained standards were loaded into the wells. The gels were run at 100V for 10 min and then at 140V for a further 60-90 min until the blue dye front of the bromophenol blue within the sample had reached the bottom of the gel and had just run into the buffer.

2.4.4 Membrane Transfer (Western Blotting)

The proteins were transferred from the gel to a nitrocellulose membrane by wet electroblotting. In this procedure, a sandwich of gel and nitrocellulose is compressed in a cassette and immersed in buffer, between two parallel electrodes. A current is passed across the gel, which causes the separated proteins to electrophorese out of the gel and onto the nitrocellulose sheet (Schleider & Schuell, Basssell, Germany). This sheet is termed the “blot”. See Figure 2.3 & 2.4.
The electrode assembly was removed from the tank, the glass plates gently separated and the stacking gel discarded. The gels were placed in transfer buffer (See Appendix) to equilibrate. A “sandwich” was made up in a plastic cassette of the following layers: (see also Figure 2.4)

1. Sponge
2. 4 sheets of filter paper soaked in transfer buffer
3. Gel
4. nitrocellulose membrane
5. 4 sheets of filter paper soaked in transfer buffer
6. Sponge

Any air bubbles were rolled out and the layers were made up to ensure even transfer of the proteins. The cassette was closed and placed in a transfer cell ensuring that the gel was on the side of the negative electrode (cathode), as the proteins will transfer from the cathode to the anode. The transfer cell was placed in a tank containing transfer buffer and allowed to run at 26V overnight (see Figure 2.4.)
2.4.5 Immunodetection of Antigen

Post-transfer, the blotting cassette was opened and the membranes placed in blocking solution (See Appendix) in a 50ml centrifuge tube on a roller for 1h. This blocks remaining hydrophobic binding sites on the membrane, to prevent any non-specific binding of the antibody. After 1h, the blots were washed 3 times for 10 min each in Phosphate Buffered Saline-Tween 20 (PBS-T; See Appendix). The primary antibody was added in 2ml of PBS-T to the tubes and incubated on the roller at room temperature for 1h. The wash step was repeated and the horseradish peroxidase labelled secondary antibody was added in 2ml to the tube and incubated for 30 min. The wash step was repeated and the membranes processed by enhanced chemiluminescence.
Development of Antigen

Enhanced chemiluminescence (ECL) is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase (HRP) labelled antibodies. An oxidation reaction occurs between the HRP molecules and the ECL reagent, which causes light to be emitted, which is detected on radiographic film.

![Figure 2.5 Principles of ECL Western Blotting](image)

The reaction and principle behind enhanced chemiluminescence.

ECL is carried out in a darkroom under safe light. Excess PBS-T was blotted, and the membrane placed on a sheet of acetate. 1ml of ECL reagent ‘1’ was mixed with 1ml of ECL reagent ‘2’ and pipetted over the blot and left for 1 min. The excess was drained off, a second sheet of acetate placed over the blot and put face-up into a film cassette. A piece of x-ray film was added and the cassette closed for 1 min. The film was then immersed in developer until dark bands were clearly seen, if the bands were too faint or too strong, then a second piece of film was exposed to the membrane in the cassette for a longer or shorter period of time, respectively. The developed film was then placed in the fixer washed and dried. The film was analysed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at [http://rsb.info.nih.gov/nih-image/](http://rsb.info.nih.gov/nih-image/)). The position of the molecular weight markers was used to determine the weight of the antigen.
2.5 Immunocytochemistry

2.5.1 Introduction

Immunocytochemistry is a method that can be used to visualise proteins on or within a cell using fluorescently labelled antibodies. An indirect method of detection was used with the monoclonal primary antibody being detected by a FITC or Cy3 conjugated secondary antibody and viewed using epi-fluorescence, using a microscope.

2.5.2 Preparing and Coating Glass Coverslips

13mm diameter glass coverslips (BDH, UK) were coated with poly-l-lysine to create a charged surface for the cells to adhere to. The coverslips were soaked in 70% ethanol for 24 hours. They were then removed and placed in a clean pyrex petri dish using forceps and a needle. A small volume of tissue grade water was added to prevent the coverslips from aggregating. The petri dish was wrapped in foil and baked at 200°C for 2h. Cooled coverslips were then transferred to a new plastic petri dish covered with 0.01% high molecular weight poly-l-lysine and incubated on the shaker for 3h. The poly-l-lysine was aspirated and stored for re-use. The coverslips were then washed with dH₂O overnight on a shaker. The coverslips were air-dried in a fume hood, and stored in a sterile petri dish sealed with parafilm tape.

2.5.3 Preparing and Fixing the Cells.

A prepared 13mm coverslip was placed into each well of a 24 well cell culture plate. HOF and HDF were seeded onto the coverslips at a density of 1.33 x 10⁴ cells in 300μl FGM per well and left to grow for 4 days (until they were near confluence). The medium was replaced with serum free medium for 24h and the cells were treated on day 5. After 3 days, the medium was aspirated and the cells were fixed by adding
500\mu l of 4% paraformaldehyde (See Appendix) to each well and the plate left on ice for 10 min. The paraformaldehyde was then removed, the coverslips were washed twice with PBS, covered with ice-cold methanol and the plate left on ice for a further 10 min. The methanol was then removed, the coverslips were washed twice with PBS, covered with 0.025% Triton X-100 (see Appendix) to permeabilise the membranes and left on ice for 10 min. The Triton X-100 was then removed, the coverslips were washed twice with PBS.

2.5.4 Immunodetection of Antigen

The PBS was removed from the wells and the primary antibody, diluted in PBS, was added to each coverslip and incubated on ice for 1h. The coverslips were washed in PBS, an anti-mouse biotin added and incubated for a further 45 min. The coverslips were washed in PBS and a FITC or Cy3-streptavidin conjugated, secondary antibody diluted (1:200) in PBS containing DAPI was added and incubated for 30 min on ice. The coverslips were washed and mounted onto glass slides with Citifluor anti-fade media and the edges sealed with clear nail varnish.

2.5.5 Visualising Antigen

The slides were examined under epi-fluorescence using a LEICA DMIRB inverted microscope, linked to a computer running the LEICA software FW4000. In fluorescence microscopy, a cell is stained with a dye and the dye is illuminated with filtered light at the absorbing wavelength. The light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen. The dye glows brightly against a dark background because only the emitted wavelength is allowed to reach the eye piece or camera port of the microscope.
Chapter Two: Materials and Methods

2.6 Collagen Gel Contraction Assay

2.6.1 Introduction

Several in vitro models of wound contraction have been developed using fibroblasts cultured in collagen or fibrin matrices. Elsdale and Bard (1972) showed that fibroblasts cultured in collagen matrices acquire tissue-like phenotypic characteristics not typically observed in cells in monolayer culture. Here we have used fibroblasts cultured in a type-I collagen gel lattice, and will refer to the collagen gels as fibroblast populated collagen lattices, or FPCL’s.

2.6.2 Contraction Assay

Collagen type-1 solution was prepared by dissolving 10 adult rat tail tendons in 500ml of 3% acetic acid overnight at 4°C. Non-dissolved tissue was removed by centrifugation (3000rpm at 4°C for 2h) and the supernatant dialysed exhaustively against distilled water for 48h. The collagen concentration was adjusted to 3.2 mg/ml with distilled water, 1% penicillin-streptomycin added and the acidity adjusted to approximately pH 4 using NaOH, prior to storage at 4°C.

A single cell suspension was prepared by mixing $1.5 \times 10^6$ fibroblasts with or without TGF-β1 and integrin blocking antibodies with 85% of collagen solution, 10% of 10X MEM and 5% sodium bicarbonate (7.5% solution) to yield 3 gels of 2ml volume containing $5 \times 10^5$ fibroblasts. The gel solution (2ml per well of a 6 well culture plate) was allowed to polymerise at 37°C in 95% air/5% CO₂ for 30 min. Immediately after polymerisation, 2 ml of FGM was added to each well; gels were detached from the well, using a sterile, plastic P200 pipette tip and left free-floating in the medium. Gel (FPCL) contraction was monitored over 7 days by analysis of images of the gels alongside a ruler captured using the Alpha Imager documentation and analysis system (Alpha Innotech Corp., Flowgen/Novara, Ashby-de-la-Zouch,
UK). The area of each gel was measured using the public domain NIH Image program.


2.7 Scratch Assay

HOF and HDF were grown to confluence in 6 well plates. The medium was replaced with serum free medium for 24 h to quiesce the cells and they were then treated with or without TGF-β1 and integrin blocking antibodies. After 3 days the monolayer of cells in each well was scratched to create a cell-free zone of the configuration ‘#’ using a plastic P1000 pipette tip (See Figure 2.6). The cells were incubated with further integrin blocking antibody for 48h at 37°C and 5% CO2. In vitro wound closure was monitored by the repopulation of the cell-free area over time. Images were taken immediately after wounding (0h). The fields were recorded (See Figure 2.6) and the same fields were photographed again 24h and 48h later. The gap between the migrating fibroblasts was measured and compared with the treated cells to determine whether the treatment had any affect on wound closure.

Figure 2.6 Scratch assay configuration

a: Black Circle represents one well of a 6-well plate. Grid represents denuded or ‘scratched’ area made by pipette tip. Red boxes indicate the images taken at 0hr, 24hr and 48hr. b: Blue arrows represent the four measurements of the wound gap taken for each image.
2.8 Gelatin Zymography

2.8.1 Introduction

This technique was used to investigate and quantify the expression of matrix-degrading metalloproteinases (MMPs). The two MMPs of interest were the gelatinases MMP-2 and MMP-9. Gelatin zymography involves the electrophoresis of secreted protease enzymes through discontinuous polyacrylamide gels containing enzyme substrate (type III gelatin). After electrophoresis, removal of SDS from the gel by washing in 2.5% Triton X-100 solution allows enzymes to renature and degrade the protein substrate. Staining of the gel with commassie blue allows the bands of proteolytic activity to be detected as clear bands of lysis against a blue background.

2.8.2 Preparation and Electrophoresis of Supernatant Samples

HOF and HDF were treated and the supernatant collected. Cell supernatants were mixed with an equal volume of 2X non reducing sample buffer (See Appendix) and 15\(\mu\)l loaded per well. The samples were electrophoresed through a 12% gelatine-substrate acrylamide separating gel (See Appendix) as described in section 2.3.3 (‘SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of proteins’).

2.8.3 Renaturing and Developing of Gels

Following electrophoresis, the SDS was removed from the gel by washing 2 x 15 min in renaturing buffer (See Appendix), then incubated overnight in developing buffer (See Appendix) at 37°C. This allows the MMPs to renature and digest the surrounding substrate. After incubation, the gel was stained with a solution of 0.25%
Coomassie blue R250, 40% methanol and 10% acetic acid for 1h at room temperature and destained with 40% methanol, 10% acetic acid until the bands of lysis became clear.

The gel was analysed using the Alpha Imager documentation and analysis system and the domain NIH Image program (http://rsb.info.nih.gov/nih-image/). The position of the molecular weight markers was used to determine the weight of the protease.
2.9 Enzyme Linked Immunosorbant Assay (ELISA)

The enzyme linked immunosorbant assay (ELISA), was originally developed by Engvall and Perlman in 1971 to quantify the amount of immunoglobulin (Ig) G present in a mixed Ig sample. Since then, this assay has been modified and developed to detect a wide range of antigens and antibodies. All cytokine detection assays performed in this project were two-site ELISA’s. This procedure involved detection of the protein of interest using two antibodies to the protein. Quantikine® ELISA kits to HGF and TGF-β1 were purchased from R&D Systems, the plates were pre-coated with either a monoclonal antibody specific to HGF, or TGF-β soluble receptor type II, which binds to TGF-β1. Standards and samples were pipetted into the wells and any cytokine present was bound to the immobilized antibody or immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody, specific for either HGF or TGF-β1 was added to the wells. Following a wash step to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of cytokine bound in the initial step. The colour development is stopped and the intensity of the colour is measured using a microplate reader set to 450nm.
2.10 Statistical Analysis

Data were calculated and are expressed as the mean +/- standard deviation (sd) of a given number of observations. Where appropriate, a 2 independent, non-parametric Mann-Whitney U test was used to compare multiple groups. A p value of 0.05 or lower was considered to be significant.
Chapter Three

Results One

Integrin Involvement in Myofibroblast Differentiation

3.1 Introduction

The differentiation from fibroblast to myofibroblast plays an important role in many aspects of tissue repair and pathology. Fully differentiated myofibroblasts are characterised by, amongst other structural features, the presence of the cytoskeletal protein α-smooth muscle actin (α-sma), contained within stress fibres (Serini and Gabbiani, 1999). A number of growth factors, cytokines and extracellular matrix molecules have been identified that can drive the transition of fibroblasts to myofibroblasts; of these, TGF-β1 has been most widely studied. The cytoskeletal expression of α-sma can be induced in vitro in fibroblasts from a variety of tissues by TGF-β1, which is also well known for stimulating the accumulation of ECM. Furthermore, high levels of TGF-β1 are consistently associated with myofibroblast-containing lesions in vivo (Desmouliere et al., 1993; Lewis and Norman, 1998; Moulin et al., 1998; Serini et al., 1998; Sieuwerts et al., 1998; Tuan and Nichter, 1998; Yokozeki et al., 1997). These data suggest a dynamic situation in which myofibroblast differentiation, ECM deposition and TGF-β1 are intimately connected. One mechanism by which changes in ECM and changes in cytoskeleton may be linked is via the integrin receptors.

The integrins are a family of heterodimeric transmembrane, cell surface ECM receptors, composed of non-covalently linked α and β chains. In addition to their...
adhesive function, integrins mediate important signalling pathways regulating a
diverse range of cell functions including motility, proliferation and apoptosis.
Specific integrin signals can affect the composition of connective tissue by
modulating expression of ECM proteins (Wu et al., 1995b), matrix degrading
enzymes and their inhibitors (Huhtala et al., 1995; Larjava et al., 1993; Niu et al.,
1998; Tremble et al., 1995). Moreover integrins of the αv family can activate latent
TGF-β1 by binding to the growth factor-bound Latency Associated Peptide (LAP-
β1; Munger et al., 1999). We therefore hypothesize that differential expression of
integrins may be a feature of the transition from fibroblast to myofibroblast. Indeed,
in vitro data indicate that differential regulation of subsets of integrin receptors
(particularly the αv integrins) occurs concomitantly with cellular differentiation in
models of osteoclast (Rodan and Rodan, 1997), oligodendrocyte (Milner et al.,
1999), keratinocyte (Thomas et al., 2001a) and myoblast differentiation (Blaschuk et
al., 1997).

The hypothesis that, changes in integrin expression and integrin-mediated signalling
induce the differentiation of fibroblasts to myofibroblasts implies that there are
differences in integrin expression and/or function of the two cell types. There is
currently very little data on changes in integrin expression that occur when
fibroblasts differentiate into myofibroblasts.

In terms of wound healing, adult dermal wound healing is a restorative process
involving the formation of a collagen rich scar. In contrast, fetal wound healing, is a
regenerative process characterised by the absence of any such scar (Schor et al.,
1996). Oral wounds have been noted for their ability to exhibit this privileged ‘fetal-
like’ method of healing (Stephens et al., 2001a). While this is no doubt related to the
differing environments of the cell types, phenotypic differences may exist between
the cells themselves.

The aims of this chapter were therefore to investigate the role of cell-matrix
interactions, via the interaction with integrin receptors, in the myofibroblast
differentiation process in both human oral and dermal fibroblasts. We have used 3
sets of matched primary fibroblasts from the mouth and skin, to establish whether
there are any phenotypic differences - with respect to myofibroblast differentiation, between the cells that could contribute to the lack of scarring seen in the oral mucosa.

Primarily, integrins from the αv family, α5 and β1 have been targeted for blocking to establish whether these integrins are involved in fibroblast to myofibroblast differentiation and the effect this differentiation has on wound contraction.
3.2 Methods

Myofibroblast differentiation was induced by the addition of TGF-β1 to cultured cells and subsequent incubation prior to analysis. By using time course and dose dependant studies, along with previously published data (Evans et al., 2003) we found that the treatment of oral and dermal fibroblasts with TGF-β1 at a concentration of 10ng/ml for 72h caused maximal myofibroblast differentiation. These dose and time points were used throughout the rest of the studies. Throughout the thesis, HOF and HDF that had been treated with TGF-β1 for 72h at 10ng/ml will be referred to as myofibroblasts.

The integrin subunits present on the cell surface of oral and dermal fibroblasts and myofibroblasts were analysed by flow cytometry and Western blotting. Initially, 3 integrin subunits (αv, α5, and β1) were targeted for blocking studies due to their upregulation upon TGF-β1 treatment, and therefore, their possible involvement in the differentiation process. The blocking antibody was added to the cells at the same time as the TGF-β1 treatment and after 72h incubation, the cells were analysed for their α-sma content by Western blotting and immunocytochemistry. α-sma was used as the marker for myofibroblast differentiation and analysis after 72 h was performed to determine whether blocking of the particular integrin receptor/integrin subunit had prevented the TGF-β1 induced myofibroblast differentiation.

Subsequently, the effects of the specific subunits of αv (β3 and β5) were also investigated by function blocking studies. The concentration at which to use the anti-integrin antibodies was determined either experimentally (for α5 integrin blocking using the P1D6 antibody; data not shown), from previously published data (for the β1 and αv integrin blocking antibodies (Marshall et al., 1995; Thomas et al., 2001b)) or by manufacturers recommendations (for Chemicon antibodies used, see section 2.1.2 - Antibodies, for a list)

To discover whether the effects of the differentiation from fibroblast to myofibroblast, and integrin involvement in such process, had an effect on wound
 contraction, collagen gel contraction assays were used as an in vitro wound model. (See also Section 2.5.1 - Introduction to Collagen Gel Contraction Assay).

In all experiments the cells were incubated in serum free growth medium for 48h to quiesce the cells prior to treatment with growth factor and/or antibody.
3.3 Results

3.3.1 Expression of α-sma by Human Oral and Dermal Fibroblasts

To determine the effect of TGF-β1 on α-sma levels in HOF and HDF, increasing concentrations of the growth factor (0-20ng/ml) were added to the cells and incubated for 72h prior to analysis by Western blotting. Similarly, a time course study from 0-96h of TGF-β1 treatment (10ng/ml) was carried out. It was noted that a significant increase in the presence of α-sma and thereby differentiation to myofibroblast, occurred at 10ng/ml concentration (Figure 3.1 b; green bar, p=0.05), and 72 hours incubation (Figure 3.1 a; dark blue bar, p=0.05) with TGF-β1. Data shown are for HDF, similar results were also obtained for HOF.

Myofibroblast differentiation (α-sma upregulation) can be illustrated in Figures 3.2-3.4. Immunocytochemistry (Figure 3.2) illustrates that when α-sma expression is upregulated, on addition of TGF-β1 (Green staining), it appears in prominent stress fibres extending the length of the cell and localising to points of contact between the cell and substrate (Figure 3.2; b&d). In control, untreated cells, the basal levels of α-sma appear as a more diffuse globular, weak signal within the cytoplasm of the cells (Figure 3.2; a&c).

This increased level of expression seen upon TGF-β1 treatment was quantified by Western blotting (Figure 3.3). The TGF-β1 treated (α-sma positive) cells (Figure 3.4; b, d) appear larger, flattened and more stellate than the control, untreated (α-sma negative) cells (Figure 3.4; a, c) as illustrated by the modulation contrast microscopy images seen in Figure 3.4. See also Figures 4.1 & 4.2; 48hr panel for comparison)
Figure 3.1 Myofibroblast differentiation in response to TGF-β1 is both dose and time dependant.

Western blot analysis showing the induction of α-sma by HDF over time (a; 0-96h) when treated with TGF-β1 (10ng/ml) and that such induction is TGF-β1 dose dependant (b; 0-20ng/ml; 72h).

Figure shows representative bands from one experiment of HDF, similar results were obtained for HOF (data not shown). Bar charts show density analysis from an experiment run in duplicate. Error bars represent standard deviation. The experiment was run 3 times in duplicate.
Figure 3.2 TGF-β1 induces α-sma expression in HOF and HDF.

HOF and HDF, grown on glass coverslips were treated for 72h with either FGM (a&c) or FGM containing TGF-β1 (10ng/ml; b&d). α-sma (green staining) was detected using mouse monoclonal antibody 1A4 with a FITC-conjugated rabbit anti-mouse secondary antibody. Nuclei (blue staining) were visualised using DAPI. Cells were imaged on an inverted microscope using epi-fluorescence (LEICA DMRB; Leica, Milton Keynes, UK) and images captured via a Cohu CCD camera and LEICA FW4000 software (Leica, Cambridge, UK). Images taken at x63. Scale bar = 20μm.

Figure shows weak diffuse cytoplasmic staining in control oral and dermal fibroblasts treated with FGM only (a&c) and strong expression of α-sma incorporated into stress fibres in oral and dermal fibroblasts treated with TGF-β1 (b&d).
Figure 3.3 TGF-β1 induces α-sma expression in HOF and HDF.

Western blot analysis showing the induction of α-sma by HOF and HDF after treatment with TGF-β1 (10ng/ml; 72h). Figure shows a representative blot of an experiment run in triplicate with the density analysis shown in the bar chart. Statistical comparisons are shown in the text box. Error bars represent standard deviation. The experiment was performed a total of 3 times.
Figure 3.4 Changes in Morphology of HOF and HDF after treatment with TGF-β1.
Modulation contrast microscopy illustrating the morphology of HOF and HDF without (a&c) and with (b&d) treatment with TGF-β1 (10ng/ml; 72 hours). Figure illustrates the thin, spindle-like shape of untreated cells (a&c; dotted outlined cell) compared to the flat, stellate nature of TGF-β1 treated cells (b&d; dotted outlined cell). Images taken x20, scale bar = 50μm.
3.3.2 Integrin Expression on Control and TGF-β1 Treated HOF and HDF

Flow cytometry and Western blotting were used to determine the individual integrin subunits present on control and TGF-β1 treated HOF and HDF. This was used as a selection process for the subunits that were to be targeted for blocking studies. Figure 3.5 and 3.6 show the flow cytometry profiles for the integrin subunits α1, α2, α3, α4, α5, α6, αv and β1 on HOF and HDF. The mean fluorescence of each cell type (control and TGF-β1 treated HOF and HDF) stained with the specific anti-integrin antibody was recorded by the FACScan cytometer (See Appendix 2 (Figure 3.5 & Figure 3.6)) and the values used to compare the levels of integrin subunits on the surface of the cells. It was found that both HOF and HDF expressed all of the integrin subunits analysed except α6, which was negative on both cell types (Figure 3.5b & Figure 3.6b; α6). The flow cytometry data showed the presence of the proteins, but after 4 experiments, each run in duplicate, results were inconclusive as to whether there was any up- or down-regulation of the individual integrin receptors. Western blotting studies using the same integrin panel revealed that the subunits αv, α5 and β1 were upregulated upon TGF-β1 treatment (Figure 3.7 & Figure 3.8). Immunocytochemistry (Figure 3.9) illustrates the presence of these 3 subunits on the cell surface of both control and TGF-β1 treated HOF and HDF. Both these data and other supporting literature led to the αv, α5, and β1 integrin subunits being initially targeted for blocking studies.
Figure 3.5a. Integrin profile of HOF.

Flow cytometric analysis of different integrins on control (a) and TGF-β1 treated (b; 10ng/ml; 72h) HOF. a Green peaks = negative control for control HOF (no primary Ab), red/orange peaks = control HOF integrin analysis. b Green peaks = negative control for treated HOF, red/orange peaks = TGF-β1 treated HOF integrin analysis. A shift to the right from the negative control indicates a positive signal, and presence of the protein on the cell surface.

Figure represents one experiment run in duplicate. The experiment was performed a total of 3 times.
Figure 3.5b. Integrin profile of HOF contd.
Flow cytometric analysis of different integrins on control (a) and TGF-β1 treated (b; 10ng/ml; 72h) HOF. a Green peaks = negative control for control HOF (no primary Ab), red/orange peaks = control HOF integrin analysis. b Green peaks = negative control for treated HOF, red/orange peaks = TGF-β1 treated HOF integrin analysis. A shift to the right from the negative control indicates a positive signal, and presence of the protein on the cell surface. Figure represents one experiment run in duplicate. The experiment was performed a total of 3 times.
Figure 3.6a. Integrin profile of HDF.
Flow cytometric analysis of different integrins on control (a) and TGF-β1 treated (b; 10ng/ml; 72h) HDF. a Green peaks = negative control for control HDF (no primary Ab), red/orange peaks = control HDF integrin analysis. b Green peaks = negative control for treated HDF, red/orange peaks = TGF-β1 treated HDF integrin analysis. A shift to the right from the negative control indicates a positive signal, and presence of the protein on the cell surface. Figure represents one experiment run in duplicate. The experiment was performed a total of 3 times.
Figure 3.6b. Integrin profile of HDF contd.
Flow cytometric analysis of different integrins on control (a) and TGF-β1 treated (b; 10ng/ml; 72h) HDF. a Green peaks = negative control for control HDF (no primary Ab), red/orange peaks = control HDF integrin analysis. b Green peaks = negative control for treated HDF, red/orange peaks = TGF-β1 treated HDF integrin analysis. A shift to the right from the negative control indicates a positive signal, and presence of the protein on the cell surface.
Figure represents one experiment run in duplicate. The experiment was performed a total of 3 times.
Figure 3.7 αv and β1 integrin expression in HOF and HDF

Western blot analysis of integrin receptors αv and β1 on HOF and HDF. Cells were treated with either FGM (C) or FGM containing TGF-β1 (T; 10ng/ml) for 72h before being lysed and analysed by Western blotting. Figure shows representative bands from an experiment run in triplicate. The experiment was performed a total of 3 times. Bar charts represent density analysis of bands. Error bars represent standard deviation. Statistical comparisons can be seen on the graphs.
**Figure 3.8 α5 integrin expression in HOF and HDF**

Western blot analysis of the integrin receptor α5 on HOF and HDF. Cells were treated with either FGM (C) or FGM containing TGF-β1 (T; 10ng/ml) for 72h before being lysed and analysed by Western blotting. Figure shows representative bands from an experiment run in triplicate. The experiment was performed a total of 3 times. Bar charts represent density analysis of bands. Error bars represent standard deviation. Statistical comparisons can be seen on the graphs.
Chapter Three: Results One

Figure 3.9 Integrin expression on HOF and HDF.

HOF and HDF, grown on glass coverslips were treated for 72h with either FGM (C; Control) or FGM containing TGF-β1 (T; Treated; 10ng/ml). Integrins (red staining) were visualised with specific antibodies (αv:L230, β1: A11B2 and α5b1D6) followed by incubation with an anti-mouse biotin step and a streptavidin-Cy3 conjugated secondary antibody. Nuclei (blue staining) were visualised using DAPI. Images were taken at x63.

Figure illustrates the integrins on the surface of both control and TGF-β1 treated HOF and HDF. Scale bar = 20μm and is representative for each image.
3.3.3 Functional Blockade of αv Integrin can Block TGF-β1 Induced Myofibroblast Differentiation

To ascertain whether up regulation of particular integrin subunits is functionally linked to myofibroblast differentiation, anti-integrin antibodies were added to both control cells and simultaneously as the TGF-β1 treatment. Changes in α-sma expression after 72 hours of incubation were analysed by Western blotting. The function-blocking anti-αv antibody (L230; 10μg/ml) had very striking effects on both HOF and HDF when it was added to the culture medium at the same time as TGF-β1. Blockade of αv integrin prevented the upregulation of α-sma expression seen on the addition of TGF-β1 to the medium, and the levels of α-sma remained at basal levels, as seen in control cells (for T v's T+L230; p=0.05 (HOF) & p=0.05 (HDF). This prevention of upregulation is illustrated in Figure 3.10, a represents results for HOF and b results for HDF.
Figure 3.10 Effect of blocking αv integrin on TGF-β1 induced expression of α-sma.

Western blot analysis showing that blocking αv integrin with the antibody L230 (10μg/ml) prevents α-sma upregulation upon TGF-β1 treatment in HOF (a) and HDF (b). Cells were grown and treated for 72h with either FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing an αv blocking antibody, L230 (10μg/ml; C+L230) or FGM containing both TGF-β1 and L230 (T+L230) before being lysed and their α-sma content analysed by Western blotting. The upper panel in each (a&b) shows individual lanes taken from a Western blot run in triplicate, and the lower panel illustrates the mean and standard deviation following density analysis of all bands from a representative experiment. The experiment was performed a total of 3 times. Statistical comparisons are shown in the text boxes.
3.3.4 Functional Blockade of β1 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation

In a similar fashion to the blockade of the αv integrin subunit (Section 3.3.3), the anti-β1 integrin antibody A11B2 (10μg/ml) was added to HOF and HDF, to both control cells and at the same time as TGF-β1 treatment. Changes in α-sma expression after 72h of incubation were measured by Western blotting. The blockade of the β1 integrin again had particularly noticeable effects on both HOF and HDF when it was added to the culture medium at the same time as TGF-β1. Blockade of the β1 integrin prevented the upregulation of α-sma expression seen on the addition of TGF-β1 and the levels of α-sma remained that of basal levels seen in control cells (for T v's T+A11B2; p=0.05 (HOF) & p=0.05 (HDF). This prevention of upregulation is illustrated by Figure 3.11, a represents results for HOF and b results for HDF.
Figure 3.11 Effect of blocking β1 integrin on TGF-β1 induced expression of α-sma.

Western blot analysis showing that blocking β1 integrin with the antibody A11B2 (10μg/ml) prevents α-sma upregulation upon TGF-β1 treatment in HOF and HDF. Cells were grown and treated for 72hs with either FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing a β1 blocking antibody, A11B2 (10μg/ml; C+A11B2) or FGM containing both TGF-β1 and A11B2 (T+A11B2) before being lysed and their α-sma content analysed by Western blotting.

The upper panel in each (a&b) show individual lanes taken from a Western blot run in triplicate, and the lower panel illustrates the mean and standard deviation following density analysis of all bands from a representative experiment. The experiment was performed a total of 3 times. Statistical comparisons are shown in the text boxes.
3.3.5 Functional Blockade of α5 Integrin can Block TGF-β1 Induced Myofibroblast Differentiation

In a similar fashion to the blockade of αv and β1 integrin subunits (Section 3.3.3 and 3.3.4 respectively), the anti-α5 integrin antibody P1D6 (2μg/ml) was added to HOF and HDF, to both control cells and at the same time as TGF-β1 treatment. Changes in α-sma expression after 72h of incubation were measured by Western blotting. The blockade of the α5 integrin again had particularly noticeable effects on both HOF and HDF when it was added to the culture medium at the same time as TGF-β1. Blockade of the α5 integrin prevented the upregulation of α-sma expression seen on the addition of TGF-β1, and the levels of α-sma remained at basal levels, as seen in control cells (for T v’s T+P1D6; p=0.05 (HOF) & p=0.05 (HDF). This prevention of upregulation is illustrated by Figure 3.12, a represents results for HOF and b results for HDF.
Figure 3.12 Effect of blocking α5 integrin on TGF-β1 induced expression of α-sma.

Western blot analysis showing that blocking α5 integrin with the antibody P1D6 (2 μg/ml) prevents α-sma upregulation upon TGF-β1 treatment in HOF and HDF. Cells were grown and treated for 72h with either FGM (C), FGM containing TGF-β1 (10 ng/ml; T), FGM containing an α5 blocking antibody, P1D6 (2 μg/ml; C+P1D6) or FGM containing both TGF-β1 and P1D6 (T+P1D6) before being lysed and their α-sma content analysed by Western blotting.

The upper panel in each (a&b) show individual lanes taken from a Western blot run in triplicate, and the lower panel illustrates the mean and standard deviation following density analysis of all bands from a representative experiment. The experiment was performed a total of 3 times. Statistical comparisons are shown in the text boxes.
3.3.6 Functional Blockade of the $\alpha v\beta 3$ Integrin can Block TGF-β1 Induced Myofibroblast Differentiation

Use of the pan-$\alpha v$ blocking antibody (L230) indicated that $\alpha v$ integrins play a role in promoting the myofibroblast phenotype (Figure 3.10). Heterodimer specific antibodies against $\alpha v\beta 3$ (LM609; 10μg/ml) and $\alpha v\beta 5$ (P1F6; 10μg/ml) were used to determine the role, if any, of the individual $\alpha v$ containing receptors in mediating these myofibroblastic changes.

In a similar fashion to the blockade of $\alpha v$, $\beta 1$ and $\alpha 5$ integrins (Section 3.3.3, 3.3.4 and 3.3.5 respectively), the anti-$\alpha v\beta 3$ integrin antibody LM609 was added to HOF and HDF, to both control cells and at the same time as TGF-β1 treatment. Changes in $\alpha$-sma expression after 72h of incubation were measured by Western blotting. The blockade of the $\alpha v\beta 3$ integrin with the function-blocking anti-$\alpha v\beta 3$ antibody (LM609; 10μg/ml) again had particularly noticeable effects on both HOF and HDF when it was added to the culture medium at the same time as TGF-β1. Blockade of the $\alpha v\beta 3$ integrin prevented the upregulation of $\alpha$-sma expression seen on the addition of TGF-β1 and the levels of $\alpha$-sma remained at basal levels, as seen in control cells (for T $\rightarrow$ T+LM609; $p=0.05$ (HOF) & $p=0.05$ (HDF). This prevention of upregulation is illustrated by Figure 3.13, a represents results for HOF and b results for HDF.
Figure 3.13 Effect of blocking \( \alpha\nu\beta3 \) integrin on TGF-\( \beta1 \) induced expression of \( \alpha\text{-sma} \).

Western blot analysis showing that blocking \( \alpha\nu\beta3 \) integrin with the antibody LM609 (10\( \mu \)g/ml) prevents \( \alpha\text{-sma} \) upregulation upon TGF-\( \beta1 \) treatment in HOF and HDF. Cells were grown and treated for 72h with either FGM (C), FGM containing TGF-\( \beta1 \) (10ng/ml; T), FGM containing an \( \alpha\nu\beta3 \) blocking antibody, LM609 (10\( \mu \)g/ml; C+LM609) or FGM containing both TGF-\( \beta1 \) and LM609 (T+LM609).

The upper panel in each (a&b) show individual lanes taken from a Western blot run in triplicate, and the lower panel illustrates the mean and standard deviation following density analysis of all bands from a representative experiment. The experiment was performed a total of 3 times. Statistical comparisons are shown in the text boxes.
3.3.7 Functional Blockade of $\alpha v\beta 5$ Integrin can Block TGF-$\beta 1$ Induced Myofibroblast Differentiation

In a similar fashion to the blockade of $\alpha v$, $\beta 1$, $\alpha 5$ and $\alpha v\beta 3$ integrin subunits (Section 3.3.3 - 3.3.6 respectively), the anti-$\alpha v\beta 5$ integrin antibody P1F6 was added to HOF and HDF, to both control cells and at the same time as TGF-$\beta 1$ treatment. Changes in $\alpha$-sma expression after 72h of incubation were measured by Western blotting. The blockade of the $\alpha v\beta 5$ integrin with the function-blocking anti-$\alpha v\beta 5$ antibody (P1F6; 10$\mu$g/ml) again had particularly noticeable effects on both HOF and HDF when it was added to the culture medium at the same time as TGF-$\beta 1$. Blockade of the $\alpha v\beta 3$ integrin prevented the upregulation of $\alpha$-sma expression seen on the addition of TGF-$\beta 1$ and the levels of $\alpha$-sma remained at basal levels, as seen in control cells (for T v's T+P1F6; $p=0.05$ (HOF) & $p=0.05$ (HDF). This prevention of upregulation is illustrated by Figure 3.14, a represents results for HOF and b results for HDF.
Figure 3.14 Effect of blocking αvβ5 integrin on TGF-β1 induced expression of α-smooth muscle actin (α-sm). Western blot analysis showing that blocking αvβ5 integrin with the antibody P1F6 (10 μg/ml) prevents α-sm upregulation upon TGF-β1 treatment in HOF and HDF. Cells were grown and treated for 72 hours with either FGM (C), FGM containing TGF-β1 (10 ng/ml; T), FGM containing an αvβ5 blocking antibody, P1F6 (10 μg/ml; C+P1F6) or FGM containing both TGF-β1 and P1F6 (T+P1F6). Figure shows representative lanes from an experiment run in triplicate. Error bars represent standard deviation.
3.3.8 $\alpha$-sma Expression Correlates with Collagen Gel Contraction

To determine the functional consequence of TGF-β1 induced myofibroblast differentiation in terms of a wound model, HOF and HDF were cultured with or without TGF-β1 (10ng/ml) within 3D collagen type-I gels (fibroblast populated collagen lattices -FPCL’s) (See Section 2.5.2 for collagen gel composition). The FPCL’s were left to polymerise at 37°C in 95% air/5% CO$_2$ for 30 min before being overlain with FGM (Figure 3.15; Control) or FGM containing TGF-β1 (Figure 3.15; TGF; 10ng/ml). The polymerised gels were detached from the well and left free-floating in the medium. Contraction was measured over a 7 day study period. Images of the FPCL’s were captured using the Alpha Imager documentation and analysis system (Alpha Innotech Corp., Flowgen/Novara, Ashby-de-la-Zouch, UK). The area of each gel was measured using the public domain NIH Image program.

In both HOF and HDF, TGF-β1 treatment (10ng/ml) induced a significant increase in contraction, compared to the control, non-TGF-β1 treated cells after 7 days (Figure 3.15).

To determine whether changes in $\alpha$-sma expression induced by TGF-β1 and its inhibition by the $\alpha\nu$ family, $\alpha$5 and $\beta$1 integrin blocking antibodies had any effect on gel contraction, HOF and HDF were embedded within collagen gels, with or without TGF-β1 and/or function blocking antibodies to the integrins $\alpha\nu$ (L230), $\alpha$5 (P1D6), $\alpha\nu$β3 (LM609), $\alpha$νβ5 (P1F6) and $\beta$1 (A11B2). Figure 3.16 illustrates the results for HOF and Figure 3.17, the results for HDF. When an anti-$\alpha\nu$ integrin blocking antibody was added to the gels at the same time as TGF-β1, the degree of contraction resembled that seen in control, untreated cells (Figure 3.16 & Figure 3.17, T+$\alpha\nu$). Similarly, antibodies to $\alpha$5 (Figure 3.16 & Figure 3.17, T+$\alpha$5), $\alpha$νβ3 (Figure 3.16 & Figure 3.17, T+$\alpha$νβ3) or $\alpha$νβ5 (Figure 3.16 & Figure 3.17, T+$\alpha$νβ5) blocked TGF-β1 induced gel contraction (Figure 3.16 & Figure 3.17, T) to that of control levels.
The anti-β1 integrin blocking antibody completely prevented both basal and TGF-β1-induced contraction of the collagen gels. (Figure 3.16 & Figure 3.17, T+β1).

Modulation contrast microscopy (Figure 3.18 illustrates the results for HOF and Figure 3.19, the results for HDF) revealed that the cells treated with the αν (Figure 3.18 & Figure 3.19, T+αν) α5 (Figure 3.18 & Figure 3.19, T+α5), ανβ3 (Figure 3.18 & Figure 3.19, T+ανβ3) and ανβ5 (Figure 3.18 & Figure 3.19, T+ανβ5) blocking antibodies were well spread exhibiting multiple cell processes within the gel, as in the control (Figure 3.18 & Figure 3.19, C) and TGF-β1-treated gels (Figure 3.18 & Figure 3.19, T). However, in the gels where the function blocking anti-β1 integrin antibody was added, the cells were rounded showing no cell processes or spreading within the gel (Figure 3.18 & Figure 3.19, T+β1).
Chapter Three: Results One

Figure 3.15 *3D gel contraction correlates with α-sma expression.*

HOF and HDF were trypsinised and incubated with (TGF) or without (Control) TGF-β1 (10ng/ml) for 30 min prior to culturing within 3D collagen type-I gels. The gels were left at 37°C for 30 min to polymerise before being overlain with FGM (Control) or FGM containing TGF-β1 (TGF; 10ng/ml). Contraction was measured over a 7 day study period. Contraction of the collagen gels correlated with TGF-β1 induced α-sma upregulation seen in both HOF and HDF (See Figure 3.4).

Figure shows representative photomicrographs of gels from one experiment. Bar charts show gel area (mm²) from a representative experiment that was run in triplicate. Error bars represent standard deviation. Statistical comparisons are shown in the text box. The experiment was repeated a total of 3 times.

<table>
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<th>Comparison</th>
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<tr>
<td>Control HDF v’s Treated HDF</td>
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<tr>
<td>Control HOF v’s Control HDF</td>
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<tr>
<td>Treated HOF v’s Treated HDF</td>
<td>p=0.05</td>
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Figure 3.16 Effect of integrin blocking antibodies on TGF-β1 induced collagen gel contraction in HOF.

HOF were trypsinised and incubated on ice either in FGM (Control) or FGM containing TGF-β1 (TGF; 10ng/ml) and integrin blocking antibodies to αv (L230, 10µg/ml; T+αv), α5 (P1D6, 2µg/ml; T+α5), αvβ3 (LM609, 10µg/ml; T+αvβ3), αvβ5 (P1F6, 10µg/ml; T+αvβ5) or β1 (A11B2, 10µg/ml; T+β1) for 30 min prior to culturing within 3D collagen type-I gels. The gels were left at 37°C for 30 min to polymerise before being overlain with FGM. Contraction was measured over a 7 day study period.

Representative photomicrograph of gels illustrates contraction of control HOF (Control), TGF-β1 induced increased contraction (TGF) and the restriction of contraction partially or completely with the addition of specific integrin blocking antibodies. Figure shows representative gels from one experiment. Bar charts show gel area (mm²) from an experiment run in triplicate. Error bars represent standard deviation. Statistical comparisons are shown in the text box.
Figure 3.17 Effect of integrin blocking antibodies on TGF-β1 induced collagen gel contraction in HDF.
HDF were trypsinised and incubated on ice either in FGM (Control) or FGM containing TGF-β1 (TGF; 10ng/ml) and integrin blocking antibodies to αv (L230, 10μg/ml; T+αv), α5 (P1D6, 2μg/ml; T+α5), αvβ3 (LM609, 10μg/ml; T+αvβ3), αvβ5 (P1F6, 10μg/ml; T+αvβ5) or β1 (A11B2, 10μg/ml; T+β1) for 30 min prior to culturing within 3D collagen type-I gels. The gels were left at 37°C for 30 min to polymerise before being overlain with FGM. Contraction was measured over a 7 day study period.
Representative photomicrograph of gels illustrates contraction of control HDF (Control), TGF-β1 induced increased contraction (TGF) and the restriction of contraction partially or completely with the addition of specific integrin blocking antibodies. Figure shows representative gels from one experiment. Bar charts show gel area (mm²) from an experiment run in triplicate. Error bars represent standard deviation. Statistical comparisons are shown in the text box.
Figure 3.18  Modulation contrast microscopy illustrating morphology of HOF cultured within collagen gel lattices.

Modulation contrast microscopy of gels seen in Figure 3.16 illustrates that the control HOF (Control), and cells treated with TGF-β1 (TGF; 10ng/ml) and integrin blocking antibodies to αv (L230, 10μg/ml; T+αv) α5 (P1D6, 2μg/ml; T+α5), αvβ3 (LM609, 10μg/ml; T+αvβ3) and αvβ5 (P1F6, 10μg/ml; T+αvβ5) were well spread and attached within the gel. In contrast, cells treated with TGF-β1 and the anti-β1 integrin antibody (A11B2, 10μg/ml; T+β1) remained rounded and unattached.
Figure 3.19  Modulation contrast microscopy illustrating morphology of HDF cultured within collagen gel lattices.
Modulation contrast microscopy of gels seen in Figure 3.17 illustrates that the control HDF (Control), and cells treated with TGF-β1 (TGF; 10ng/ml) and integrin blocking antibodies to αv (L230, 10pg/ml; T+αv) α5 (P1D6, 2pg/ml; T+α5), αvβ3 (LM609, 10pg/ml; T+αvβ3) and αvβ5 (P1F6, 10pg/ml; T+αvβ5) were well spread and attached within the gel. In contrast, cells treated with TGF-β1 and the anti-β1 integrin antibody (A11B2, 10pg/ml; T+β1) remained rounded and unattached.
3.3.9 α-sma Expression in Oral Compared to Dermal Fibroblasts

It has been shown that oral fibroblasts have a higher level of α-sma both basally and upon TGF-β1 treatment (Figure 3.20; a) when compared to a matched sample of dermal fibroblasts. This increased expression correlates with the oral fibroblasts having an increased ability to contract collagen gels (Figure 3.20 b), and confirms previous findings that wound contraction is mediated by the myofibroblasts (Arora et al., 1999; Moulin and Plamondon, 2002).

Throughout the thesis oral and dermal fibroblasts have been used and phenotypic comparisons made. Other than the differences in the levels of α-sma, it has also been observed that oral fibroblasts grow and proliferate at a faster rate than the dermal fibroblasts. This was a qualitative observation made during the constant maintenance of the cells in culture and that the oral fibroblasts became confluent 24-48 hours before the dermal fibroblasts. However no qualitative assays were performed.

The oral fibroblasts also appear to have a higher rate of migration. This can be seen by comparing 2 graphs in Chapter 4 (Figure 4.1 and Figure 4.2). The oral fibroblasts repopulated the wound area and closed the gap more than the dermal fibroblasts.
Figure 3.20 α-sma expression and gel contraction
Panel a, taken from Figure 3.8 and Panel b, taken from Figure 3.15, illustrating that HOF have a higher level of α-sma than HDF both basally (HOF Control & HDF Control; p=0.05) and upon TGF-β1 treatment (HOF + TGF-β1 & HDF + TGF-β1; p=0.05), and that this expression correlates with HOF having an increased ability to contract FPCL’s (HOF + TGF-β1 & HDF + TGF-β1; p=0.05)
3.3.10 Integrin Binding to ECM Ligands can cause Myofibroblast Differentiation via Integrin Signalling Mechanisms

Each integrin receptor can bind to their various ligands with differing affinities. Therefore, to ascertain whether the ECM proteins themselves differentially regulate myofibroblast differentiation, HOF (Figure 3.21; a) and HDF (Figure 3.21; b) were plated onto matrix coated plates (collagen type I, fibronectin and Matrigel), grown to confluence and their \( \alpha \)-sma content analysed by Western blotting. They were compared to cells grown on plastic and those treated with TGF-\( \beta \)(10ng/ml) for 72h. Figure 3.21 illustrates the basal levels of \( \alpha \)-sma vary significantly depending on the ECM protein upon which the cells are grown. Cells grown on fibronectin (FN; green bar) express a significantly higher level of \( \alpha \)-sma compared to control cells (\( p=0.05 \) (HOF) & \( p=0.05 \) (HDF)). In contrast, the \( \alpha \)-sma expressed by the cells grown on type I collagen (col-1; yellow bar; \( p=0.127 \) (HOF) & \( p=0.127 \) (HDF)) and Matrigel (MG; dark blue bar; \( p=0.827 \) (HOF) & \( p=0.513 \) (HDF)) is not significantly different to that from control cells (Control; aqua blue bar). This data suggests that stimulation of integrin signalling pathways by matrix binding can lead to myofibroblast differentiation. Integrins can activate many intracellular signalling molecules (see Section 1.4.6 - Integrin Signalling), examples of which are FAK and Src kinases. To determine whether FAK/Src kinases are involved in the TGF-\( \beta \) induced differentiation to myofibroblast the FAK/Src kinase inhibitor PP2 (10\( \mu \)M; (Thannickal et al., 2003)), or it’s inactive analog PP3 (10\( \mu \)M; (Thannickal et al., 2003)) were added at the same time as TGF-\( \beta \) treatment, and the cells analysed by western blot for their \( \alpha \)-sma content after 72h incubation. Figure 3.22 illustrates that PP2 can inhibit the TGF-\( \beta \) induced upregulation of \( \alpha \)-sma (\( p=0.05 \) (HOF) & \( p=0.05 \) (HDF)). These results illustrate that FAK/Src kinases, and thus intracellular integrin signalling pathways are involved in myofibroblast differentiation, and that binding to fibronectin can initiate such pathways, therefore binding to fibronectin may be involved in the myofibroblast differentiation step.
Chapter Three: Results One

Figure 3.21 Expression of α-sma by HOF and HDF grown on different matrices

Western blot analysis showing the expression of α-sma by HOF (a) and HDF (b) grown on plastic (Control), plastic plus treated with TGF-β1 (TGF;10ng/ml), collagen type I coated plates (col-1), fibronectin coated plates (FN) or matrigel coated plates (MG).

Figure shows representative lanes of a single experiment. The experiment was performed twice in triplicate. The density analysis of the bands from one experiment is shown in the bar chart. Error bars represent standard deviation. Statistical comparisons are shown in the text box.
Figure 3.22 Effect of inhibition of FAK on TGF-β1 induced expression of α-sma

Western blot analysis showing that inhibition of FAK signalling with PP2 (10μM) prevents α-sma upregulation upon TGF-β1 treatment in HOF and HDF. Cells were grown and treated for 72 hours with either FGM (Control), FGM containing TGF-β1 (10ng/ml; TGF), FGM containing TGF-β1 and a FAK inhibitor, PP2 (10μM; TGF/PP2) or FGM containing TGF-β1 and PP3 (inactive analog of PP2; 10μM; TGF/PP3).

Figure shows representative lanes from an experiment run in triplicate. Error bars represent standard deviation. The experiment was performed a total of 2 times. Statistical comparisons are shown in the text box.
3.4 Results Summary

- Optimal differentiation of HOF and HDF to myofibroblast and expression of α-sma by TGF-β1 occurs at 10ng/ml after 72h.

- The integrin subunits α1, α2, α3, α4, α5, αv and β1 are expressed on the surface of control and TGF-β1 treated HOF and HDF.

- Blocking the integrin receptors αv, or α5, or β1, or αvβ3 or αvβ5 at the same time as TGF-β1 treatment inhibits (either partially or fully, depending on the subunit blocked) the increased expression of α-sma seen upon TGF-β1 treatment.

- The level of α-sma expressed by a cell correlates with the degree of collagen gel contraction. The higher the amount of α-sma, the higher the level of contraction.

- Oral fibroblasts have a higher basal and TGF-β1 induced upregulated level of α-sma when compared to dermal fibroblasts.

- Integrin binding to certain ECM Ligands can caused increased α-sma expression and myofibroblast differentiation via integrin signalling pathways.
Chapter Four

Results Two

Functional Consequences of Integrin Blockade of Myofibroblast Differentiation.

4.1 Introduction

In Chapter 3, it has been shown that by blocking integrin receptors both specific to fibronectin and vitronectin (αvβ3, αvβ5 and α5β1), and a more general subunit (β1) in the apparent absence of ligands for these receptors, we can either suppress, or prevent the TGF-β1 induced upregulation of α-sma, or differentiation to the myofibroblast phenotype. In this chapter the functional consequences that such a blockade has on the role that fibroblasts and myofibroblasts play have been investigated, specifically in terms of wound migration and matrix metalloproteinase production.

Upon wounding, fibroblasts are known to migrate into the wound site, differentiate to myofibroblasts and also produce abundant amounts of ECM proteins. Fibroblast migration begins shortly after wounding, possibly due to chemoattractive factors secreted by cells present in the wound site, such as macrophages and platelets.

Every step of wound healing requires a tightly controlled balance between matrix production, and matrix degradation. Disturbance in the balance between the two can lead to pathological conditions such as chronic ulcers, rheumatoid arthritis, osteoarthritis, atherosclerosis, tumour invasion and tumour metastasis (the result of excessive ECM degradation) or fibrosis, keloid and hypertrophic scarring (the result of excessive ECM production and accumulation). This balance is maintained by
different proteases, two important groups being the matrix metalloproteinases (MMPs) and serine proteases.

The MMPs are a well described family of structurally related zinc-dependant endopeptidases that are collectively capable of degrading all components of the ECM. There are at least 20 members of the human MMP family and they are characterised into groups according to their structure and substrate specificity, known as the collagenases, gelatinases, stromelysins and membrane type MMPs. They are all proteinases which can degrade one or more components of the ECM, they contain a Zn$^{2+}$ ion and are inhibited by chelating agents, they are synthesized in a latent, proenzyme form and they are inhibited by TIMPs (Jones et al., 2003; Lewis et al., 2000; Lewis et al., 2001; Ravanti and Kahari, 2000; Thomas et al., 1999).

The aims of this chapter were to look at differences in (i) migratory capacity in response to wounding and (ii) secretion of MMP-2 and MMP-9 by human oral and dermal fibroblasts and myofibroblasts. Furthermore, it was investigated whether the involvement of αv, β1 and α5 integrins in myofibroblast differentiation had an impact on both functionalities.
4.2 Methods

For the wound, or ‘scratch’ assay experiments HOF and HDF were grown to confluence in a 6 well plate. The medium was replaced with serum free medium for 24h to quiesce the cells and the cells were then treated with or without TGF-β1 (10ng/ml) and one of the integrin blocking antibodies. After 3 days incubation, the monolayer of cells in each well was scratched to create a cell-free zone using the configuration ‘#’ with a sterile plastic P1000 pipette tip (See figure 2.6; a; black grid). Fresh medium plus growth factor/antibody was added to the cells for 48 hours at 37°C and 5% CO₂. In vitro wound closure was monitored by the repopulation of the cell-free area over time. Images were taken immediately after wounding (0h). The fields were recorded (See figure 2.6; red boxes) and the same fields were photographed again 24h and 48h later. The gap between the migrating fibroblasts/myofibroblasts was measured and compared with the treated cells to determine whether the treatment had any affect on wound closure. Measurements of the width of the wound gap were normalised relative to the widest measurement (termed 100%) to account for the ‘scratch’ variability of using the plastic pipette tip.

Four equally spaced measurements of the wound gap were taken for each image (See figure 2.6; b; blue arrows). Four images were taken per well (See figure 2.6; a; red boxes) and triplicate wells were run per experiment. The experiment was repeated 3 times. To ensure consistency between the ‘scratches’ the widest gap measured at time 0hr was given a value 100% and all values normalised to that, so wound closure was measured as a percentage width of the wound gap, 100% being the widest gap and 0% being no gap. See also section 2.6 - Scratch Assay.

The supernatants from cells (HOF and HDF) treated with TGF-β1 and/or integrin blocking antibodies to αv, β1 and α5 (Refer to Chapter 3.3 - 3.5) were collected and analysed for their MMP-2 and MMP-9 content by gelatin zymography. A full protocol is detailed in section 2.7 - Gelatin Zymography.
4.3 Results

4.3.1 Migration of Fibroblasts and Myofibroblasts in Response to Wounding

To determine the effect that differentiation to myofibroblast (by addition of TGF-β1 to HOF and HDF) had on migration of cells in response to wounding, scratch assays on confluent monolayers of cells were performed and repopulation of the denuded area was monitored over time. It was seen that cell migration of fibroblasts (HOF = Figure 4.1, C & Dark Blue Line; HDF = Figure 4.2, C & Dark Blue Line), was high and the cells repopulated the wound gap within 48h. In contrast, the migration rate of the myofibroblasts - oral (Figure 4.1, T & Pink line) and dermal (Figure 4.2, T & Pink line), was significantly slower than the fibroblast controls (HOF: p<0.0001 and HDF: p<0.0001) and the wound area was never completely repopulated.
**Figure 4.1 Cell migration/wound closure is decreased upon treatment with TGF-β1 in HOF**

HOF grown in 6 well plates were treated with FGM (C; Dark Blue Line) or FGM containing TGF-β1 (10ng/ml; T; Pink line). After 72h, the monolayer was ‘scratched’ to create a cell free area and repopulation monitored over 48h. Control fibroblasts migrated to close the wound and fill the gap, in contrast to TGF-β1 treated fibroblasts where migration was slow and the wound area was not completely repopulated.

Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
Figure 4.2 Cell migration/wound closure is decreased upon treatment with TGF-β1 in HDF

HDF grown in 6 well plates were treated with FGM (C; Dark Blue Line) or FGM containing TGF-β1 (10ng/ml; T; Pink Line). After 72h, the monolayer was 'scratched' to create a cell free area and repopulation monitored over 48h. Control fibroblasts migrated to close the wound and fill the gap, in contrast to TGF-β1 treated fibroblasts where migration was slow and the wound area was not completely repopulated.

Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
4.3.2 Functional Blockade of αv Integrin can Block Fibroblast Migration

To discover whether αv integrin is involved in the migration of fibroblasts in response to wounding, a function blocking anti-αv integrin antibody (L230; 10μg/ml) was added to control cells and at time of TGF-β1 treatment. Following a 72h incubation period, the area was scratched and migration monitored over 48h.

Blockade of the αv integrin significantly decreased cell migration of both HOF (Figure 4.3; C+L230; Yellow Line; p<0.0001) and HDF (Figure 4.4; C+L230; Yellow Line; p<0.0001) when compared to control cells, without antibody blockade (HOF: Figure 4.3 & HDF: Figure 4.4; C; Dark Blue Line).

Blockade of the αv integrin at the time of TGF-β1 treatment should prevent the myofibroblast differentiation (as seen in section 3.3.3). Therefore the cells should act as fibroblasts, and again, as seen above, the blockade of the αv integrin prevents fibroblast migration in response to wounding (HOF: Figure 4.3 & HDF: Figure 4.4; T+L230; Aqua Blue Line; p<0.0001).
Figure 4.3 Blocking αv integrin prevents cell migration of HOF

HOF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink Line), FGM containing L230 (10μg/ml; C+L230; Yellow Line) or FGM containing TGF-β1 & L230 (T+L230; Aqua Blue Line). After 72h, the monolayer was 'scratched' to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with either the αv blocking antibody alone, TGF-β1 or a combination of the two was decreased when compared to control cell migration. Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
Figure 4.4 Blocking αv integrin prevents cell migration of HDF

HDF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink line), FGM containing L230 (10μg/ml; C+L230; Yellow line) or FGM containing TGF-β1 & L230 (T+L230; Aqua Blue Line). After 72h, the monolayer was ‘scratched’ to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with either the αv blocking antibody L230, TGF-β1 or a combination of the two was decreased when compared to control cell migration.

Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
4.3.3 Functional Blockade of β1 Integrin can Block Fibroblast Migration

In a similar fashion to αv, to discover whether β1 integrin is involved in the migration of fibroblasts in response to wounding, a function blocking anti-β1 integrin antibody (A11B2; 10μg/ml) was added to control cells and at time of TGF-β1 treatment. Following a 72h incubation period, the area was scratched and migration monitored over 48h.

Blockade of the β1 integrin significantly decreased cell migration of both HOF (Figure 4.5; C+A11B2; Yellow line; p<0.0001) and HDF (Figure 4.6; C+A11B2; Yellow line; p<0.0001) when compared to control cells, without antibody blockade (HOF: Figure 4.5 & HDF: Figure 4.6; C; Dark Blue Line).

Blockade of the β1 integrin at the time of TGF-β1 treatment should prevent the myofibroblast differentiation (as seen in section 3.3.4). Therefore the cells should act as fibroblasts, and again, as seen above, the blockade of the β1 integrin prevents fibroblast migration in response to wounding (HOF: Figure 4.3 & HDF: Figure 4.4; T+A11B2; Aqua Blue Line; p<0.0001).
Figure 4.5 Blocking β1 integrin prevents cell migration of HOF

HOF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink Line), FGM containing A11B2 (10μg/ml; C+A11B2; Yellow Line) or FGM containing TGF-β1 & A11B2 (T+A11B2; Aqua Blue Line). After 72h, the monolayer was ‘scratched’ to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with either the β1 blocking antibody A11B2, TGF-β1 or a combination of the two was decreased when compared to control cell migration. Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
Figure 4.6 Blocking β1 integrin prevents cell migration of HDF

HDF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink Line), FGM containing A11B2 (10μg/ml; C+A11B2; Yellow Line) or FGM containing TGF-β1 & A11B2 (T+A11B2; Aqua Blue Line). After 72h, the monolayer was 'scratched' to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with either the β1 blocking antibody A11B2, TGF-β1 or a combination of the two was decreased when compared to control cell migration.

Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
4.3.4 Functional Blockade of α5 Integrin has no Effect on Fibroblast Migration

In a similar fashion to αv and β1, to discover whether α5 integrin is involved in the migration of fibroblasts in response to wounding, a function blocking anti-α5 integrin antibody (P1D6; 2 μg/ml) was added to control cells and at time of TGF-β1 treatment. Following a 72h incubation period, the area was scratched and migration monitored over 48h.

Blockade of the α5 integrin had no effect on cell migration of both HOF (Figure 4.7; C+P1D6; Yellow line) and HDF (Figure 4.8; C+P1D6; Yellow line) when compared to control cells, without antibody blockade (HOF: Figure 4.7 & HDF: Figure 4.8; C; Dark Blue Line).

Blockade of the α5 integrin at the time of TGF-β1 treatment should prevent the myofibroblast differentiation (as seen in section 3.3.5). Therefore the cells should act as fibroblasts, and again, as seen above, the blockade of the α5 integrin has no effect on fibroblast migration in response to wounding (HOF: Figure 4.3 & HDF: Figure 4.4; T+A11B2; Aqua Blue Line) and the cells close the gap as in control, untreated cells.
Figure 4.7 Blocking α5 integrin has no effect on cell migration of HOF

HOF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink line), FGM containing P1D6 (2μg/ml; C+P1D6; Yellow Line) or FGM containing TGF-β1 & P1D6 (T+P1D6; Aqua Blue Line). After 72 h, the monolayer was ‘scratched’ to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with TGF-β1 was slow when compared to control cells. Incubation of the cells with either P1D6 or P1D6 and TGF-β had no effect on cell migration and wound closure was that of control cells.

Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
Figure 4.8 Blocking α5 integrin has no effect on cell migration of HDF

HDF grown in 6 well plates were treated with FGM (C; Dark Blue Line), FGM containing TGF-β1 (10ng/ml; T; Pink Line), FGM containing P1D6 (2μg/ml; C+P1D6; Yellow line) or FGM containing TGF-β1 & P1D6 (T+P1D6; Aqua Blue Line). After 72 hours, the monolayer was ‘scratched’ to create a cell free area (dotted line represents original scratch at time 0h) and repopulation monitored over 48h. Migration of fibroblasts incubated with, TGF-β1 was slow compared to control cells. Incubation of the cells with either P1D6 or P1D6 and TGF-β had no effect on cell migration and wound closure was the same as control cells. Figure shows representative experiment which was run in triplicate. The experiment was performed a total of 3 times. Error bars represent standard deviations. Statistical comparisons are shown in the text box.
4.3.5 Differentiation to Myofibroblast has no Effect on the Production of Matrix Metalloproteinases

To determine the effect that differentiation to myofibroblast (by addition of TGF-β1 to HOF and HDF) had on MMP production, gelatin zymography was performed on the supernatants of the control and TGF-β1 treated cells. It was demonstrated (Figure 4.9) that differentiation to myofibroblast in both HOF and HDF had no effect on the levels of MMP-2 (Aqua Blue Bars; HOF: p=0.121; HDF: p=0.827) and MMP-9 was not detected.
Figure 4.9 Treatment with TGF-β1 had no effect on MMP-2 and MMP-9 production by HOF and HDF
HOF grown in 6 well plates were treated with FGM (C) or FGM containing TGF-β1 (10ng/ml; T). After 72h, the supernatant was aspirated and analysed by zymography for it’s MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
4.3.6 Functional Blockade of $\alpha v$ Integrin has no Effect on the Production of Matrix Metalloproteinases

To ascertain whether the blockade of the $\alpha v$ integrin had an effect on MMP production, an anti-integrin antibody (L230; 10$\mu$g/ml) was added to both control cells and at the same time as TGF-β1 treatment. After 72h incubation, the supernatant was removed and analysed for its MMP-2 and MMP-9 content by gelatin zymography. The function-blocking anti-$\alpha v$ antibody L230 had no effect on MMP-2 production by control cells (HOF: $p=0.827$; HDF: $p=0.827$) or those treated with TGF-β1 at 10ng/ml (HOF: $p=0.275$; HDF: $p=0.827$). Again, MMP-9 was not detected. The experiment was performed 3 times and each experiment in triplicate.
Figure 4.10 Treatment with αv blocking antibody had no effect on MMP-2 and MMP-9 production by HOF

HOF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing L230 (10µg/ml; C+L230) or FGM containing L230 and TGF-β1 (T+L230). After 72h, the supernatant was aspirated and analysed by zymography for its MMP-2 and MMP-9 content.

Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
Figure 4.11 Treatment with αv blocking antibody had no effect on MMP-2 and MMP-9 production by HDF
HDF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing L230 (10µg/ml; C+L230) or FGM containing L230 and TGF-β1 (T+L230). After 72h, the supernatant was aspirated and analysed by zymography for it’s MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
4.3.7 Functional Blockade of β1 Integrin has no Effect on the Production of Matrix Metalloproteinases

To ascertain whether the blockade of the β1 integrin had an effect on MMP production, an anti-integrin antibody (A11B2; 10μg/ml) was added to both control cells and at the same time as TGF-β1 treatment. After 72h incubation, the supernatant was removed and analysed for its MMP-2 and MMP-9 content by gelatin zymography. The function-blocking anti-β1 antibody A11B2 had no effect on MMP-2 production by control cells (HOF: p=0.121; HDF: p=0.567) or those treated with TGF-β1 at 10ng/ml (HOF: p=0.121; HDF: p=0.439). Again, MMP-9 was not detected. The experiment was performed 3 times and each experiment in triplicate.
Figure 4.12 Treatment with β1 blocking antibody had no effect on MMP-2 and MMP-9 production by HOF
HOF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing A11B2 (10µg/ml; C+A11B2) or FGM containing A11B2 and TGF-β1 (T+A11B2). After 72h, the supernatant was aspirated and analysed by zymography for it’s MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
Figure 4.13 Treatment with β1 blocking antibody had no effect on MMP-2 and MMP-9 production by HDF

HDF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing A11B2 (10µg/ml; C+A11B2) or FGM containing A11B2 and TGF-β1 (T+A11B2). After 72h, the supernatant was aspirated and analysed by zymography for it's MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
4.3.8 Functional Blockade of α5 Integrin has no Effect on the Production of Matrix Metalloproteinases

To ascertain whether the blockade of the α5 integrin had an effect on MMP production, an anti-integrin antibody (P1D6; 2μg/ml) was added to both control cells and at the same time as TGF-β1 treatment. After 72h incubation, the supernatant was removed and analysed for it's MMP-2 and MMP-9 content by gelatin zymography. The function-blocking anti-α5 antibody P1D6 had no effect on MMP-2 production by control cells (HOF: p=1.000; HDF: p=1.000) or those treated with TGF-β1 at 10ng/ml (HOF: p=0.827; HDF: p=0.121). Again, MMP-9 was not detected. The experiment was performed 3 times and each experiment in triplicate.
Figure 4.14 Treatment with α5 blocking antibody had no effect on MMP-2 and MMP-9 production by HOF

HOF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing P1D6 (2μg/ml; C+P1D6) or FGM containing P1D6 and TGF-β1 (T+P1D6). After 72h, the supernatant was aspirated and analysed by zymography for it’s MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
Figure 4.15 Treatment with α5 blocking antibody had no effect on MMP-2 and MMP-9 production by HDF

HDF grown in 6 well plates were treated with FGM (C), FGM containing TGF-β1 (10ng/ml; T), FGM containing P1D6 (2μg/ml; C+P1D6) or FGM containing P1D6 and TGF-β1 (T+P1D6). After 72h, the supernatent was aspirated and analysed by zymography for it’s MMP-2 and MMP-9 content. Figure represents result for one experiment run in triplicate. The experiment was performed a total of 3 times. Error Bars represent standard deviations. ND = Not Detected.
4.4 Results Summary

• Differentiation of fibroblast to myofibroblast slowed down in vitro cell repopulation in response to wounding in both HOF and HDF.

• Blocking the integrin receptors αv and β1 inhibited fibroblast wound repopulation of both HOF and HDF.

• Blocking the integrin receptor α5 blocked myofibroblast differentiation and allowed normal wound repopulation to occur in both HOF and HDF.

• Differentiation of fibroblast to myofibroblast had no effect on MMP-2 secretion and MMP-9 secretion was undetected, in both HOF and HDF grown on a 2D rigid surface.

• Blocking the integrin receptors αv, α5 and β1 has no effect on MMP-2 secretion and MMP-9 secretion was undetected, in both HOF and HDF grown on a 2D rigid surface.
4.4 Results Summary

- Differentiation of fibroblast to myofibroblast slowed down in vitro cell migration in response to wounding in both HOF and HDF.

- Blocking the integrin receptors αv and β1 inhibited fibroblast migration of both HOF and HDF.

- Blocking the integrin receptor α5 had no effect on fibroblast migration of both HOF and HDF.

- Differentiation of fibroblast to myofibroblast had no effect on MMP-2 secretion and MMP-9 secretion was undetected, in both HOF and HDF.

- Blocking the integrin receptors αv, α5 and β1 has no effect on MMP-2 secretion and MMP-9 secretion was undetected, in both HOF and HDF.
Chapter Five

Results Three

Tumour Derived TGF-β Modulates Myofibroblast Differentiation and Promotes Scatter Factor Dependant Invasion of Oral Squamous Carcinoma Cells.

5.1 Introduction

Generation of a reactive surrounding stromal environment occurs in many different types of cancer (Tuxhorn et al., 2002). Originally the stroma was thought to play a passive role in supporting the tumour, however, it is now thought that it actively contributes towards malignant progression (Liotta and Kohn, 2001). The specific mechanisms of activation and the degree to which the stroma regulates the biology of tumourigenesis is not fully understood. Cancer cell proliferation and invasion through the basement membrane into the surrounding stroma can be considered as a disruption in tissue homeostasis. Such a disruption would lead to a repair process being initiated by these surrounding cells to try and restore tissue integrity. It has been suggested that the stromal response to carcinoma is a modified wound healing response, therefore the surrounding reactive environment is likely to mimic that of granulation tissue (Orimo et al., 2001; Ruiter et al., 2002; Tuxhorn et al., 2001). The reactive stroma surrounding carcinomas such as breast and colon, has been described as being a mix of fibroblasts, myofibroblasts, endothelial cells and immune cells (Liotta and Kohn, 2001). The presence of myofibroblasts is of particular interest in terms of their ability to produce ECM, proteases, including uPA, fibroblast activating
protein (FAP), and MMPs. Production of these results in ECM remodelling, which may be a stimulus for cancer cell growth and migration (Tuxhorn et al., 2002). Myofibroblasts, therefore appear to play a critical role and be a key cell type in the creation of an activated stromal environment for tumourigenesis.

The aims of this chapter were to investigate the role of OSCC cells in myofibroblast differentiation, to determine the effect of such cells on OSCC invasion, and to identify the possible mechanisms involved in these processes.
5.2 Methods

The work described in this chapter was carried out in collaboration with colleagues of the Richard Dimbleby/CRUK labs at St. Thomas’ Hospital, London. The immunohistochemistry and invasion assays were performed by Dr Gareth Thomas at St. Thomas’ and were not done by myself. All other experimental data were generated by myself.

5.2.1 Immunohistochemistry

15 archival OSCC’s were chosen at random. Sections (3μm) were de-waxed, brought to absolute alcohol and endogenous peroxidase neutralised with 0.5% methanolic hydrogen peroxide for 10 min. Sections were washed in water, followed by 0.05% Tween 20 in TBS pH 7.4 (TBS/Tween). Primary anti-smooth muscle actin antibody was applied for 60 min at a dilution of 1:150. Sections were again washed in TBS/Tween and secondary antibody applied for 30 min (Dako K5001 ChemMate HRP/DAB kit). Sections were washed in TBS/Tween and peroxidase-labelled streptavidin was applied for 30 min (Dako K5001). The peroxidase was visualised using DAB (Dako K5001) for 7 min and counterstained in Mayer’s haematoxylin.

5.2.2 Cell Culture

HOF were grown and maintained in FGM (See Appendix 1) as described in section 2.2 (Routine Cell Culture). A panel of 3 OSCC cell lines were used. The invasive VB6 cell line was generated previously by transfection and retroviral infection of integrin subunits to express high levels of the integrin αvβ6 (Thomas et al., 2001a). CA1 and 5PT were kind gifts from Professor I.C Mackenzie (Cardiff Dental School, UK). Cells were grown in KGM (See Appendix 1)
5.2.3 Preparation and Use of Medium Conditioned by Oral Squamous Carcinoma Cells

OSCC cells were grown to around 70% confluence in KGM (See Appendix 1) in 80cm² flasks, washed twice with PBS and incubated for 72h with 10ml of α-MEM. This squamous cell carcinoma conditioned medium (SCCM) from each cell line was collected, clarified by centrifugation and the cells detached with trypsin/EDTA and counted. Concurrently, 1.5x10³ HOF/cm² were plated in FGM in 80cm² flasks or on glass coverslips for 3 days then washed twice with PBS and incubated for 72h with either α-MEM, the SCCM (at equal volumes/keratinocyte cell number), or α-MEM containing TGF-β1 (10ng/ml). For blocking studies, anti-TGF-β1 antibody (1µg/ml) was added to the SCCM for 30 min prior to incubation on the fibroblasts. After a further 3 days, the HOF were analysed for their α-sm content by Western blotting or immunocytochemistry. All experiments were performed in triplicate and the experiments were performed 3 times.

5.2.4 Preparation and Use of Medium Conditioned by Fibroblasts and Myofibroblasts

1.5x10³ HOF/cm² were plated in FGM in 80cm² flasks for 3 days then washed twice with PBS. To induce a myofibroblast phenotype, HOF were incubated for 72h with α-MEM containing TGF-β1 (10ng/ml). Control HOF were cultured in α-MEM alone. Some cells (HOF) were incubated for 72h with each SCCM (at equal volumes / keratinocyte cell number). The HOF were washed twice with PBS, treated and cultured for a further 72h in α-MEM. The control fibroblast conditioned medium (FCM; α-MEM treated) or myofibroblast-conditioned medium (MCM; TGF-β1 or SCCM treated) was collected, clarified by centrifugation and the fibroblasts detached and counted. The volumes of FCM and MCM were corrected for cell number, adjusted to a total volume of 500µl and used in the lower chamber of a Transwell invasion assay as a chemoattractant, or assayed by ELISA for HGF/SF.
5.2.5 Analysis of Conditioned Medium for TGF-β1 and HGF/SF Content

ELISA kits for TGF-β1 and HGF/SF were purchased from R&D Systems and the conditioned medium from both OSCC (SCCM) and HOF (FCM and MCM) were analysed for TGF-β1 and HGF/SF respectively according to the manufacturers instructions (See also Section 2.9)

5.2.6 Invasion Assays

Cell invasion assays were performed using Matrigel-coated polycarbonate filters (8 μm pore size, Transwell®, BD BioSciences, Oxford, UK) as previously described (Thomas et al., 2001b). Matrigel (70μl; 1:2 dilution in α-MEM) was added to the upper membrane and allowed to gel for 1h at 37°C. FCM or MCM were corrected for cell number, adjusted to a final volume of 500μl with α-MEM, and used as a chemo-attractant in the lower chamber of the Transwell. For blocking experiments, the conditioned media were incubated with anti-HGF/SF antibody (10μg/ml) for 30 min at 4°C prior to placing in the assay. An irrelevant antibody (W632; anti MHC-type I; 10μg/μl) was used as a control. OSCC cells were plated in the upper chamber of quadruplicate wells at a density of 5x10⁴ in 200μl of α-MEM and incubated at 37°C for 72h to allow cell invasion through the matrigel. The cells in the lower chamber (including those attached to the undersurface of the membrane) were then trypsinised and counted on a Casy 1 counter (Sharfe System GmbH, Germany). Experiments were performed four times in quadruplicate.
5.3 Results

5.3.1 Myofibroblasts are seen in the Stroma of Oral Squamous Cell Carcinomas \textit{in vivo}.

Figure 5.1 (A-D) illustrates the prominence of myofibroblasts in the tumour stroma. Such cells were usually concentrated at the invasive margin of the tumour, directly abutting malignant epithelial cells (Figure 5.1, C). Stromal $\alpha$-sma expression often demarcated the margin of the tumour (Figure 5.1, C) and was only observed in close proximity to the tumour mass (Figure 5.1 D), even in tumours containing a diffuse inflammatory infiltrate. This suggests that in OSCC, myofibroblasts may be induced primarily by tumour cells.
Figure 5.1 Immunohistochemistry showing α-sma expression in myofibroblasts in oral squamous cell carcinoma (OSCC).
A = Islands of OSCC scattered throughout a myofibroblastic stroma. Magnification = x100. B = A single island of OSCC surrounded by myofibroblastic stroma. Magnification = x400. C&D = Strong α-sma expression by myofibroblasts is only detected in the near vicinity of the tumour. Magnification = x100 & x400.
5.3.2 OSCC Cells Induce Myofibroblast Differentiation Through Secretion of TGF-β1

HOF expressed low levels of α-sma in culture (Figure 5.2, C, & Figure 5.4, a). Immunostaining showed occasional cells with weak, diffuse cytoplasmic expression of the protein (Figure 5.4, a). Treatment of HOF with exogenous TGF-β1 (10ng/ml) produced a significant increase in α-sma expression (Figure 5.2, T, & Figure 5.4, b). Figure 5.4, b illustrates the increased intensity of α-sma staining and also shows that the protein is now associated with cytoplasmic stress fibres. α-sma upregulation was also observed when HOF were cultured in conditioned medium from VB6, CA1 and 5PT squamous carcinoma cell lines (SCCM) (Figure 5.2, VB6, CA1 & 5PT, and Figure 5.4, c, d & e). ELISA confirmed that the OSCC cell lines produced TGF-β1 (Figure 5.5), the highest levels secreted by 5PT cells. To demonstrate that the generation of a myofibroblastic phenotype was TGF-β1-dependent we carried out blocking studies using a TGF-β1 inhibitory antibody. Figure 5.3 (a, b & c) demonstrates that when TGF-β1 inhibitory antibody was added to SCCM from VB6, CA1 and 5PT cells prior to incubation with HOF, the induction of α-sma expression was reduced significantly (as determined by densitometric scanning; p=<0.0005, p=0.0028, p=0.0297 respectively). These data were confirmed by immunofluorescence (Figure 5.4, f, g & h), which showed only a weak diffuse cytoplasmic staining for α-sma when TGF-β1 was inhibited.
Figure 5.2 Squamous carcinoma conditioned medium induces HOF α-sma expression.

Western blot showing α-sma expression by fibroblasts compared with myofibroblasts, which had been generated by culture with exogenous recombinant TGF-β1 or SCCM from the SCC cell lines. β-actin was used as a loading control. Figure shows representative blots of an experiment run in triplicate. Error bars represent standard deviation.
**Figure 5.3 Fibroblast α-sma expression is TGF-β1 dependent.**

Western blots and densitometric quantitation showing that the induction of α-sma by SCCM from VB6 (a), CA1 (b) and 5PT (c) cells can be significantly inhibited by addition of a TGF-β1 blocking antibody. This confirmed that the generation of the myofibroblast phenotype was TGF-β1 specific. β-actin was used as a loading control.

Figure shows representative experiment performed in duplicate. Error bars represent standard deviations.
Figure 5.4 Squamous carcinoma conditioned medium induces fibroblast α-sma expression, which is TGF-β1 dependent.

HOF, grown on glass coverslips were treated for 72 hours with α-MEM, α-MEM containing TGF-β1 (10ng/ml) or SCCM from each cell line. For blocking studies, anti-TGF-β1 antibody (1μg/ml) was added to the SCCM 30 min prior to treatment. α-sma (green staining) was detected using mouse monoclonal antibody 1A4 with a FITC-conjugated rabbit anti mouse secondary antibody. Nuclei (blue staining) were visualised using DAPI. Cells were imaged on an inverted microscope using epi-fluorescence (Leica DMIRB; Leica, Milton Keynes, UK) and images captured via a CoHu CCD camera and LEICA FW4000 software (Leica, Cambridge, UK). Images were taken at x20 (a-e) & x63 (f-h).

Figure shows weak diffuse cytoplasmic α-sma staining in control fibroblasts treated with α-MEM only (a). α-sma expression is strongly upregulated in HOF treated with TGF-β1 (b) or SCCM from each of the 3 cell lines (c= VB6; d=CA1; e=5PT). Addition of the TGF-β1 inhibitory antibody to SCCM prevented α-sma upregulation (f= VB6; g=CA1; h=5PT), confirming that the induction of the myofibroblast phenotype was TGF-β1 specific.
Figure 5.5 OSCC cell lines secrete TGF-β1.
ELISA confirming that the OSCC cell lines secrete TGF-β1. OSCC cell lines – VB6 (Pink Bar), CA1 (Yellow Bar) and 5PT (Green Bar) were cultured in α-MEM for 72h. This conditioned medium was adjusted for cell number and analysed for TGF-β1 by ELISA. α-MEM was used as a control (Aqua Blue Bar). Figure shows representative experiment performed in triplicate. Error bars represent standard deviations.
5.3.3 Myofibroblast Conditioned Medium Promotes Invasion of OSCC Cells

To determine whether myofibroblasts secrete factors, which stimulate invasion of OSCC cell lines, Transwell® assays were carried out through Matrigel®. Myofibroblasts were generated by treating HOF with either TGF-β1 or SCCM from each cell line, and then cultured for a further 72h in α-MEM. The myofibroblast conditioned media (MCM) was used as a chemoattractant in the lower chamber of the Transwell, and OSCC cells were allowed to invade towards this stimulus for 72h before being counted. Fibroblast conditioned medium (HOF grown in FGM and then incubated in α-MEM for 72h) was used as a control.

It was discovered that MCM (myofibroblasts generated with OSCCM) significantly promoted invasion of VB6, CA1 and 5PT cells (Figure 5.6a, Yellow Bars) when compared with FCM (Figure 5.6a, Aqua Blue Bars), \( p < 0.0001 \), \( p < 0.0001 \) & \( p = 0.0005 \) respectively). MCM from myofibroblasts generated using exogenous recombinant TGF-β1 (10ng/ml; Figure 5.6a, Pink Bars) produced a similar level of invasion as MCM from myofibroblasts generated by SCCM from each of the cell lines (Figure 5.6a, Yellow Bars) If TGF-β1 was inactivated using a blocking antibody added to SCCM, no differentiation of fibroblast to myofibroblast was seen (Figure 5.3, a-c and Figure 5.4, f-h) and conditioned medium from such cells (remaining as fibroblasts) no longer promoted invasion of the three OSCC cell lines (Figure 5.6b, Green Bars).
Figure 5.6a. Myofibroblast conditioned medium (MCM) promotes invasion of OSCC cells.

Cell invasion assays were performed over 72h using matrigel coated polycarbonate filters. Conditioned medium from myofibroblasts, generated either with recombinant TGF-β1 (Pink Bars), or SCCM (Yellow Bars) was used as a chemoattractant in the lower chamber of the Transwell® and the cells allowed to invade. This was compared with fibroblast conditioned medium (Aqua Blue Bars). Following incubation, the cells in the lower chamber (including those attached to the undersurface of the membrane) were trypsinised and counted. Myofibroblast conditioned medium significantly promoted invasion of VB6, CA1 and 5PT cell lines. In comparison, little invasion was seen when fibroblast conditioned medium was used. Results are expressed relative to VB6 invasion using SCCM-generated myofibroblast conditioned medium (=100). Figure shows a representative experiment performed in quadruplicate. Error bars represent standard deviation.
Figure 5.6b  Myofibroblast conditioned medium (MCM) promotes invasion of OSCC cells.
Fibroblasts treated with SCCM containing an inhibitory antibody to TGF-β1 did not show myofibroblast differentiation (Figure 5.3, a-c & Figure 5.4, f-h). Conditioned medium from such cells (Green Bars) did not promote invasion compared with MCM (Yellow Bars).
Results are expressed relative to VB6 invasion using SCCM generated myofibroblast conditioned medium (=100). Figure shows a representative experiment performed in quadruplicate. Error bars represent standard deviation.
5.3.4 Myofibroblasts Upregulate Secretion of Hepatocyte Growth Factor (HGF/SF)

Previously, it has been demonstrated that myofibroblasts may secrete HGF/SF (Goke et al., 1998). This cytokine acts to promote epithelial cell growth and migration and has been shown to stimulate invasion of prostate carcinoma cells. To determine whether induction of a myofibroblastic phenotype was associated with increased production of HGF/SF, we examined conditioned medium by ELISA. Myofibroblasts were generated using either exogenous TGF-β1 or SCCM from each OSCC cell line. Untreated primary fibroblasts were used as a control. Figure 5.7 illustrates that MCM contained significantly higher levels of HGF/SF compared with FCM (up to 35-fold higher). Myofibroblasts, which had been generated using conditioned medium from the 3 OSCC cell lines (Figure 5.7; VB6: Yellow Bar, CA1: Green Bar, 5PT: Dark Blue Bar) consistently showed a significant upregulation of HGF/SF secretion when compared with primary fibroblast controls (Figure 5.7; aqua blue bar; VB6 p=0.0063; CA1 p=0.0003; 5PT p=<0.0001). Secreted HGF/SF levels were generally higher in myofibroblasts, which had been generated with conditioned medium from the 5PT cell line. This was probably due to the higher levels of TGF-β1 produced by this line (Figure 5.5, Green Bar).
Figure 5.7 Myofibroblasts secrete scatter factor/hepatocyte growth factor (SF/HGF).
Myofibroblasts were generated either with recombinant TGF-β1 (Pink Bar) or SCCM from each cell line; VB6 (Yellow Bar), CA1 (Green Bar), 5PT (Dark Blue Bar) and the supernatants analysed for secretion of SF/HGF. Untreated fibroblasts (Aqua Blue Bar) were used as a control. Supernatants were collected after 72h, and corrected for cell number before analysis. ELISA results demonstrated that myofibroblasts generated either by the addition of recombinant TGF-β1 or SCCM secrete significantly higher levels of SF/HGF than fibroblast controls. Figure shows a representative experiment performed in triplicate. Error bars represent standard deviations.
5.3.5 Inactivation of HGF/SF in Myofibroblast Conditioned Medium Inhibits Invasion of OSCC Cells

To determine whether the HGF/SF produced by the myofibroblasts had an effect in promoting tumour invasion, invasion assays were carried out using inhibitory antibodies directed against HGF/SF, which were added to the MCM. Figure 5.8 demonstrates that inactivation of HGF/SF (Dark Blue Bars) significantly reduced invasion (Yellow Bars) of VB6, CA1 and 5PT cells through Matrigel ($p=0.0014$, $p=0.0159$, $p=0.0012$ respectively). Following HGF/SF inhibition, the level of invasion was similar to that produced by FCM (Aqua Blue Bars) suggesting that the invasion-promoting effect of MCM was mediated by the presence of HGF/SF.
Figure 5.8  Inactivation of HGF/SF in MCM inhibits invasion of OSCC cells.
Conditioned medium from myofibroblasts which had been generated with SCCM was treated with or without an HGF/SF inhibitory antibody and used as a chemoattractant in the lower chamber of a Transwell® invasion assay. Conditioned medium from such cells (MCM + HGF/SF Ab) (dark blue bars) did not promote invasion compared with MCM alone (yellow bars). Results are expressed relative to VB6 invasion using SCCM generated myofibroblast conditioned medium (=100).
5.4 Results Summary

- Myofibroblasts can be seen in the surrounding stroma of oral carcinomas *in vivo*, concentrated at the invasive margin of the tumour.

- The invasive OSCC cell lines VB6, CA1 and 5PT secrete TGF-β1

- The TGF-β1 produced by the OSCC cell lines causes myofibroblast differentiation in HOF

- Myofibroblasts produce HGF/SF

- The HGF/SF produced by the myofibroblasts promotes increased OSCC invasion through basement membrane proteins
Chapter Six

Discussion

6.1 Introduction

Myofibroblasts, characterised by the expression of the cytoskeletal protein α-sma, are observed in normal wound healing; they are present in the granulation tissue and play an essential role in wound contraction and scar formation (Grinnell, 1994; Hakkinen et al., 2000; Thannickal et al., 2003). Myofibroblasts are also seen in pathological conditions such as organ fibrosis (e.g. in kidney, lung and liver) (Grupp et al., 2001; Lewis and Norman, 1998), keloids and hypertrophic scars (Lee and Eun, 1999; Tuan and Nichter, 1998) and in carcinomas (Sieuwerts et al., 1998; Tuxhorn et al., 2002).

Transformation from fibroblast to the myofibroblast phenotype can be brought about by a number of different stimuli, such as ECM proteins, TGF-β, GM-CSF, PDGF and FGFs, of which TGF-β1 is the most widely studied and accepted as the primary mediator for differentiation. The precise mechanisms which drive differentiation however, remain to be identified. An understanding of such mechanisms will provide a valuable insight into the development of new therapeutic approaches for the treatment of the pathological disorders associated with this cell phenotype.

The model used throughout this study for fibroblast to myofibroblast differentiation was treatment of cells grown on tissue culture plastic with TGF-β1. After incubation the cells were lysed and analysed for their α-sma content. Supernatant from cells treated with TGF-β1 and/or integrin blocking antibodies was removed and analysed for its MMP-2 content.
Myofibroblasts are thought to be the primary mediators of wound contraction, and scar tissue production. Wound contraction is the result of interactions between cells and their surrounding matrix, primarily myofibroblasts and collagen. To model wound contraction, tethered and floating FPCLs have been previously used as an in vitro model, with floating gels mimicking early phase wound contraction and tethered gels, late phase wound contraction (Arora et al., 1999; Brown et al., 2002; Grinnell et al., 1999; Moulin et al., 1996; Moulin et al., 1998; Stephens et al., 1996; Vaughan et al., 2000). In our system, floating gels were used throughout the studies to mimic early stage wound contraction and designed to demonstrate integrin involvement in myofibroblast differentiation step and in vitro wound contraction.

The effect of differentiation to myofibroblast and integrin involvement in cell migration in response to wounding was analysed by the so-called ‘scratch assay’. Confluent monolayers of cells were ‘scratched’ to induce a wound and repopulation was monitored over 48h microscopically. This model has also been used previously as a method to study wound repopulation (Stephens et al., 2001b), as was used over other migration assays to investigate monolayer cell migration in response to wounding.

The presence of myofibroblasts in the surrounding stroma of a panel of OSCC’s was demonstrated in vivo immunohistologically. To determine whether these squamous carcinoma cells, or substances that they may secrete can directly induce a myofibroblast phenotype, OSCC conditioned medium was cultured on fibroblasts and their α-sma content analysed after 72h. Furthermore, conditioned medium from such differentiated myofibroblasts was seen to promote OSCC invasion through basement membrane proteins by secretion of HGF/SF.

### 6.2 The Role of Integrin receptors in Myofibroblast Differentiation

In Chapter Three, it was demonstrated that a diverse range of integrin receptors were present on the surface of both fibroblasts and myofibroblasts. Also, that some, but
not all of the receptors were upregulated on the cell surface when differentiation to myofibroblast by treatment with TGF-β1 occurs. Integrin receptors that were upregulated include αv, α5 and β1. To ascertain whether the upregulation of such receptors played a role in the differentiation step, function-blocking antibodies were added to confluent monolayers of cells, grown on tissue culture plastic, at the time of treatment with TGF-β1. Blockade of these receptors, and furthermore, the specific αv receptors αvβ3 and αvβ5 restricted the upregulation of α-sma, thus preventing the differentiation to myofibroblast. Addition of the blocking antibodies (in particular β1 due to it’s promiscuous nature of pairing with most α subunits) had no effect on the adhesion of the cells to the plastic, and the cells remained adhered. This indicates that cell adhesion to the tissue culture plastic is controlled by a number of factors including the charge of the plastic, as well as receptor attachment, and that blockade of one individual integrin subunit had no overall effect on the adhesion of the cells to the plastic surface, possibly due to the cell compensating by adhering through other integrin receptors. Addition of the blocking antibodies had no effect on the cell morphology of both HOF and HDF. This can be seen in Chapter 4, Figures 4.3-4.8, C+L230, C+A11B2 & C+P1D6.

αv integrins have previously been implicated in cellular differentiation in a variety of systems including osteoclasts, (Rodan and Rodan, 1997), whereby inhibiting the αvβ3 receptor prevented bone resorption in human osteoclasts. Milner et al have shown that the β1 integrins are vital for maturation of blood vessels in the developing mouse CNS (Milner et al., 1999; Milner and Campbell, 2002). Bader et al have shown that the αv integrin is essential for normal vasculogenesis, angiogenesis and organogenesis, causing lethality in αv knock out mice (Bader et al., 1998). αv integrins have also been implicated in tumour progression and development, Kumar et al, 2001 showed that αvβ3 and αvβ5 integrins are necessary for tumour growth of mouse melanoma cells (Kumar et al., 2001) and Thomas et al showed that αvβ6 integrin has been implicated in epithelial cell differentiation, in terms of increased capacity for migration, promoting the invasive behaviour of epithelial cells in oral carcinoma (Thomas et al., 2001a). The study of αv integrins in many differentiation processes appears therefore to be important generally in many different systems.
Prevention of myofibroblast differentiation, brought about by the addition of TGF-β1 by blocking the function of specific integrin matrix receptors, suggests that there is a dual role for both the integrin receptors and the TGF-β receptors, and their downstream signalling pathways in the myofibroblast differentiation process. It has been well documented that fibroblasts and/or myofibroblasts are capable of producing abundant amounts of ECM proteins (Clark, 1996; Grinnell, 1994; Lewis and Norman, 1998; Singer and Clark, 1999), therefore as blocking the integrin receptor prevented differentiation, it was hypothesised that the action of the TGF-β1 was for the cell to produce ECM proteins and these ECM proteins activated the integrin receptors, which caused the differentiation to myofibroblast. This was investigated two ways; by plating the cells directly onto different matrix molecules to discover whether myofibroblast differentiation still took place, and secondly by using an inhibitor to FAK/Src kinases, which are two intracellular signalling molecules implicated initially in the integrin signalling cascade, treating with TGF-β1 and analysing the α-sma content as a measure of myofibroblast differentiation.

To determine whether the presence of different ECM ligands caused differentiation to myofibroblast, fibroblasts were plated directly onto different matrix molecules. The cells were allowed to grow and proliferate, and their α-sma content was subsequently analysed. It was discovered that plating on different matrices induced the cells to express altered basal levels of α-sma. When the fibroblasts were plated on Matrigel (a basement membrane matrix, composed of laminin, collagen-IV and entactin) or collagen-I, the basal levels of α-sma were not significantly different to those seen in control cells, grown on tissue culture plastic. However, when the fibroblasts were plated on fibronectin, the basal α-sma levels were significantly (p=0.05) higher than control cells. This result, and our observation that a receptor for fibronectin (α5 integrin) is upregulated upon differentiation, is not surprising, due to the requirements of fibroblasts being able to respond to the quantities of plasma fibronectin present in the wound bed.

FAK and Src are two protein tyrosine kinases which are implicated initially in integrin signalling pathways, and their phosphorylation initiates a cascade of intracellular signals (Martin et al., 2002; Schlaepfer and Hunter, 1998; Stupack and
Cheresh, 2002). FAK has also recently been implicated in the adhesion dependant myofibroblast differentiation of fetal lung fibroblasts (Thannickal et al., 2003). To determine whether the integrin signalling cascade was involved in the TGF-β1 induced differentiation to myofibroblast of oral and skin fibroblasts, inhibitors to FAK and Src were added at the time of TGF-β1 treatment. Addition of these inhibitors prevented the phenotypic differentiation to myofibroblast, thus indicating that blockade of the integrin receptors and subsequent blockade of their intracellular signalling pathways prevented the differentiation.

This implies that specific integrin-ECM interactions may regulate the differentiation process, and that the action of the TGF-β1, in this process is secretion of matrix proteins for the integrin receptors to adhere to, causing activation of integrin downstream pathways and subsequent differentiation to myofibroblast. Analysis of the supernatant from the fibroblasts and myofibroblasts for their ECM molecule content, in particular fibronectin would prove interesting to confirm previously published observations regarding the increased amount of matrix molecules secreted by myofibroblasts. Figure 6.1 illustrates a proposed summary of the process of myofibroblast differentiation. Our experiments have confirmed that HOF and HDF binding to ECM molecules such as fibronectin is capable of causing myofibroblast differentiation (Figure 6.1; ⊗), and that functional blockade of specific integrin receptor complexes, or inhibition of integrin signalling pathways prevents TGF-β1 induced myofibroblast differentiation (Figure 6.1; ⊘). This suggested mechanism has also been proposed recently (Thannickal et al., 2003) in a similar system. However, although we have no data to the contrary, a mechanism by which TGF-β1 intracellular signalling interacts directly with integrin intracellular signalling could also be a possible mechanism for differentiation and can by no means be ruled out.

It is also important to note that αvβ6 integrin has been implicated in the activation of latent TGF-β1 and that TGF-β1 LAP is in fact a ligand for αvβ6 via its’ RGD sequence (Munger et al., 1999). In our system, the TGF-β1 added at the time of treatment is pre-activated, therefore integrin involvement in activation of the TGF-β1 is of no relevance to this model.
ESPE

ECM production by fBMMF.
Look @ TGF pathway & more work
on integrin pathway.
Look @ genes upregulated.
Figure 6.1 Proposed method of myofibroblast activation by treatment with TGF-β1

TGF-β1 activates TGF-β receptors (①), causing Smad phosphorylation, translocation to target genes in the nucleus (②) and secretion of ECM molecules extracellularly (③). The secretion of matrix molecules, such as fibronectin activate integrin receptors on the cell surface (④). This causes activation of a complex network of downstream signalling pathways, including FAK activation, translocation to target genes in the nucleus (⑤) and production of the cytoskeletal protein α-sma (⑥), thus differentiation to myofibroblast.


6.3 The Role of Myofibroblasts and Cell Adhesion in a Wound Contraction Model

To discover whether the effects of the differentiation from fibroblast to myofibroblast, and integrin involvement in such a process, had an effect on wound contraction, collagen gel contraction assays were used as an *in vitro* wound model. Previous data implicating myofibroblasts as the cell type involved in contraction were confirmed (Racine-Samson et al., 1997; Vaughan et al., 2000). This was achieved by culturing oral and dermal fibroblasts alone, and comparing them to those treated with TGF-β1. The TGF-β1 treated (myofibroblast differentiated) fibroblasts exhibit a higher degree of contraction of collagen lattices than the control cells. It was also observed that the oral fibroblasts and myofibroblasts have an increased ability to contract collagen lattices when compared to their dermal equivalents, confirming previously published data that suggest oral and dermal fibroblasts are phenotypically different from each other (al Khateeb et al., 1997; Germain et al., 1994; Stephens et al., 1996), with oral fibroblasts more closely representing fetal fibroblasts. This increased ability of oral fibroblasts to contract collagen lattices correlates with our findings that oral fibroblasts express higher levels of α-sma both basally and upon treatment with TGF-β1 when compared to dermal fibroblasts, and that the expression of this protein drives contraction.

Upon addition of integrin blocking antibodies to the fibroblasts cultured within the collagen lattices, two different effects are seen. We have demonstrated that by separately blocking αv, α5, β1, αvβ3 and αvβ5 integrins, myofibroblast differentiation can be restricted, or prevented. Blockade of the β1 integrin (of which collagen is a known ligand for) at the time of culturing the cells within the collagen lattices prevents cell attachment to the matrix. The cells remain rounded within the collagen lattice, and contraction is impossible. This suggests that the complete inhibition of contraction by blockade of the β1 integrin is entirely an adhesion related phenomenon, rather than an α-sma regulated phenomenon. Our β1 data is consistent with previous studies indicating involvement of this integrin in collagen gel contraction (Arora et al., 1999; Cooke et al., 2000). In fact, it has been shown
recently (Thannickal et al., 2003), that cell adhesion is required for myofibroblast differentiation by TGF-β1.

The αv blockade of TGF-β1-induced α-sma expression is also seen on cells grown on plastic. However, in contrast to our β1 findings, the αv blocking antibody still allows adhesion of the cells to the collagen, this is due to the main ligands for the αv integrin being fibronectin and vitronectin, not collagen (Hynes, 1992). Gel contraction of cells treated with TGF-β1 and αv blocking antibody is restricted to that of control levels. This level of contraction is due to the prevention of α-sma upregulation in the αv blocked cells, rather than prevention of adhesion of the cells to the matrix, as in the β1 blockade.

The method by which αv disrupts the TGF-β1-induced increase of α-sma is unlikely to be disruption of αv-mediated adhesion, as the cells remain adhered in both the 2D and 3D cultures after blocking antibody treatment, also the experiments were not performed on known αv ligands (e.g. fibronectin, vitronectin), however, these molecules can be produced quite quickly in fairly large amounts by the cells themselves. This suggests that the dominant function of the αv receptor in these experiments is signalling rather than adhesion.

Further investigation of the αv blocking effect was performed by the use of the heterodimeric specific antibodies LM609 (αvβ3), and P1F6 (αvβ5). As there is no antibody commercially available to target αvβ1 integrin specifically, a pan-β1 antibody was the only one available for use, therefore any conclusions with regards to the blockade of this receptor is for all β1 containing integrins, and no specific conclusions for αvβ1 can be made at this stage. In a similar fashion to the αv blocked cells, both HOF and HDF remained adhered to the plastic and collagen gels after treatment with the αvβ3 and αvβ5 receptor blocking antibodies. The restriction of contraction to that of control levels is again (as with the αv blocked cells), due to the inhibition of α-sma upregulation seen upon TGF-β1 treatment by blocking the integrin receptors αvβ3 and αvβ5, rather than prevention of adhesion to the matrix, as in the β1 blocked cells.
Blockade of the α5 integrin at the same time as TGF-β1 treatment prevented the upregulation of α-sma expression seen by the cells when treated with TGF-β1 alone. As with the results from the αv blocking studies the α5 receptor blocked cells still attach to the matrix, and prevention of TGF-β1 induced upregulation of α-sma is due to a blockade in signalling pathway rather than matrix adhesion. Again, the α5 integrin blocked cells attach to the collagen within the gels and the plastic of the plates as neither are known ligands for α5 integrin (fibronectin). Gel contraction was restricted to that of control levels, as the blockade of α5 integrin prevents the TGF-β1 induced upregulation of α-sma and the cells behave in the same way as untreated, control fibroblasts.

It has been shown previously that the presence of vitronectin or fibronectin is required for contraction of collagen lattices by myofibroblasts (Ehrlich and Wyler, 1983; Gullberg et al., 1990; Kurosaka et al., 1998; Schaffer et al., 1997; Taliana et al., 2000), and that serum components play a critical role in activation of fibroblasts and wound contraction. Bearing this in mind, all collagen lattice experiments were done in the presence of serum. The precise mechanisms of contraction are yet to be determined, although suggestions that fibronectin and vitronectin may also act as a bridge between the cell and the matrix have been proposed, as in the absence of fibronectin and vitronectin, myofibroblasts were unable to effectively cause collagen lattice contraction (Taliana et al., 2000).

6.4 The Role of Fibroblasts and Myofibroblasts in Cell Migration and Protease Production

In wound healing situations, fibroblasts are known to migrate into the wound site, proliferate, differentiate and produce abundant amounts of ECM proteins. In fact, it is the fibroblasts that are responsible for the synthesis, degradation and remodelling of the ECM (Hakkinen et al., 2000; Singer and Clark, 1999). In Chapter 3, it was confirmed that integrin receptors of the αv family, α5 and β1 are involved in
fibroblast to myofibroblast differentiation, and that blocking integrin signalling either directly or by inhibiting specific receptors, differentiation is prevented.

In Chapter Four, the effect of differentiation to myofibroblast on cellular migration in response to wounding and MMP-2 and MMP-9 production was examined. Following on from that, the prevention of differentiation by the blockade of the integrin receptors αv, α5 and β1, and it's effect in migration and protease production was also analysed.

Myofibroblast differentiation has a massive implication on the migratory capacity of the cells. The cells become flattened and stellate upon differentiation and migrate significantly less than fibroblasts (HOF & HDF P<0.0001). This inhibition of migration coincides with in vivo differentiation of the cells in wound healing, where the fibroblasts migrate into the wound site, before differentiating to myofibroblasts and laying down ECM molecules such as type-I collagen (Lewis and Norman, 1998; Singer and Clark, 1999).

Cell movement into an in vivo wound site, consisting of abundant amounts of different growth factors, cytokines, cells, proteases, blood vessels, as well as cross-linked fibrin and ECM molecules etc may require up-and/or down-regulation of different cell surface receptors (such as integrin receptors) and the activation of one, or several proteolytic pathways to create a pathway for the migrating fibroblast.

Scratch assay analysis of the migration of fibroblasts and myofibroblasts confirmed that fibroblasts migrate more quickly than myofibroblasts. By addition of an integrin blocking antibody (to either α5, αv or β1) at the time of TGF-β1 treatment, as seen in Chapter Three, differentiation to myofibroblast would expected to be restricted or prevented, and therefore migration of the cells would presumably mimic control fibroblasts and be relatively fast. This was exactly the case when α5 integrin was blocked at the time as TGF-β1 treatment and fibroblast cells treated with either α5 integrin blocking antibody alone, or α5 blocking antibody and TGF-β1 migrated to close the wound gap.
However, in terms of the blockade of αv and β1 integrins, this was not the case. When αv or β1 integrin was blocked at the time as TGF-β1 treatment, again differentiation to myofibroblast would expected to be restricted or prevented, and therefore migration of the cells would presumably mimic control fibroblasts and be relatively fast. However, the migration rate of the cells when treated with either αv/β1 integrin blocking antibody alone, or αv/β1 blocking antibody and TGF-β1 was slow and impaired.

This data suggests that αv and β1 integrins are involved in both monolayer cell migration on plastic in response to wounding, as well as the TGF-β1 induced differentiation to myofibroblast, and that the dominant function of the αv and β1 receptor in these scratch experiments is adhesion rather than signalling.

Blockade of α5 integrin had no effect on fibroblast migration in both HOF and HDF, suggesting that in contrast to αv and β1, the main function of α5 integrin is the prevention of differentiation via signalling mechanisms rather than the prevention of migration via adhesion mechanisms. This was perhaps an unexpected finding, considering α5 integrin is one of the main integrins for the ECM protein fibronectin, of which there is abundant amounts in an in vivo wound, however, the assay was performed on plastic uncoated monolayer cell cultures, so an in vivo model of fibroblast migration may produce opposing results.

In Chapter Four it was shown that the HOF and HDF did not produce MMP-9, confirming previous published observations (Lewis and Norman, 1998; McKaig et al., 2003; Ravanti and Kahari, 2000; Stephens et al., 2001a). MMP-2 production was not significantly different between fibroblasts and myofibroblasts, between oral and dermal cells, nor was the secretion affected by the addition of function-blocking antibodies to the integrins α5, αv or β1, when the cells were grown and treated on plastic. It has also been documented (Sieuwerts et al., 1998) that differentiation to myofibroblasts by TGF-β1, secreted by, in this case surrounding tumour cells, caused a decrease in the production of other proteases such as uPA by myofibroblasts, therefore production of other proteases by fibroblasts and myofibroblasts may be of interest to look at.
6.5 The Role of Myofibroblasts in Oral Squamous Cell Carcinoma Invasion

Accumulation of fibroblast-like cells including myofibroblasts is frequently observed associated with the edge of an actively expanding tumour mass (Emura et al., 2000; Martin et al., 1996). Such a phenomenon has been demonstrated, to different extents, in a variety of tumours and there is increasing evidence that tumour stroma may promote tumour progression (Liotta and Kohn, 2001; Pupa et al., 2002). Interactions between epithelial cells and fibroblasts have a major role in many biological processes and it follows that the interactions between tumour cells and neighbouring myofibroblasts may be biologically significant, probably mediated by soluble factors such as growth factors and cytokines. This has been demonstrated previously in breast cancer where TGF-β1 produced by breast cancer cells activates normal breast stromal fibroblasts and promotes them to produce proteases (Ronnov-Jessen and Petersen, 1993; Sieuwerts et al., 1998). Similar interactions have been shown in prostatic carcinomas (Olumi et al., 1999; Webber et al., 1999), and in the fibrosis observed in organs such as the kidney (Lewis and Norman, 1998) and liver (Andrade et al., 1999).

In Chapter Five, the potential interactions between OSCC cells and primary oral fibroblasts were examined. It was shown that stromal cells in OSCC in vivo often express α-sma indicating a myofibroblastic phenotype (Figure 5.1). Such cells are most commonly found at the invasive margin, directly abutting tumour cells but are absent in areas distant from tumour. These data are consistent with the possibility of a tumour-derived, diffusible factor that promotes fibroblast-to-myofibroblast differentiation.

A number of cytokines including PDGF, IL-4, insulin-like growth factor II and TGF-β1 may be involved in the differentiation of fibroblasts to myofibroblasts, and these can be derived from several cell types. TGF-β1 is frequently detectable in OSCC, particularly in the more advanced stages of tumour progression, and relatively high concentrations of TGF-β1 are usually found in tumour stroma (Pasche, 2001).
Chapter Six: Discussion

Recently it has been shown that keratinocytes genetically modified to produce activated TGF-β1, induced collagen type I gene expression in dermal fibroblasts in a co-culture system (Bauer et al., 2002). The role of TGF-β1 in OSCC is complex and studies suggest that TGF-β1 has biphasic actions on tumour cells, having an important negative growth effect in the early stages of carcinogenesis, but at later stages enhancing invasion and metastasis through epigenetic mechanisms (Akhurst and Balmain, 1999; Akhurst and Derynck, 2001). However, most studies have concentrated on the direct effect of TGF-β1 on tumour cells. The data shown here suggest that a possible indirect tumour-promoting effect of OSCC-derived TGF-β1 may be in generating a myofibroblastic stroma, which in turn modulates invasion in a paracrine manner.

Myofibroblasts may promote tumour progression in a number of different ways. They upregulate expression of serine proteinases, such as uPA which degrade and remodel extracellular matrix, possibly potentiating cell invasion and migration (Sieuwerts et al., 1998). Also it has been shown that peritumour fibroblast-conditioned medium upregulated expression of the integrin αvβ6 in OSCC cells (Ramos et al., 1997), and that de novo expression of this integrin promotes invasion of oral carcinoma (Thomas et al., 2001a; Thomas et al., 2001b). Myofibroblasts also secrete interstitial matrix as well as numerous soluble mediators of inflammation and growth factors, including HGF/SF (Powell et al., 1999a; Powell et al., 1999b). HGF was originally identified as a potent mitogen for hepatocytes, but was also identified independently as scatter factor (SF), a secretory protein of fibroblasts and smooth muscle cells that dissociates and induces motility of epithelial cells. SF and HGF were later found to be identical, hence the current name HGF/SF. HGF/SF may induce invasive growth by affecting the activity and expression of cadherins, integrins and MMPs. This results in disruption of intercellular junctions, dissolution of epithelial basement membrane and altered integrin interactions with extracellular matrix (Trusolino and Comoglio, 2002). Fibroblast-derived HGF/SF has been shown to stimulate invasion and migration in a number of tumour types including squamous cell carcinoma (Matsumoto et al., 1994; Uchida et al., 2001) and it has been demonstrated previously that exogenous HGF/SF induces expression of the type IV collagenases MMP-2 and -9 in squamous carcinoma cells (Bennett et al.,
This latter observation suggesting a possible mechanism for the HGF/SF-dependent invasion through basement membrane-like Matrigel described in Chapter Five. It has also been shown recently that HGF/SF regulates integrin function in OSCC cells (Poomsawat et al., 2003).

Several other paracrine interactions between keratinocytes and fibroblasts have been demonstrated previously. For example, it has been suggested that PDGF-activated stromal cells maintain elevated keratinocyte proliferation via a paracrine mechanism (Skobe and Fusenig, 1998). IL-1 produced by epidermal keratinocytes induced expression of keratinocyte growth factor by dermal fibroblasts, which in turn stimulated keratinocyte proliferation (Maas-Szabowski et al., 2000). Paracrine interactions have also been demonstrated between squamous carcinoma cells and other cell types, it has been found that tumour-derived TGF-β1 and monocyte chemotactic protein-1 attracted and activated monocytes (Liss et al., 2001) - macrophages secreted TNF-alpha and IL-1, which in turn stimulated tumour cells to produce IL-8 and VEGF, the latter cytokine inducing angiogenesis.

Chapter 5 illustrates that a double paracrine interaction between OSCC cells and HOF can exist that results in enhanced tumour invasion. We show that OSCC-derived TGF-β1 induces a myofibroblastic phenotype and that such cells secrete significantly higher levels of HGF/SF compared with primary HOF controls. In turn, HGF/SF promotes invasion of OSCC cells through basement membrane proteins. We also confirm that the myofibroblast population is usually located adjacent to the invasive front of OSCC. These clinical observations are consistent with the suggestion that the paracrine interactions observed in vitro between OSCC and oral fibroblasts may also occur in vivo, and emphasises the importance of the stromal contribution to tumour development.

### 6.6 Conclusion

Much of the published data concerning the differentiation of fibroblast to myofibroblast concludes that TGF-β1 is a potent mediator of such differentiation.
There is very little data, however, on exactly how this occurs and the precise mechanisms involved in the TGF-β1 controlled differentiation to myofibroblast. The data presented in this thesis illustrates the added involvement of the integrin ECM receptors in the TGF-β1 regulated differentiation step. It has been proposed that production of ECM molecules and activation of integrin intracellular signalling pathways are vital components of this differentiation step. Identification of the mechanism for differentiation could in the future lead to specific targets for the prevention of production of myofibroblasts, which could have massive benefits for a number of pathological conditions. The importance of myofibroblasts within pathological conditions is illustrated by the data showing that a paracrine interaction between OSCC cells and myofibroblasts present in the immediate surrounding stroma of a tumour exists, and that this interaction can lead to increased in vitro invasion of tumours cells and thus progression of disease.

6.7 Future Studies

Interesting future work emanating from the results published in this thesis, (in a bid to try to understand the mechanisms involved in myofibroblast differentiation, with the ultimate aim being to be able to control this cell phenotype in it’s pathological state) could include a more in depth study of how the integrin and TGF-β intracellular signalling pathways are involved in the myofibroblast differentiation step and whether other integrin receptors are involved in the differentiation step.
Appendix

1. Media and Solutions

2.1.1 - Fibroblast Growth Medium (FGM)

- For 500ml
  - 89% DMEM 455ml
  - 10% FCS 50ml
  - 1% Penicillin/streptomycin 5ml

Keratinocyte Growth Medium (KGM)

- For 500ml
  - 64% DMEM 320ml
  - 22% Ham’s F12 112ml
  - 10% FCS 50ml
  - 1.8 x 10^{-4}M Adenine 5ml of 1.8x10^{-4}M stock solution
  - 0.5 μg/ml Insulin 250μl of 1mg/ml stock solution
  - 0.4 μg/ml Hydrocortisone 2ml of 100μg/ml stock solution
  - 10 ng/ml Epidermal growth factor 0.5ml of 10μg/ml stock solution
  - 1% Penecillin/streptomycin 5ml
  - 10^{-10}M Cholera toxin 500μl of 10^{-7}M stock solution
  - 2.5 μg/ml Fungizone 5ml

2.1.4 - Freezing Medium

- Per ml
  - 90% FCS 900μl
  - 10% DMSO 100μl

2.2.2 - FACS Buffer

- For 150ml
  - 90% PBS 135ml
  - 10% FCS 15ml

2.3.2 - Protein Lysis Solution

- For 50ml
  - 1% SDS 0.5g
  - 10mM Tris, pH 7.4 0.06g
  - dH2O ⇒ 50ml

Add protease inhibitor cocktail prior to lysis at 1:20 dilution

2.3.3 - 12.5% Acrylamide Separating Gel

- 30% Acrylamide ProtoGel™ 4ml
### Appendix

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
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<td>1.5M Tris-Cl (pH 8.8)</td>
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<tr>
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<td>10% SDS</td>
<td>100μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
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<tr>
<td>TEMED</td>
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<tr>
<td>dH₂O</td>
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### 5X Sample Buffer

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<td>10% SDS</td>
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<tr>
<td>50% Glycerol</td>
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<tr>
<td>β-mercaptoethanol</td>
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<tr>
<td>bromophenol blue</td>
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<td>dH₂O</td>
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### 4% Acrylamide Stacking Gel

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<tr>
<td>10% APS</td>
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<tr>
<td>TEMED</td>
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### 0.5M Tris-Cl

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<td>3g</td>
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<tr>
<td>dH₂O</td>
<td>⇒50ml</td>
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### 10X Running Buffer

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<tr>
<td>0.025M Tris, pH 8.3</td>
<td>30g</td>
</tr>
<tr>
<td>0.192M Glycine</td>
<td>144g</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>1g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>⇒1 litre</td>
</tr>
</tbody>
</table>
2.3.4 - Transfer Buffer

0.025M Tris, pH 8.3 3.03g
0.2M Glycine 14.42g
dH₂O ⇒ 1 litre

2.3.5 - Wash Buffer (PBS-T)

PBS (non-sterile) 10 tablets
0.05% Tween 500μl
dH₂O ⇒1 litre

Blocking Solution

5% Non-Fat Milk powder 5g
PBS-T 100ml

2.4.3 - 4% Paraformaldehyde

Paraformaldehyde For 100ml
dH₂O 4g
A pellet of NaOH 50ml
Warm the solution in a water-bath at 60°C until dissolved.
Add 50ml 2X PBS.

2.4.4 - 0.025% Triton-X 100

Triton-X 100 For 100ml
2.5ml of a 1% stock solution
dH₂O ⇒ 100ml

2.7.2 - 2X Sample Buffer

0.5M Tris-Cl 1ml
dH₂O 3ml
Glycerol 0.8ml
10% SDS 3.2ml
0.5% bromophenol blue 0.016g

12% Gelatine-Substrate Acrylamide Separating Gel

30% Acrylamide ProtoGel™ 4.0ml
1.5M Tris-Cl (pH 8.8) 2.5ml
dH₂O 3.2ml
10% SDS 100μl
10% Gelatin 100μl
10% APS 100μl
TEMED 4μl

2.7.3 - Renaturing Buffer

0.0625% Triton-X 100 5ml of a 2.5% stock solution
dH₂O 200ml

Developing Buffer

1M Tris-Cl, pH 7.5 5ml
5M NaCl 4ml
1M CaCl₂ 500μl
2.5% Triton-X 100 800μl
dH₂O 90ml
2. Antibody Concentrations Used Throughout the Study

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<thead>
<tr>
<th>Antibody</th>
<th>Flow Cytometry</th>
<th>Western Blotting</th>
<th>Function Blocking</th>
<th>Invasion Assay</th>
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<td></td>
<td></td>
<td>1:1000</td>
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<td>1:100 of 1mg/ml stock</td>
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<td>β-actin, clone AC-15</td>
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<td>FITC. F(ab')2 Rabbit anti-mouse</td>
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<tr>
<td>HGF/SF antibody, clone 24612</td>
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<td>MHC-class I, clone W632</td>
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3. Data From Results Chapters

**Figure 3.5**
Geometric mean fluorescence of flow cytometry traces (HOF)

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<tr>
<th>HOF</th>
<th>Integrin cont</th>
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<tr>
<td>negative</td>
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<td>10.52</td>
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<tr>
<td>Control</td>
<td>88.41</td>
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</tr>
<tr>
<td>α1</td>
<td>95.65</td>
<td>209.6</td>
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<tr>
<td>α2</td>
<td>98.39</td>
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**Figure 3.6**
Geometric mean fluorescence of flow cytometry traces (HDF)

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<td>65.47</td>
<td>102.11</td>
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References


References


References


in extracellular matrix reorganization and matrix metalloproteinase activity. Br. J.
Dermatol. 144, 229-237.

involvement in actin cable formation in an in vitro model of events associated with

(2001b). Phenotypic variation in the production of bioactive hepatocyte growth
factor/scatter factor by oral mucosal and skin fibroblasts. Wound. Repair Regen. 9,
34-43.

Opin. Cell Biol. 11, 634-640.


fibronectin is required for corneal fibroblast-seeded collagen gel contraction. Invest


Thannickal, V.J., Lee, D.Y., White, E.S., Cui, Z., Larios, J.M., Chacon, R.,
Transforming Growth Factor-beta 1 Is Dependent on Cell Adhesion and Integrin

AlphaVbeta6 integrin promotes invasion of squamous carcinoma cells through up-


117, 67-73.


Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat. Rev.


stromal myofibroblast cell line WPMY-1: a model for stromal-epithelial interactions in prostatic neoplasia. Carcinogenesis 20, 1185-1192.


