Molecular Mechanism of Podosome Formation and Proteolytic Function in Human Bronchial Epithelial Cells

By

Helan Xiao

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Physiology

University of Toronto

© Copyright by Helan Xiao 2009
Molecular Mechanism of Podosome Formation and Proteolytic Function in Human Bronchial Epithelial Cells

Helan Xiao

Doctor of Philosophy

Graduate Department of Physiology

University of Toronto

2009

Abstract

In the lung, epithelial cell migration plays a key role in both physiological and pathophysiological conditions. When the respiratory epithelium is injured, the epithelial lining in the respiratory system can be seriously damaged. Spreading and migrating of the surviving cells neighboring a wound are essential for airway epithelial repair. When the repair process is affected, aberrant remodelling may occur, which is important in the pathogenesis of lung diseases. However, in comparison with other cellular and molecular functions in the respiratory system, our understanding on lung epithelial cell migration and invasion is limited.

To gain insight into the molecular mechanisms that govern these cellular processes, I asked whether normal (non-cancerous) human airway epithelial cells can form podosomes, a cellular structure discovered from cancer and mesenchymal cells that controls cell migration and invasion. I found that phorbol-12, 13-dibutyrate (PDBu), a protein kinase C (PKC) activator, induced podosome formation in primary normal human bronchial epithelial cells, and in normal human airway epithelial BEAS2B cells. PDBu-induced podosomes were capable of degrading
fibronectin-gelatin-sucrose matrix. PDBu also increased the invasiveness of these epithelial cells. I further demonstrated that PDBu-induced podosome formation was mainly mediated through redistribution of conventional PKCs, especially PKC\(\alpha\), from the cytosol to the podosomes, whereas atypical PKC\(\zeta\) played a dominant role in the proteolytic activity of podosomes through recruitment of MMP-9 to podosomes, and MMP-9 secretion and activation. I also found that PDBu can activate PI3K/Akt/Src and ERK1/2 and JNK but not p38. PI3K, Akt and Src were critical for podosome formation, whereas ERK1/2 and JNK mediated the proteolytic activity of podosomes via MMP-9 recruitment, gene expression, release and activation without affecting podosome assembly.

Podosomes are important for epithelial cell migration and invasion, thus contributing to respiratory epithelial repair and regeneration. My thesis work unveils the molecular mechanisms that regulate podosomal formation and proteolytic function in normal human bronchial epithelial cells. These novel findings may enhance our understanding of cell migration and invasion in lung development and repair. Similar mechanisms may be also applicable to other cell types in distinct organs.
Acknowledgements

The thesis is to my beloved parents Mr. Qingcai Xiao and Ms. Dong Li. I sincerely appreciate their love and support throughout my life.

First and foremost, I would like to give my greatest gratitude to my supervisor Dr. Mingyao Liu, who has provided me opportunity, knowledge, mentorship and assistance in all these six years. As an international student and non-native English speaker, I met numerous obstacles when I first came to Toronto. Dr. Liu always encourages me and tries his best to cheer me up whenever I had troubles. Thanks to all of my current and previous academic committee members, Dr. Andras Kapus, Dr. Alan S. Mak, Dr. Wei-Yang Lu, Dr. John F. MacDonald and Dr. Daniela Rotin, for their continuous guidance, help, support, thoughtful discussion, friendship and inspiration. I will always remember their serious scientific attitudes, passion for research, dedication to science, and industrious work.

I would like to give my special gratitution to Dr. Shaf Keshavjee, Dr. Thomas Waddell, Dr. Marc de Perrot and Dr. David Hwang for their support, leadership and help. I would like to especially express my thanks to Dr. Jing Xu, Dr. Monika Lodyga, Dr. Feng Xu, Dr. Bing Han, Ms. Xiao-Hui Bai, and Dr. Jeya Nadesalingam who have provided me their technical expertise, suggestion, kindly assistance, thoughtful discussion, encouragement and friendship. Special thanks to Ms. Xiao-Hui Bai to teach me Real-time RT-PCR and PKC assay, to Mr. Rob Eves to teach me in situ zymography and gelatin gel zymography assay. I am also fortunate the dear friendship, help and support from all of my current and previous lab members: Mr. Peter S. Tang, Dr. Atsushi Shiozaki, Ms. Yu Zhang, Dr. Xiaolin He, Dr. Marco Mura, Dr. Takeshi Oyaizu, Ms. Roli Bawa, Mr. Matthew Rubacha, Dr. Yongfang Yuan, Dr. Shane Fung and Ms.
Ivone Ornelas. Many thanks to the help from all of my summer students: Mr. David Laugren, Mr. Wayne Kan, Ms. Christina Yep, Ms. Debbie Li, and Mr. Wasim Kagzi. I would give my special acknowledgements to our collaborators in Queen’s University for their contributions to my publications, technical support, contributive and thoughtful discussions: Dr. Alan S. Mak, Mr. Rob Eves, Ms. Lily Jia, and Dr. Bradley Webb. Great thanks to our entire research team members and local collaborators: Dr. Lowell Langille, Ms. Hanna Zhihong Yun, Dr. Amy Wong, Dr. Masaaki Sato, Dr. Kota Ishizawa, Dr. Masashi Gotoh, Dr. Shin Hirayama, Dr. Licun Wu, Dr. Macelo Cypel, Dr. Masaki Anruku, Mr. Pascal Dunchesneau, Mr. Paul Chartrand, Dr. Rongyu Jin, Dr. Yingzhe Zhou, Dr. Peter Sabatini, Mr. Marc L. Chretien, Dr. Rosalind Silverman and Dr. Lorelei Silverman.

I would like to give my sincere gratitude to the current and previous graduate coordinators, graduate administrators and graduate assistants in Department of Physiology for their help, kind suggestion, consultant and encouragement, Dr. Milton P. Charlton, Dr. Martin Wojtowicz, Dr. Denise Belsham, Ms. Julie Weedmark, Ms. Rosalie Pang and Ms. Colleen Shea. Great thanks to all professors and teaching assistants in the courses I have taken. And many thanks to all the people who ever kindly helped me during my PhD study.

I am also grateful for the financial support from Peterborough K. M. Hunter Graduate Studentship for cancer research.
Table of Contents

ABSTRACT .................................................................................................................................. II

ACKNOWLEDGEMENTS ....................................................................................................... IV

TABLE OF CONTENTS ........................................................................................................... VI

LIST OF FIGURES .................................................................................................................. XII

LIST OF TABLES ...................................................................................................................... XVI

LIST OF ABBREVIATIONS ............................................................................................... XVII

LIST OF PUBLICATIONS ................................................................................................. XXIII

1. CHAPTER ONE. GENERAL BACKGROUND - EPITHELIAL CELL MIGRATION
IN HUMAN LUNG PHYSIOLOGY, PATHOPHYSIOLOGY AND RESPIRATORY
DISEASES ............................................................................................................................. 1

1.1 Overview - Lung epithelial cell migration ................................................................. 3

1.2 Respiratory epithelial cell migration is an early event in the repair process .......... 4

1.2.1 Epithelial cells in the airway and lung parenchyma .............................................. 4

1.2.2 Airway epithelial cell migration occurs after tissue injury as an early event .... 6

1.3 Regulation of airway epithelial cell migration ......................................................... 10

1.4 Alveolar epithelial cell migration occurs after tissue injury .................................. 13

vi
1.5 Perspective: insight from epithelial cell migration to understanding of injury and repair of lung diseases ................................................................................................................................. 15

2. CHAPTER TWO. GENERAL BACKGROUND - CELLULAR STRUCTURES FOR CELL ADHESION, MIGRATION AND INVASION ................................................................................................................................. 17

2.1 Molecular mechanisms of cell migration ................................................................................................................................. 18

2.2 Focal adhesion ........................................................................................................................................................................ 21

2.3 Podosome ............................................................................................................................................................................. 24

2.4 Invadopodia ........................................................................................................................................................................... 28

2.5 Molecular components of podosomes ........................................................................................................................................ 28

2.5.1 Src .................................................................................................................................................................................. 29

2.5.2 PI3K/Akt ........................................................................................................................................................................ 31

2.5.3 MAPKs .......................................................................................................................................................................... 34

2.5.4 Matrix metalloproteinases ................................................................................................................................................ 36

2.6 Focal adhesion vs. podosome: what is the difference? ........................................................................................................... 42

2.7 Podosome vs. invadopodia: what is the difference? ................................................................................................................ 42

3. CHAPTER THREE. GENERAL BACKGROUND - PROTEIN KINASE C FAMILY IN CYTOSKELETON REARRANGEMENT AND CELL MOTILITY .................................................................... 47

3.1 Introduction of PKC family ...................................................................................................................................................... 48

3.1.1 Molecular structure of PKC family ........................................................................................................................................ 49

3.1.2 Mechanism of PKC activation ........................................................................................................................................... 51

3.2 The role of PKCs in cell motility ................................................................................................................................................ 54

3.2.1 Classical PKCs in cell motility ............................................................................................................................................. 54
3.2.2 Novel PKCs in cell motility ................................................................. 56
3.2.3 Atypical PKCs in cell motility.............................................................. 59
3.2.4 PKCµ in cell motility ........................................................................... 62

4. CHAPTER FOUR. RATIONALE, HYPOTHESIS AND SPECIFIC AIMS.............. 64
4.1 Rationale.................................................................................................. 65
4.2 Hypothesis.............................................................................................. 66
4.3 Specific aims .......................................................................................... 66

5. CHAPTER FIVE. MATERIALS AND METHODS ............................................. 68
5.1 Reagents and antibodies ....................................................................... 69
5.2 Cell culture ............................................................................................. 70
5.3 siRNA transfection ................................................................................ 71
5.4 Immunofluorescence staining ................................................................. 71
5.5 Microscopy and image analysis ............................................................... 72
5.6 Live cell imaging .................................................................................... 73
5.7 Fibronectin based in situ zymography .................................................. 74
5.8 Gelatin based in situ zymography .......................................................... 75
5.9 Cell invasion assay ................................................................................ 75
5.10 Western blotting .................................................................................. 76
5.11 Gelatinase zymography gel ................................................................. 76
5.12 Real-Time quantitative RT-PCR ........................................................... 77
5.13 Statistics ............................................................................................... 78
6. CHAPTER SIX. PHORBOL ESTER-INDUCED PODOSOMES IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS.................................................................................................................. 79

6.1 Summary.............................................................................................................................................................................. 80

6.2 Introduction......................................................................................................................................................................... 80

6.3 Results ......................................................................................................................................................................................... 82

6.3.1 PDBu induces reorganization of cytoskeleton in normal human bronchial epithelial cells .................................................................................................................................................................................. 82

6.3.2 Identification of ventral podosomes and circular dorsal ruffles ............................................................ 86

6.3.3 Formation of podosomes in primary normal human bronchial epithelial cells... 89

6.3.4 Accumulation of actin-associate proteins and regulators in podosome-like structures .................................................................................................................................................................................. 93

6.3.5 Recruitment of integrin and associated proteins in podosome-like structures .... 96

6.3.6 Lack of vinculin in PDBu-induced small dots in normal human lung epithelial cells ................................................................................................................................................................................................ 99

6.3.7 Increased protein tyrosine kinases in podosome-like structures ...................... 102

6.3.8 Distribution of MMPs in podosome-like structures ................................................. 102

6.3.9 PDBu-induced podosome-like structures can degrade extracellular matrix .... 107

6.4 Discussion......................................................................................................................................................................................... 110

7. CHAPTER SEVEN. PKCZ REGULATES RECRUITMENT OF MMP-9 TO PODOSOMES, AND ITS RELEASE AND ACTIVATION ................................................................. 115

7.1 Summary......................................................................................................................................................................................... 116

7.2 Introduction......................................................................................................................................................................................... 117

7.3 Results ......................................................................................................................................................................................... 118
7.3.1 PDBu-induced PKC phosphorylation and translocation to podosomes and circular ruffles ................................................................. 118
7.3.2 PDBu-induced podosome assembly depends on cPKCs .................. 124
7.3.3 PDBu-induced matrix degradation can be blocked by siRNAs of multiple PKC isoforms ................................................................. 127
7.3.4 PKCζ mediates PDBu-induced proteolytic activity of podosomes, MMP-9 recruitment to podosome, and MMP-9 release and activation ...................... 130
7.3.5 PDBu-induced MMP-9 is involved in degradation of gelatin matrix .......... 136
7.3.6 Recruitment of PKCζ to PDBu-induced posodosmes depended on nPKC ... 139
7.4 Discussion ......................................................................................... 144

8. CHAPTER EIGHT. COORDINATED REGULATION OF PKC ACTIVATION-
INDUCED PODOSOME FORMATION AND PROTEOLYTIC ACTIVITIES OF PI3K,
SRC AND MAPK PATHWAYS IN HUMAN BRONCHIAL EPITHELIAL CELLS ...... 148
8.1 Summary .......................................................................................... 149
8.2 Introduction ....................................................................................... 150
8.3 Results .................................................................................................. 151
8.3.1 PDBu-induced protein phosphorylation and translocation of PI3K/Akt related signaling molecules ......................................................... 151
8.3.2 Effects of PDBu-stimulation on phosphorylation and distribution of Src and MAPKs ............................................................................... 153
8.3.3 PI3K/Akt and Src regulates podosome assembly and proteolytic activity .... 155
8.3.4 ERK and JNK mediate proteolytic activity of PDBu-induced podosomes without affecting podosome formation .............................................. 160
8.3.5 Crosstalk among PI3K/Akt, Src and MAPK pathways ........................................... 164
8.4 Discussion ...................................................................................................................... 169

9. CHAPTER NINE. SUMMARY, GENERAL DISCUSSION AND PERSPECTIVES .. 172
9.1 Summary .................................................................................................................... 173
9.2 Discussion ................................................................................................................... 175
  9.2.1 The role of podosomes in normal bronchial epithelial cells in lung physiology and
  pathophysiology ........................................................................................................... 175
  9.2.2 PKCs and cell motility in lung physiology and pathophysiology ...................... 176
  9.2.3 PKC isoforms and isoform redundancy ............................................................... 177
  9.2.4 Cell migration vs. cell invasion ......................................................................... 177
  9.2.5 Podosome formation vs. podosomal proteolytic function ................................. 178
  9.2.6 MMP-9 gene expression vs. MMP-9 recruitment ............................................. 179
  9.2.7 Study approaches and their limitations .............................................................. 182
    9.2.7.1 Cell lines and primary cells ................................................................. 182
    9.2.7.2 Chemical inhibitor and siRNA .............................................................. 183
    9.2.7.3 In vitro vs. in vivo .............................................................................. 183
  9.3 Conclusions .............................................................................................................. 184
  9.4 Future directions ...................................................................................................... 186

10. REFERENCES ............................................................................................................ 188
List of Figures

| Figure 1-1     | Epithelial cells in the lung     | 5  |
| Figure 1-2     | Lung epithelial repair after injury | 7  |
| Figure 1-3     | Cellular mechanism of lung epithelial repair after injury | 8  |
| Figure 2-1     | Steps of cell migration           | 19 |
| Figure 2-2     | Structure of focal adhesion       | 22 |
| Figure 2-3     | Morphology of lamellipodia, podosomes and invadopodia | 26 |
| Figure 2-4     | Structure of podosome             | 27 |
| Figure 2-5     | PI3K/Akt signaling pathway        | 33 |
| Figure 2-6     | MAPKs signaling pathway           | 35 |
| Figure 2-7     | Molecular structure of MMPs       | 38 |
| Figure 3-1     | Molecular structure of PKCs       | 50 |
| Figure 3-2     | The serine/threonine phosphorylation sites of PKCs in the catalytic domain | 52 |
| Figure 3-3     | Molecular mechanism of PKCs activation | 53 |
| Figure 6-1     | PDBu induces reorganization of cytoskeletal structure in BEAS2B cells | 84 |
| Figure 6-2     | Characterization of ventral podosomes and circular dorsal ruffles in BEAS2B cells | 87 |
| Figure 6-3     | Characterization of ventral podosomes and circular dorsal ruffles in primary normal human bronchial epithelial cells | 90 |
| Figure 6-4     | PDBu induces reorganization of cytoskeletal structure in A549 cells | 92 |
Figure 6-5  Localization of actin associated proteins in podosomes and circular dorsal ruffles  94

Figure 6-6  PDBu-induced redistribution of integrin and integrin associated proteins  97

Figure 6-7  Lack of translocation of vinculin to actin-rich dots in normal human airway epithelial cells after PDBu stimulation  100

Figure 6-8  Protein tyrosine kinases were recruited to PDBu-induced podosomes and circular dorsal ruffles  103

Figure 6-9  Matrix metalloproteases are associated with podosomes and circular dorsal ruffles  105

Figure 6-10  BEAS2B cells with podosomes can degrade matrix and are invasive  108

Figure 7-1  PDBu-induced PKC phosphorylation  120

Figure 7-2  PDBu-induced PKC translocation to podosomes  122

Figure 7-3  PDBu-induced podosome formation is mainly mediated through classical PKC, but matrix degradation can be blocked by multiple PKC inhibitors  125

Figure 7-4  PDBu-induced podosome formation is reduced by PKCα siRNA but matrix degradation can be blocked by multiple PKC siRNA  128

Figure 7-5  PKCζ regulates proteolytic activity of PDBu-induced podosomes and MMP-9 translocation to podosomes  131

Figure 7-6  PKCζ regulates MMP-9 release and activation  134

Figure 7-7  Role of MMP-9 and MMP-14 in PDBu-induced proteolytic activity of podosomes  137
Figure 7-8  Rottlerin reduced PDBu-induced translocation of PKCζ to podosomes 140

Figure 7-9  PKCδ controls recruitment of PKCζ and MMP-9 to podosomes 142

Figure 8-1  PDBu-induced phosphorylation and translocation of PI3K/Akt related proteins 152

Figure 8-2  PDBu-induced phosphorylation and translocation of Src and MAPK signaling proteins 154

Figure 8-3  PI3K and Akt regulate podosome assembly and its proteolytic activity through MMP-9 expression, release and activation 156

Figure 8-4  Src is involved in podosome formation and proteolytic activity 158

Figure 8-5  ERK and JNK mediate proteolytic activity of PDBu-induced podosomes 161

Figure 8-6  Inhibitors for ERK and JNK pathways prevented MMP-9 translocation to PDBu-induced podosomes 163

Figure 8-7  Confirmation of ERK pathway in PDBu-induced gelatin matrix degradation, MMP-9 translocation and gene expression with another MEK inhibitor 166

Figure 8-8  Effects of multiple inhibitors on phosphorylation status of PI3K/Akt, Src and MAPKs 168

Figure 9-1  Role of PKCs in podosome formation and podosomal proteolytic function 174

Figure 9-2  MAPK signaling pathways regulate MMP-9 181

Figure 9-3  PKC, PI3K, Akt, Src and MAPK in podosome formation and 185
podosomal proteolytic function
List of Tables

Table 1-1  Factors inhibit airway epithelial cell migration  11
Table 1-2  Factors promote airway epithelial cell migration  12
Table 1-3  Factors affect alveolar epithelial cell migration  14
Table 2-1  Comparison podosome and invadopodia in 2D vs. 3D culture  44
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABR</td>
<td>Actin binding repeat; cortacin domain containing the repeat region</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>Arp</td>
<td>actin related protein</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Arp2/3 complex; complex of 7 proteins including Arp2 and Arp3, which nucleate dendritic actin filaments</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovarian cell line</td>
</tr>
<tr>
<td>cPKC</td>
<td>Conventional protein C; classical PKC</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac1 interacting binding domain</td>
</tr>
<tr>
<td>Csk</td>
<td>Carboxyl-terminal Src kinase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Dyn2</td>
<td>Dynamin 2</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial-cadherin</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetra acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene-bis (oxyethylenenitrilo) tetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin; a two-stranded polymer comprised of actin monomers</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin; monomeric actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GBD</td>
<td>GTPase binding domain</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
</tbody>
</table>
GDI  Guanine nucleotide dissociation inhibitor
GEF  Guanine nucleotide exchange factor
GFP  Green fluorescent protein
GIT1 G-protein-coupled receptor kinase-interacting target 1
GITBD GIT1-binding domain of PIX
GPCR G-protein-coupled receptor
GST  Glutathione S-transferase
HB-EGF Heparin-binding EGF-like growth factor
HEPES N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]
HCl  Hydrochloric acid
HDL  High-density lipoproteins
HG-DMEM High glucose Dulbecco's modified Eagle's medium
HGF  Hepatocyte growth factor
HIF-1 Hypoxia inducible factor-1
ICAM-1 Intercellular adhesion molecule-1
IGF  Insulin growth factor
IKK  I kappa B kinase
IL   Interleukin
JNK  c-Jun N-terminal kinase
kDa  Kilo Dalton
LDL  Low-density lipoproteins
LPS  Lipopolysaccharide
MAPK Mitogen-activated protein kinase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARCKS</td>
<td>Myristlated alanine-rich C kinase substrate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Membrane type 1-matrix metalloproteinases; MMP-14</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nPKC</td>
<td>Novel protein kinase C</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDBu</td>
<td>Phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH domain</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PICK1</td>
<td>Protein interacting with C kinase 1</td>
</tr>
<tr>
<td>PIX</td>
<td>PAK-interacting exchange factor</td>
</tr>
<tr>
<td>PKB</td>
<td>Proteins kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetoate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferators activated receptor</td>
</tr>
<tr>
<td>PS region</td>
<td>Phosphatidylserine region</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome ten</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>pTyr</td>
<td>Phosphorylated tyrosine</td>
</tr>
<tr>
<td>RACK</td>
<td>Receptors for activated C kinase</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RICK</td>
<td>Receptors for inactive C kinase</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time transcriptase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology domain 3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SSeCKS</td>
<td>Src-suppressed C kinase substrate</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiole cyanate</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>t-octylphenoxy polyethoxyethanol</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>VCR</td>
<td>Verprolin, central and acidic domain of the WASp family of NPFs</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott Aldrich Syndrome protein</td>
</tr>
<tr>
<td>WIP</td>
<td>WASp interacting protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
List of Publications

Publications included in PhD thesis:

Xiao H, Mura M, Li D, Liu M. Epithelial cell migration in respiratory diseases. (Review) (Submitted to *American Journal of Physiology-Lung Cellular and Molecular Physiology*) (Chapter 1)

Xiao H, Liu M. PKC in cell motility. (Review) (In preparation) (Chapter 3)


Xiao H, Bai XH, Kapus A, Mak AS, Lu WY, Liu M. PKCζ regulates recruitment of MMP-9 to podosomes, and its release and activation. (2nd revision of *Molecular Biology of the Cell*) (Chapter 7)

Xiao H, Bai XH, Mak AS, Liu M. Coordinated regulation of PKC activation-induced podosome formation and proteolytic activities of PI3K, Src and MAPK pathways in human bronchial epithelial cells. (In preparation for *Journal of Cell Science*) (Chapter 8)

Publications not included in PhD thesis:


Han B*, Xiao H*, Xu J, Bai XH, Liu M. Regulation of Src activity by AFAP leading to SRE/AP-1 transcriptional activation. (Under revision of *Biochemical Journal*, * contribute equally)

Xiao H, Liu M. XB130, Fish/Tks5 and Src interaction in podosome formation and proteolytic function. (In preparation)
1. Chapter One. General Background - Epithelial cell migration in human lung physiology, pathophysiology and respiratory diseases

Part of the content of this chapter was prepared as a review article for *American Journal of Physiology-Lung Cellular and Molecular Physiology*:

**Xiao H**, Mura M, Li D, Liu M. Epithelial cell migration in respiratory diseases. (Review) (Submitted)
1. Chapter One. General background - Epithelial cell migration in human lung physiology, pathophysiology and respiratory diseases

Respiratory epithelial cells are more than a structural component in the lung and may contribute to normal lung physiology such as lung development and homoeostasis, and lung repair after injury (1) through cell migration, differentiation and proliferation (2). These processes are temporally strictly regulated by specific intracellular signaling pathways. My PhD studies focus on the discovery of podosomes, a cellular structure responsible for cell invasion and migration, formed by normal human bronchial epithelial cells. I have found that several protein kinase C (PKC) isozymes are involved in podosome formation and proteolytic activities in a coordinated fashion. I further found that PKC activated other signaling pathways, which also contributed to the podosome functions. Firstly, in this chapter, I will review the role of epithelial cell migration in human lung physiology, pathophysiology and various respiratory diseases. Secondly, in Chapter two, I will introduce the cellular structures especially podosomes involved in cell adhesion, migration and invasion. Thirdly, in Chapter three, I will review the role of protein kinase C (PKC) family in cell motility.

In this chapter, I will focus on the role of epithelial cell migration in lung physiology, pathophysiology and human lung diseases. Firstly, I will review the sub-types of epithelial cells in the lungs and their specific physiological functions. Secondly, I will introduce the role of airway epithelial cell motility and their regulatory factors under physiological and pathophysiological conditions. Thirdly, I will summarize the role of alveolar epithelial cell migration and their specific mediatory factors in normal lung physiology and respiratory diseases.
1.1 Overview - Lung epithelial cell migration

Cell migration is a central process in the development and maintenance of multicellular organisms. In the adult, cell migration is central to homeostatic processes such as mounting an effective immune response and the repair of injured tissues. Cell migration has been described in many organs and systems, contributing to many pathologic conditions, such as chronic inflammatory diseases, vascular diseases, tumour formation and metastasis. In wound repair, migration of epithelial cells and other tissue cells is required to heal the injury.

In the lung, epithelial cell migration plays a key role in both physiological and pathophysiological conditions. Epithelial cell migration is involved in embryo and fetal lung development, lung growth, maintenance of the air-blood barrier and homeostasis. When the respiratory epithelium is injured by various insults, such as inhaled dusts, toxins, cigarette smoking, infection of airborne micro-organisms, physical injuries (e.g. endotracheal intubation and mechanical ventilation), the epithelial lining in the respiratory system can be seriously damaged. In these pathophysiological conditions, cell migration as well as cell proliferation is required for proper tissue repair, in order to restore proper barrier and gas exchange functions. When the repair process is impaired, aberrant remodelling may occur, which plays an important role in the pathogenesis of several lung disorders. However, in comparison with other cellular and molecular processes in the respiratory system, our understanding on lung epithelial cell migration is limited. The objective of this chapter is to promote interests in this fundamental research area.

Although epithelial cell migration also plays a very important role in tumour cell invasion and metastasis, in this chapter we will only focus on respiratory epithelial cell migration in lung physiology and various non-tumour disorders. To facilitate the discussion, we will focus on the
role of cell migration in the repair process in the context of airways and lung parenchymal diseases. Gaining a better understanding of the regulatory mechanisms of respiratory epithelial cell migration, along with its clinical applications, may be of vital importance in promoting normal respiratory function and healing, as well as treating many respiratory disorders that are associated with great morbidity and mortality.

1.2 Respiratory epithelial cell migration is an early event in the repair process

1.2.1 Epithelial cells in the airway and lung parenchyma

The respiratory epithelium consists of highly differentiated epithelial cells exerting different physiological functions at different pulmonary compartments (Figure 1-1). The majority of the upper respiratory tree, excluding the vocal cords, is lined by pseudo-stratified, tall columnar, ciliated epithelial cells. In the trachea and main bronchi, airway epithelial cells provide physical protection and also participate in the innate immunity (3). Bronchial epithelial cells provide a physical barrier with intercellular junctions against microorganisms; other defensive mechanisms include secretion and clearance of mucus and the homeostasis of ion and water transport (4). In the proximal bronchioles, epithelial cells take on a more cuboidal shape, with both ciliated cells, and secretory nonciliated Clara cells. In the distal bronchioles, only Clara cells can be identified.
Figure 1-1. Epithelial cells in the lung.

The respiratory epithelium is covered by highly differentiated epithelial cells. The upper respiratory tree is lined by pseudostratified, tall columnar, ciliated epithelial cells. In the trachea and main bronchi, bronchial epithelial cells provide a physical barrier against microorganisms. In the proximal bronchioles, epithelial cells take on a more cuboidal shape, with both ciliated cells, and secretory nonciliated Clara cells. In the distal bronchioles, both Clara and non Clara cells can be identified. Alveolar epithelial type I and type II cells cover the surface of alveoli. Type I pneumocytes are flattened squamous epithelial cells.
Alveolar epithelial cells cover the surface of alveoli. Type I pneumocytes are flattened squamous epithelial cells, which interface with pulmonary capillaries and are highly permeable to gases, thus perfectly suitable for gas exchange at the air-blood barrier. Type II pneumocytes synthesize and secrete pulmonary surfactant, and transport sodium from apical to basolateral cell surfaces to minimize alveolar fluid. Type II cells are involved in the innate host defense by producing a variety of cytokines, chemokines and soluble mediators (5). Type II cells may act as progenitor cells for type I alveolar cells (6).

1.2.2 Airway epithelial cell migration occurs after tissue injury as an early event

The respiratory epithelium is subjected to various chemical, physical, environmental and inflammatory insults, which can vary in severity from temporary induction of surface epithelium permeability due to destruction of tight junctions, to cell death and denudation of the epithelial lining (Figure 1-2) (7).

The repair and regeneration of the epithelium is accomplished by three processes: the neighbouring basal cells migrating over the denuded area, which is followed by cell proliferation, active mitosis, squamous metaplasia, and finally, redifferentiation to pseudo-stratified mucociliary epithelium (8) (Figure 1-3). These three steps play a distinct role at the different stages of epithelium repair and regeneration.
Figure 1-2. Lung epithelial repair after injury.

The respiratory epithelium is subjected to various chemical, physical, environmental and inflammatory injuries, which can vary in severity from temporary induction of surface epithelium permeability due to destruction of tight junctions, to complete death and denudation of the epithelial cell lining.
Figure 1-3. Cellular mechanism of lung epithelial repair after injury.

Lung epithelial repair mainly depends on three processes: 1) epithelial cell migration toward the wound area; 2) proliferation of the lung epithelial progenitor cells; 3) differentiation. Epithelial cell migration is an early event of the lung repair after injury. Both the airway epithelial cells and alveolar epithelial cells can migrate to cover the wound area in vitro and in vivo.
In order to understand the sequence of events that occur during wound healing in the respiratory system, various experimental models have been developed, involving the induction of injury to the respiratory epithelium and observation of the healing process and mechanisms. Cell spreading and migration are the initial steps of wound repair in epithelial cells, as complete wound closure has been noted in cell sheets as early as within 5-8 hours, long before the effects of proliferation could be a contributing factor (7, 9-13). In fact, in one study, the repair process was seen to begin almost immediately, with the remaining basal cells being seen to flatten and migrate to cover the basement membrane within less than 20 minutes (14). Also, cell mitotic activity typically does not peak until 24-48 hours after wounding, and mainly involved cells located close to the wound edge (10, 12).

Interestingly, cells at the wound edge have been reported to travel 2.5 times more distance than cells located far from the wound area (10), and migration speed was highest for cells at the wound edge, at 35-45 µm/h (15). The reason for such variations in migration velocity near the wound edge is likely due to the lack of contact inhibition by neighbouring cells (10, 15).

Erjefält et al studied deepithelialisation, reepithelialisation and associated events in guinea-pig trachea after shedding-like epithelial denudation in vivo (12). They generated a mechanical deepithelialisation of an 800 µm wide tracheal zone using an orotracheal steel probe without bleeding or damage to the basement membrane. Immediately after epithelial removal, secretory and ciliated and presumably basal epithelial cells at the wound margin dedifferentiated, flattened and migrated rapidly about 2-3 µm/min over the denuded basement membrane. Within 8-15 h, a new, flattened epithelium covered the entire deepithelialised zone. At 30 h, a tight epithelial barrier was established and after 5 days the epithelium was fully redifferentiated (16).
Similarly, Shimizu et al investigated epithelial regeneration in mechanically injured rat trachea by using phenotypic markers that identify unique differentiated stages of epithelial cells (17).

Taken together, these studies demonstrate that airway epithelial cell migration is a critical component of wound healing post injury in airways.

1.3 Regulation of airway epithelial cell migration

Numerous respiratory diseases are associated with abnormal or insufficient repair post injury to heal the wound, leading to loss of epithelial integrity and function. For example, chronic obstructive pulmonary disease (COPD) exacerbations may lead to dysregulation of proinflammatory cytokines, matrix metalloproteinases (MMPs), tissue inhibitor of matrix metalloproteinases (TIMPs), intercellular adhesion molecules, thus inducing remodelling of airway mucosa and leakiness of epithelium (18). COPD exacerbations are often associated with pollutants, viruses and bacteria as well. It is known that remodelling of epithelium during injury and repair favours bacterial infections (18, 19).

We reviewed different groups of factors inhibiting or facilitating airway epithelial cell migration during lung repair after injury. Bacterial and viral infection, inflammatory factors, inhaled toxins, mechanical injury and alcohol abuse are major factors preventing airway epithelial cell migration (Table 1-1), whereas certain secreted cytokines, inflammatory mediators, growth factors, neuropeptides, extracellular matrix molecules are critical for prompting airway epithelial cell migration (Table 1-2).
### Table 1-1. Factors inhibit airway epithelial cell migration

<table>
<thead>
<tr>
<th>Inhibitory factors</th>
<th>Year</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bovine herpesvirus-1</td>
<td>1995</td>
<td>(20)</td>
</tr>
<tr>
<td>2. Inflammatory mediators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP-mediated activation of dual oxidase 1</td>
<td>2007</td>
<td>(21)</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>2003</td>
<td>(22)</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>2008</td>
<td>(23)</td>
</tr>
<tr>
<td>IL-4, IL-13</td>
<td>2001</td>
<td>(24)</td>
</tr>
<tr>
<td>3. Inhale factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarette smoke</td>
<td>1995</td>
<td>(25)</td>
</tr>
<tr>
<td>Hog barn dust</td>
<td>2001</td>
<td>(26)</td>
</tr>
<tr>
<td>Feedlot dust</td>
<td>2007</td>
<td>(27)</td>
</tr>
<tr>
<td>Arsenic</td>
<td>2007</td>
<td>(28)</td>
</tr>
<tr>
<td>Air pollution and ozone exposure</td>
<td>2008</td>
<td>(29)</td>
</tr>
<tr>
<td>Cold draught air</td>
<td>2005</td>
<td>(30)</td>
</tr>
<tr>
<td>Ultrafine particles</td>
<td>2006</td>
<td>(31)</td>
</tr>
<tr>
<td>High levels of ambient air pollution, particulate matter</td>
<td>2001</td>
<td>(32)</td>
</tr>
<tr>
<td>4. Mechanical ventilation</td>
<td>1998</td>
<td>(33)</td>
</tr>
<tr>
<td>5. Ethanol</td>
<td>2001</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>(36)</td>
</tr>
</tbody>
</table>
Table 1-2. Factors promote airway epithelial cell migration

<table>
<thead>
<tr>
<th>Enhancers</th>
<th>Year</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>1996</td>
<td>(37)</td>
</tr>
<tr>
<td>IL-6</td>
<td>1997</td>
<td>(38)</td>
</tr>
<tr>
<td>Chemokines to activate CXCR3</td>
<td>2006</td>
<td>(39)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>2001</td>
<td>(40)</td>
</tr>
<tr>
<td>2. Other inflammatory mediators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tachykinins</td>
<td>1995</td>
<td>(37)</td>
</tr>
<tr>
<td>uPA</td>
<td>2000</td>
<td>(38)</td>
</tr>
<tr>
<td>Trefoil factor family (TFF)/EGF</td>
<td>2001</td>
<td>(40)</td>
</tr>
<tr>
<td>TFF/PKC</td>
<td>2002</td>
<td>(41)</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>2006</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td>2007</td>
<td>(43)</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>(43)</td>
</tr>
<tr>
<td>3. Growth factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin and Insulin-like growth factor-I (IGF-I)</td>
<td>1990</td>
<td>(44)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>2006</td>
<td>(45)</td>
</tr>
<tr>
<td>4. Receptor agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulation hormone (i.e. catecholamine)</td>
<td>2002</td>
<td>(46)</td>
</tr>
<tr>
<td>β-adrenergic receptor</td>
<td>2002</td>
<td>(47)</td>
</tr>
<tr>
<td>adenosine receptors A2A</td>
<td>2002</td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td>2006</td>
<td>(49)</td>
</tr>
<tr>
<td>5. Extracellular matrix molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1989</td>
<td>(50)</td>
</tr>
<tr>
<td>Fibronectin via α5β1 integrin</td>
<td>1996</td>
<td>(51)</td>
</tr>
<tr>
<td>ECM</td>
<td>1992</td>
<td>(52)</td>
</tr>
<tr>
<td>very late adhesion integrins (VLA integrin)</td>
<td>1999</td>
<td>(53)</td>
</tr>
<tr>
<td>ECM, MMP-9</td>
<td>1999</td>
<td>(54)</td>
</tr>
<tr>
<td>6. Signaling pathways</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>1996</td>
<td>(55)</td>
</tr>
<tr>
<td></td>
<td>1997</td>
<td>(56)</td>
</tr>
<tr>
<td>GSK3β/β-catenin</td>
<td>2007</td>
<td>(57)</td>
</tr>
<tr>
<td>GP130-STAT3</td>
<td>2008</td>
<td>(58)</td>
</tr>
</tbody>
</table>
1.4 Alveolar epithelial cell migration occurs after tissue injury

Lung alveolar cells have also been shown to be capable of migration to heal the wound post-injury (57-59). Acute lung injury leads to type I alveolar epithelial cell death, denudation of the alveolar basement membrane, and formation of an alveolar provisional matrix from fibronectin, fibrinogen and type I collagen which provides a scaffold for alveolar repair (59). To restore normal lung architecture, surviving type II alveolar epithelial cells reepithelialize denuded alveoli (59). During reepithelialization, type II cells initially appear to migrate and spread over a remodelled matrix; and then a secondary proliferative phase occurs (57).

Following acute lung injury, type II alveolar epithelial cells could migrate, spread over and proliferate on a refaced matrix to repair the epithelium layer (60-62). It has been observed that type II cells locomotion can be promoted by various growth factors, proinflammatory cytokines and substrate adhesion molecules (Table 1-3). Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1), inhaled particles, cytokines (IFNγ and IL-6), and mechanical ventilation may inhibit alveolar epithelial cell migration (Table 1-3). Post acute lung injury, multipotent molecules may act in different ways, either by cooperative or counterbalancing activities, to modulate epithelial repair (57).
Table 1-3. Factors affect alveolar epithelial cell migration

<table>
<thead>
<tr>
<th>Enhancers</th>
<th>Year</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Growth Factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF, TGF-β, IGF-I, KGF</td>
<td>1996</td>
<td>(57)</td>
</tr>
<tr>
<td>TGF-α</td>
<td>1994</td>
<td>(58, 63)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>2008</td>
<td>(64)</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF)</td>
<td>1999</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>2003</td>
<td>(66)</td>
</tr>
<tr>
<td>Keratinocyte growth factor (KGF)</td>
<td>2003</td>
<td>(67)</td>
</tr>
<tr>
<td>2. Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>2000</td>
<td>(68)</td>
</tr>
<tr>
<td>IFNγ/IL-2 combination</td>
<td>2004</td>
<td>(69)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1996</td>
<td>(57)</td>
</tr>
<tr>
<td>3. Extracellular matrix molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrin/ECM</td>
<td>1997</td>
<td>(59)</td>
</tr>
<tr>
<td>ECM/Collagenase</td>
<td>1999</td>
<td>(70)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>2005</td>
<td>(71)</td>
</tr>
<tr>
<td>Vitronectin/αvβ1 integrin</td>
<td>2004</td>
<td>(72)</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. uPA and PAI-1</td>
<td>2004</td>
<td>(72)</td>
</tr>
<tr>
<td>2. Inhale Particles</td>
<td>2008</td>
<td>(73)</td>
</tr>
<tr>
<td>Ambient particle PM 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>1996</td>
<td>(57)</td>
</tr>
<tr>
<td>IL-6</td>
<td>1996</td>
<td>(57)</td>
</tr>
</tbody>
</table>
1.5 Perspective: insight from epithelial cell migration to understanding of injury and repair of lung diseases

Because respiratory epithelial injury and denudation/shedding is now known to be an important and active component of many respiratory diseases, including inflammatory airway diseases such as asthma and COPD, as well as remodelling diseases such as idiopathic pulmonary fibrosis and bronchiolitis obliterans syndrome, further understanding of the mechanisms governing epithelial repair and regeneration \textit{in vivo} holds great promise in the treatment of such diseases, which continue to hold great morbidity and mortality for its affected patients.

Previous studies used \textit{in vivo} models to study injury and repair. Later on, as cell culture and microscopic techniques advanced, there was a trend towards employing \textit{in vitro} studies to determine the complex association of factors involved in these processes, allowing for identification of molecular factors associated with the wound repair process. It will be necessary to return to \textit{in vivo} techniques once again, as the various limitations of cell culture models have been described (74). The basement membrane appears to be a key player in epithelial repair post-injury. Erjefalt et al. found the speed of cell migration in vivo to be in the range of several $\mu$m/min, much faster than the speeds seen in cell culture experiments, pointing to the existence of important \textit{in vivo} factors that influence the repair process (12, 75). Furthermore, the clinical features of cell migration and repair cannot be completely reproduced in cell culture models. Performing more \textit{in vivo} or \textit{ex vivo} experiments with contemporary and laboratory technologies such as real-time confocal microscopy, intravital imaging, two-photon microscopy, and real-time multiphoton microscopy and so on, could lead to the uncovering of a wealth of knowledge with direct clinical relevance in lung diseases.
Recent research in the area of tissue bioengineering and epithelial regeneration/transplantation holds great potential as a treatment for disorders where epithelial damage has already been incurred, with the goal to prevent abnormal healing/fibrosis and promote functional reepithelialization. Additionally, interest in the possibility of using stem cells to aid in repair and regeneration process of injured lung tissue has led to the discovery of adult bone marrow-derived stem cells that may have the plasticity and ability to differentiate into bronchial and alveolar epithelium (76-79). Circulating progenitor epithelial cells may also be recruited to participate to repair after injury (80). Wong et al. demonstrated that targeted delivery of short-term cultured bone marrow cells into a reversible airway injury milieu favored cell engraftment, and may be used for cell based therapy (81). Kim et al isolated bronchioalveolar stem cells (BASCs) from bronchioalveolar duct junction (82). BASCs are resistant to damage in the naphthalene-induced airway denudation model. BASCs started to proliferate and self renew at 30 h after nephelane treatment. The regeneration of intact airway epithelium is mediated by the migration, proliferation and differentiation of BASCs in vivo, although there is some evidence that BASCs are expanded in lung adnocarcinoma precursors and tumours (82, 83). The migration of stem cells during tissue regeneration merit further investigation in the future (83).

In summary, in this chapter, I reviewed the importance of epithelial cell migration in lung physiology, and pathophysiology and lung diseases. From this chapter, it is clear that the repair of epithelium post-injury is crucial to restore barrier function in both normal physiology and to prevent the initiation and progression of many chronic lung diseases. Research on the promotion of epithelial repair and regeneration may lead to new therapeutic strategies, allowing for the reconstitution of well-differentiated and functional airway epithelium.
2. Chapter Two. General background - Cellular structures for cell adhesion, migration and invasion
2. Chapter Two. General background - Cellular structures for cell adhesion, migration and invasion

In Chapter One, we discussed that epithelial cell migration plays an important role in human lung physiology, pathophysiology and respiratory diseases. Cell migration results from the coordination of motions generated in different parts of a cell and integrated with a directed endocytic cycle. Nowadays advanced video microscopy reveals the process of cell migration in details. In this chapter, firstly, I will review the molecular mechanisms in cell migration. Secondly, I will summarize three types of cellular structures focal adhesion, podosomes and invadopodia and their role in cell adhesion, cell migration and invasion. Thirdly, I will focus on review the role of podosomal component proteins in cell motility. Fourthly, I will compare the similarity and differences among focal adhesion, podosome and invadopodia.

2.1 Molecular mechanisms of cell migration

Cell migration involves many steps, and the ability of a cell to migrate is dependent upon cell-cell interactions and cell-matrix interactions (84). Cell migration consists on a cyclical process that can be divided into five phases: morphological polarization; membrane extension; formation of cell-substratum attachments or adhesions; contractile force and traction; release or breaking of cell attachments (Figure 2-1) (15, 85-87).
Figure 2-1. Steps of cell migration.

Cell migration is a cyclical process that can be divided into five phases: 1) morphological polarization, which has been described as a clear distinction between cell front and rear; 2) membrane extension, which is an initial protrusion or extension of the plasma membrane at the leading edge of the cell, driven by polymerization of the cytoskeletal network of actin filaments, and stabilized by adhesive complexes; 3) adhesion, which is formation of cell-substratum attachments or adhesions at the leading edge; 4) translocation, movement of the cell body; 5) de-adhesion, also known as release or breaking of cell attachments, of adhesions located at the rear of the cell, thereby allowing for net movement in the forward direction.
The processes of cell migration can be turned on and off by quantitative changes in the concentrations of various molecular components, including adhesion molecules, cytoskeletal-linking proteins, and extracellular matrix (ECM) ligands, as well as by the changes of the physicochemical properties surrounding the cells. The mechanisms that regulate these processes into directional migration of the cell are still unclear, although many proteins have been identified, such as the Rho/Rac family of signalling molecules, the integrin family of transmembrane adhesion molecules linking the ECM to the cell’s cytoskeletal actin filaments and microtubules (88-90).

Cdc42 and Rac are two members of the Rho family of GTPases that have been shown in particular to be involved in regulating the formation of new cellular protrusions and focal adhesions. Cdc42 has been shown to stimulate actin polymerization to form filopodia, and activation of Rac stimulates lamellipodia formation. Additionally, these molecules have been shown to regulate contractile forces at the leading edge of the cell by regulation of the myosin light chain phosphorylation (91), via its interactions with PAK (p21 activated kinase) (92), and via Rho kinase (93, 94).

Modification of proteins by tyrosine phosphorylation is involved in the formation of adhesive structures. Focal adhesion kinase (FAK), paxillin, and tensin are among the phosphoproteins known to constitute adhesive complexes (95-98). Tyrosine phosphorylation of paxillin, tensin, and FAK creates recognition sites for proteins containing Src-homology 2 (SH2) domains. Dysfunction in migration due to dysfunctional focal adhesion turnover has been reported in cells lacking focal adhesion components: Src family kinases (99), FAK (100), and calpain (101). Phosphatidylinositol 3-kinase (PI3K) and its down-stream signals, particularly Akt (also called protein kinase B, PKB) play a central role in regulation of cell adhesion and
deadhesion, cell extension, migration and invasion (102). Other regulators of actin also localize at or near the cell’s leading edge including PIP2 (103-105), WASP (106, 107), Scar (108, 109), Arp2/3 (108); along with other molecules known to function in migration, such as integrins (110), talin (111) and vinculin (112, 113).

2.2 Focal adhesion

Focal adhesions are small, dynamic protein complexes through which the cytoskeleton connects to the extracellular matrix (Figure 2-2) (114). Due to the differences in their size and shape, focal adhesions are commonly referred as focal complexes, focal contacts and fibrillar adhesions (115). Many attempts have been made to classify these cell-substrate adhesion sites using descriptive features such as shape, size, cellular location, GTPases dependency and protein components (114, 116-120). The size of focal adhesion is usually within 15 nm to 60 nm (121). The turnover rate of focal adhesion is within minutes to hours. Each cell may have many focal adhesions in the cytoplasm and at the peripheral. The morphology of focal adhesions is arrowhead shaped structures as the two end of the stress fibers (122). Focal contacts are small initial adhesions during cell attachment.
Figure 2-2. Structure of focal adhesion.
Focal adhesions serve both as the mechanical linkage to the extracellular matrix (123), and as a biochemical signaling hub to concentrate and direct numerous signaling proteins, kinases, adaptor proteins at sites of integrin binding and clustering (114). The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. The smaller focal adhesion, which also called “focal complexes”, is formed at the leading edge of a cell in lamellipodia. Many of these focal complexes fail to mature and disassembly as the lamellipodia withdraw. However, some of the focal complexes mature into larger and stable adhesions and recruit more proteins such as zyxin (124, 125). Once in place, a focal adhesion remains stationary with respect to the extracellular matrix, and the cell uses this as an anchor on which it can push or pull itself over the extracellular matrix. Although focal adhesions are quite stable under normal condition, in a motile cell, focal adhesions are being constantly assembly and disassembly as the cell establishes new contacts at the leading edge and breaks old contacts at the trailing edge of the cell. During cell attachment and spreading, initial adhesions evolve into small focal complexes that further mature into focal adhesions connected to actin stress fibers.

As anchorage of the cell, focal adhesions connect with proteins of the extracellular matrix generally through integrin (126). Integrin binds to extracellular proteins via short amino acid sequences such as the R-G-D sequence motif in proteins such as fibronectin (127), laminin and vitronectin (128), and the DGEA and GFOGER motifs in collagen (129). Integrins are heterodimers which are formed from one α and one β subunit (130-134). Focal adhesions are mostly composed of β1 and β3 integrins. The C-terminal PTB domain of tesins can interact directly with the NPXY motif in the β subunit of integrin (135). Within the cell, the intracellular domain of integrin binds to the cytoskeleton via adaptor proteins such as talin, α-actinin, filamin
and vinculin (136-140). Many signaling molecules such as FAK, Src, PKC and PI3K bind to and associate with this integrin-adaptor protein-cytoskeleton complex (136-140).

2.3 Podosome

Podosomes are unique actin-rich structures which protrude into the extracellular matrix, resulting in localized remodeling activities associated with enhanced invasiveness (Figure 2-3 and 2-4) (141, 142). Podosome, not only establishes close contact to the substratum, but can also degrade components of extracellular matrix to assist motile cells to cross tissue boundaries, thus, it has been called foot and mouth of the cell (141).

Although podosomes in different cell types share similar functions - promoting matrix degradation and cell invasion, their morphologies appear to be diverse. For example, podosomes in primary human macrophages are hundreds of small tiny dots (0.2-0.5 µm in size) distributed at the ventral surface underneath the cytoplasm at the leading edge of lamellipodia (143-145). In smooth muscle cells, podosomes appear as small dots (0.5-1 µm in size) which often aggregate to form bands (2 µm in size) along the cortex of the cell (146). In bovine aorta endothelial cells, podosome is one huge single ring (15 µm in size) beside the nucleus (147). In c-Src Y527F transformed NIH 3T3 fibroblasts podosomes are numerous rosette-like structures (2 µm in size) at the end of plasma membrane spike (148). In melanoma cells, podosomes are couple of dots (1 µm in size) in the cytoplasm (149). In rat bladder carcinoma 804G cells, podosome-like structures were identified around hemidesmosomes that make connections with intermediate filaments, as small dots surrounded by rings (150, 151). Linder (152) summarized that podosomes are dot-like structures attached to substrate, and containing actin regulators and
plaque proteins with the number of around 20-100 per cell with the maximum size of 1 µm in
diameter and 0.4 µm in depth.

So far nearly one hundred of proteins have been identified at the sites of podosomes. Six
groups of proteins were usually thought as the molecular components of podosomes (150): (i)
actin which is usually located at the core of podosomes and actin-associated proteins (e.g., F-
actin, cortactin, α-actinin, gelsolin), and regulators (e.g., Arp2/3, WASP, N-WASP, Cde42)
which work as actin linkers and serve as the architecture of “podosome bone”; (ii) integrin and
integrin-associated proteins (e.g., β1, β2, β3, αvβ2, αvβ3 -integrin, vinculin, paxilin, talin) which
form the cell-matrix anchor; (iii) protein kinases (e.g., Src, FAK, PKC, Pyk2, PI3K), which
regulate the actin cytoskeleton and membrane remodeling; (iv) signaling adaptor proteins (e.g.
Tks5/Fish, WIP, p130Cas, cofilin, AFAP110) which regulate podosome formation, dynamics
and maturation and lifespan; (v) motor proteins such as dynamin 2 and myosine which perform
as the actin-based motor; and (vi) MMPs (e.g. MMP-2, MMP-9, MMP-14), which mediate ECM
degradation. For individual podosome dot, it has an actin core enriched with F-actin, cortactin,
WASP/N-WASP, and Arp2/3 complex etc; and an ring structure composed of integrins, Src,
vinculin etc (150).

It is well known that podosomes function to degrade local extracellular matrix and
therefore promote invasion through underneath tissue boundaries. Less is known that podosomes
may also function to anchor a cell to the extracellular matrix, and to sense substrate rigidity and
transmit forces. Collin et al reported that motor protein myosin II formed circular structures at
the outside edges of the podosome actin ring to regulate podosome dynamics (153). They
proposed that podosomes are dynamic mechanosensors which interact with myosin tension and
actin network in living cells (153).
Figure 2-3. Morphology of lamellipodia, podosomes and invadopodia.

(A) Lamellipodia is a broad actin-rich membrane extension at the leading edge of the cell; (B) Podosome is an actin-rich membrane inward protrusion of the ventral surface of the cell membrane; (C) Invadopodia is an actin-rich outward protrusion of the ventral surface of the cell membrane and it is usually formed at the peri-nucleus region; (D) Ruffle is an actin-rich outward membrane protrusion of the dorsal surface of the cell membrane and it is often located at the peripheral.
Figure 2-4. Structure of podosome.
2.4 Invadopodia

Invadopodia are actin-based outward protrusions at the ventral surface of tumor cells and transformed cells which mediate proteolysis of the extracellular matrix (Figure 2-3) (154). The size of invadopodia varies from 0.1 µm to 0.8 µm in diameter and may reach 2-3 µm or greater in length (155). Invadopida usually assemble into clusters around membrane invaginations proximal to the Golgi complex in the cytoplasm (156). Invadopodia were first observed in the oncogene v-Src transformed fibroblasts (157, 158). Invadopodia show stable once it formed and prolonged protease secretion to degrade the extracellular matrix (159). Usually a cell can form less number of invadopodia than podosomes.

Generally, invadopodia share similar molecular protein components with podosomes. However, the same protein in different structure may play a distinct role. For example, cortactin is commonly considered as a protein marker for podosome, although it does exist at invadopodia as well. Cortactin as a regulator of the Arp2/3 complex is also of particular important in invadopodia function (160). While most of studies have been on the possible role of cortactin in actin assembly for direct formation of actin-rich invadopodia puncta (161), Emily et al found that the primary role of cortactin in invadopodia is to promote protease secretion (160, 162). Thus cortactin may link vesicular trafficking and dynamic branched actin assembly to regulate protease secretion for invadopodia-associated ECM degradation (162).

2.5 Molecular components of podosomes

Podosomes are punctate actin-rich structures located at the ventral surface of the cell membrane (163). A non-exhaustive list of component that has been identified in and around podosomes as summarized above. Podosome formation is dependent on PKC, Src and
RhoGTPases (141, 142) and other kinases. I will introduce the roles of signal transduction pathways involved in podosome assembly including Src, PI3k/Akt and MAPK in this chapter, focusing their roles in cytoskeleton rearrangement, podosome/invadopodia assembly and cell motility. The role of PKC in podosome formation and cell motility will be discussed in detail in the next chapter.

2.5.1 Src

Src family kinases, as a family of cytoplasmic non-receptor protein tyrosine kinases, are involved in various signaling pathways regulating diverse cell events such as cell adhesion, migration, invasion, proliferation and cell cycle (164). The Src family consists of 8 members: c-Src, Fyn, Lyn, Hck, c-Yes, Blk, Fgr and Lck. Many cell types express multiple Src family kinases (165).

Src activation may lead to cytoskeleton reorganization. Podosomes are found as organized F-actin rich structures on the ventral surface of v-Src-transformed cells (141, 166, 167). An inhibitory mutant of N-WASP impairs podosome formation as well as v-Src-induced matrix degradation, indicating a probable link between control of actin assembly and the formation of structures that mediate Src-induced matrix remodelling (168).

Src not only play a critical role in the process of podosome formation, but also regulate podosomal dynamics, turnover rate, life span and proteolytic function. Podosome number and the podosome-associated actin cloud were decreased in Src-/- osteoclasts (169). The life span of podosomes in Src-/- osteoclasts was increased fourfold and that the rate of actin flux in the core was decreased by 40%. Rescue of these cells with Src mutants showed that both the kinase activity and either the SH2 or the SH3 domain are required for Src to restore normal podosome
organization and dynamics (169). When osteoclasts adhere onto bone surfaces, they condense their actin-rich podosomes in tight belts to establish sealing zones (170). ARF (ADP-ribosylation factor) GTPase-activating protein GIT2 (G protein-coupled receptor kinase-interactor 2), which localizes to sealing zones, depends on Src phosphorylation for maintaining osteoclast polarity (170). In smooth muscle A7r5 cells, the phosphorylation of Ser277 of actin filament associated protein (AFAP) regulated podosome lifespan in a Src- and PKCα-dependent manner (171). Courtneidge et al showed that the adaptor protein tyrosine kinase substrate 5 or five SH3 containing protein (Tks5/Fish) was required for podosome/invadopodia formation, degradation of ECM, and cancer cell invasion in vitro (148) and in vivo (172). Recently they demonstrated that Tks4, a protein contains an amino-terminal PX domain, four SH3 domains, and several proline-rich motifs, is tyrosine phosphorylated and predominantly localized to rosettes of podosomes in Src-transformed fibroblasts. In the absence of Tks4, MT1-MMP was not recruited to the incomplete podosomes in these cells (173). Takenawa’s group reported that Src-expression stimulated podosome formation at the sites of focal adhesion after PI(3,4)P2 accumulation in NIH3T3 cells. The adaptor protein Tks5/Fish, which is essential for podosome formation, was found to form a complex with Grb2 at adhesion sites in a Src-dependent manner. Further, it was found that N-WASP bound to all SH3 domains of Tks5/Fish, which facilitated circular podosome formation (174).

Src is involved in FGF induced activation of STAT5 during vascular endothelial morphogenesis in mouse microvascular endothelial cell (175). The activated FAK forms a binary complex with Src family kinases which can phosphorylate other substrates and trigger multiple intracellular signaling pathways to regulate cancer cell migration, invasion, epithelial mesenchymal transition (EMT) and angiogenesis (176). In Ras-mediated transformation, the
amoeboid movement of tumor cells in the three-dimensional matrix, and transmigration of tumor cells through the mesothelial monolayer, mDia, is potentially linked to Rac activation and membrane ruffle formation through c-Src-induced phosphorylation of focal adhesion proteins, and ROCK antagonizes this mDia action (177). By activating Src-FAK, TGFβ integrates ErbB receptor and integrin signaling to induce cell migration and survival during breast cancer progression (178). By interacting with Src kinase, S100B, a Ca2+-binding protein of the EF-hand type known as S100 that is abundantly expressed in astrocytes, contributes to reducing the differentiation potential of cells and participate in the astrocyte activation in case of brain insult and in invasive properties of glioma cells (179). In mouse embryonic fibroblast cells, Src-Associated substrate during Mitosis of 68 kDa (Sam68) localizes near the plasma membrane during cell attachment and serves as an adaptor protein to modulate Src activity for proper signaling to small Rho GTPases during cell polarization and cell migration (180).

2.5.2 PI3K/Akt

The phosphoinositide 3-kinases (PI3K) are a family of enzymes that regulate diverse biological functions in various cell types by generating lipid second messengers (181). PI3K can phosphorylate the D3 position of the inositol ring of phosphoinositides. According to the similarity of molecular structure, the PI3K family can be divided into three classes: (i) Class IA PI3Ks, which can be activated by tyrosine-kinase-associated receptors, are heterodimeric enzymes consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110α, p110β or p110δ) (182), and Class IB PI3K, PI3Kγ, which can be activated by G-protein-coupled receptors, only contain one catalytic subunit p110γ and one regulatory subunit p101 (182), (ii) class II and (iii) class III (183). The class II PI3Ks harbor human PI3K-C2α, PI3K-C2β or
human HsC2-PI3K, and PI3K-C2γ (184). The class III enzyme is a heterodimer of p150 protein kinase and the mammalian homology of the yeast VSP34 PI3K (185).

PI3K is a major signaling component that can convert phosphatidylinositol-4,5-biophosphate (PI (4,5)P2) to phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) at the inner leaflet of the plasma membrane (186). PI(3,4,5)P3 acts as a binding site for numerous intracellular enzymes that contain pleckstrin homology (PH) domains (187), such as the serine/threonine kinase Akt (188). In turn, PIP3 contributes to the recruitment and activation of a wide range of downstream targets (189). Akt binds to PI(3,4,5)P3 via its N-terminal PH domain (190, 191) and is activated by phosphorylation at T308 and S473 by PDK1 and putative PDK2, respectively (192-194). Putative PDK2 were also identified as the mTOR/Rictor complex (195). Activated Akt modulates a wide range of cellular functions, such as cell survival, proliferation, polarity formation, and cortical actin regulation. Akt can modulate activities of multiple downstream factors such as glycogen synthase kinase-3β (GSK3β), BAD, caspase 9, and the fork head transcription factors (196), through phosphorylation (197). Akt phosphorylates and inhibits GSK3β activity, thereby leading to the stabilization of β-catenin (198). The signaling map for PI3K/Akt is shown in Figure 2-5.

PI3K/Akt signaling is critical for cytoskeleton rearrangement and podosomes. Interaction of PI(4,5)P2 with gelsolin and WASP is critical for podosome assembly/disassembly and actin ring formation in osteoclasts (199). PI(4,5)P2 enhances de novo actin polymerization by increase Arp2/3 complex-induced actin nucleation through modulation of N-WASP (200). In fibroblast, The first PH domain of AFAP-110 bound to PKCα and further recruit c-Src to the site of podosome, which was dependent on PI3K activity (201).
Figure 2-5. PI3K/Akt signaling pathway.
2.5.3 MAPKs

Mitogen-activated protein kinase (MAPK) pathways constitute a large kinase network that regulates a variety of physiological processes, such as cell differentiation, apoptosis and cell adhesion, migration and invasion (202). Deregulation of MAPK activity has been implicated in several pathological situations, including inflammation, oncogenic transformation, and tumor cell invasion (202, 203). To date, several distinct groups of MAPK pathways have been characterized in mammals (204): extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/γ (ERK6)/δ (205-208) (Figure 2-6).

The ERK pathway is activated by a large variety of mitogens and by phorbol esters, whereas the JNK and p38 pathways are stimulated mainly by environmental stress and inflammatory cytokines (203, 209, 210). MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of a MAPK module (MAPKKK→MAPKK→MAPK) (Figure 2-6) (204). After MAPKs are activated either in the cytoplasm or in the nucleus, they regulate transcription by modulating the function of targeted transcription factors through serine/threonine phosphorylation (211). In addition to the transcriptional effects, MAPKs regulate cell behavior also by phosphorylating cytoplasmic target proteins, such as apoptotic or cytoskeletal proteins (212).
Figure 2-6. MAPKs signaling pathway.
Accumulating data indicated that various signaling through activation of MAPKs to control cell migration and invasion and MMPs. In vascular smooth muscle A7r5 cells, PKC-mediated MEK/ERK/Caldesmon phosphorylation and translocation has been shown to remodel actin stress fiber into F-actin-enriched podosome columns (213). MEK/ERK1/2/Caldesmon signaling cascade regulated the lifetime and size of podosomes differentially (214). In murine bone marrow-derived dendritic cells, the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) adaptor protein mediated integrin engagement to facilitate podosome distribution through ERK1/2 phosphorylation in the presence of chemokine (215). In bone marrow-derived neutrophils, CDC42 regulated horizontal directional migration and vertical directional invasion associated with podosome-like structures at the cell leading edge through ERK1/2 activity, while CDC42GAP-induced p38 MAPK phosphorylation regulated directed migration by antagonizing filopodia assembly (216). Big MAPK Erk5 may promote Src-induced podosome formation in fibroblasts by RhoGAP7 and thereby limiting Rho activity (217). In murine fibroblast cell line C3H10T1/2, oncogene v-Src transfection lead to MMP-2 expression through ERK signaling pathway and transcription activation of Sp1 promoter (218). In Cos7 cells, concanavalin A stimulated MMP-2 expression and activation through Src/ERK/p38 signaling pathway (219). In human lung adenocarcinoma A549 cells, EGF stimulated Src kinase and JNK/ERK signaling pathway through FAK at the cell membrane to increase MMP-9 expression and secretion (220).

2.5.4 Matrix metalloproteinases

Invadopodia and podosomes are able to degrade matrix proteins, such as fibronectin, collagen and laminin (221). Matrix metalloproteinases such as membrane type-1 matrix metalloproteinase (MT1-MMP, also called MMP-14), MMP-2 and -9, are thought to mediate
ECM degradation at sites of invadopodia extension (222, 223). In this section, firstly, I will review the family members of MMPs, their molecular structures and the molecular mechanism of regulation of MMP gene expression, activation and inhibition. Secondly, I will briefly summarize the role of MT1-MMP, MMP-2/-9 in podosomes/invadopodia and cell motility.

The human MMP family includes 26 (and growing) members, including both secreted and membrane-bound enzymes, characterized by their ability to degrade ECM and by their dependence on Zn$^{2+}$ binding for proteolytic activity (224, 225). MMPs are divided into five major subclasses: collagenases (e.g. MMP-1,8,13), gelatinases (e.g., MMP-2, 9), stromelysins, matrilysins, and membrane-type MMPs (e.g., MT1-MMP to MT6-MMP) (225, 226) (Figure 2-7).

The activity of MMPs is regulated by three mechanisms: transcription; secretion and activation of the latent pro-enzymes; and inhibition by their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs) (8, 226), which are able to inhibit metalloproteinase activity by forming non-covalent complexes with active MMPs. Most MMPs are secreted as inactive zymogens or pro-enzymes, and activation requires disruption of the Cys-Zn$^{2+}$ interaction (proteolytic removal of a prodomain) (227). Most pro-MMPs are thought to be activated by tissue or plasma proteinases. Pro-MMP-2 activation is typically thought to take place on the cell surface by MT1-MMP. Recently, it has been suggested that this activation process requires both active MT1-MMP and the TIMP-2-bound MT1-MMP (228, 229).
Figure 2-7. Molecular structure of MMPs.
The expression of many MMPs is transcriptionally regulated by growth factors, hormones, cytokines, chemical agents (eg. phorbol esters, actin stress-fibre inducing drugs) and cellular transformation (227). Phorbol esters have been shown to stimulate MMP- expression via PKC in several systems (230-234). Up-regulation of PKC induces secretion of MMP-9 in capillary endothelial cells (234), PKC-dependent NF-κB activation is involved in MMP-9 induction in hepatocellular carcinoma cells (235), and inhibition of NF-κB activity by synthetic compounds inhibits MMP-9 secretion (236). Different isoforms of PKC has been implicated in MMP-9 expression in various cell types (237-240). Phorbol 12-myristate 13-acetate (PMA) induces MMP-9 expression via a PKCα-dependent signaling cascade in BEAS2B human lung epithelial cells (225).

MT1-MMP, MMP-2 and MMP-9 have been suggested to be the crucial MMP’s involved in podosome/invadopodia matrix-degradation, and their regulation has been closely linked to podosome formation and function (147, 241).

MT1-MMP (Membrane-Type 1- MMP) or MMP-14 is a membrane-bound metalloproteinase with broad substrate specificity and multiple cellular functions (242). MT1-MMP activates MMP-2 and possibly MMP-13, and may proteolytically modify CD44, αV integrin, and transglutaminase, therefore playing a regulatory role in cell migration (243). Additionally, MT1-MMP itself has been shown to degrade multiple ECM components, including collagen types I, II, and III; fibronectin; and laminins 1 and 5 (242-246). Protease inhibitor studies have demonstrated that MT1-MMP is an enzyme crucial for gelatin matrix degradation in the breast carcinoma cell line MDA-MB-231 (242). It has also been localized to invadopodia of melanoma cells, and when it was overexpressed in RPM17951 human melanoma cells, cells made contact with ECM, activated soluble and ECM-bound MMP-2, and degraded the ECM for
invasion (223). The localization of MT1-MMP to invadopodia is necessary for the invadopodia’s degradative function, as in the absence of localization to invadopodia activated soluble MMP-2 failed to enable ECM degradation (223). Artym et al (159) suggested that 4 distinct invadopodial stages in a stepwise model of invadopodia formation and function: (i) membrane-cortactin aggregation at membranes adherent to matrix; (ii) MT1-MMP accumulation at region of cortactin accumulation; (iii) matrix degradation at invadopodia region; and (iv) subsequent cortactin dissociation with foci of degraded matrix.

The gelatinase MMP-2 and 9 have gelatin-binding domains. Both have been implicated in the angiogenesis and metastasis of cancer cells (247). MMP-2 (or Gelatinase A) and the MMP-TIMP-2 complex have been found to bind and localize at invadopodia plasma membrane of Src-transformed fibroblasts (248). MMP-2 is activated from its pro-MMP-2 form into active MMP-2 by MT1-MMP at the cell surface (249). It has been suggested that invadopodia direct localized degradation of the ECM by concentrating active membrane-associated collagenases at sites of cellular invasion (248). It has been hypothesized that proteinase recruitment to podosomes/invadopodia could involve trafficking of vesicles along microtubules (152), given that, exocytosis of MMP-2 and MMP-9 in melanoma cells is microtubule-dependent (250). Bound MMP-2 at invadopodia loses its propeptide, and thus becomes activated.

Previous reports suggest MMP-9 (or Gelatinase B) is overexpressed in response to injury, and is linked to reepithelialization and early repair processes (87, 251, 252), whereas MMP-2 is important during the extended remodeling phase (252, 253). In airway epithelium, MMP-9 is expressed by human bronchial epithelial cells, and blocking MMP-9 activity results in inhibition of the wound repair process (87, 251). Legrand et al found MMP-9 accumulated (via an actin-dependent pathway) in the advancing lamellipodia of migrating cells at the leading edge of a
wound, and they speculated that the active form of MMP-9 was located at the digested area of the coated extracellular matrix (87). MMP-9 has been implicated in migration of smooth muscle cells (254), human eye corneal cells (255, 256), human skin epidermal cells (257), human keratinocytes (258) and G8 mouse fetal myoblasts and KM201 and IM7.8 hybridomas (259).

MMP-9 has been shown to be involved in the migration of inflammatory cell types such as macrophages, T-lymphocytes, and eosinophils (251, 260-264). MMP-9 is also a major factor of human polymorphonuclear cell migration across the basement membrane and elastase contributes to this process by activating pro-MMP-9 (262). Induction of keratinocyte migration by EGF and HGF coincides with the induction of MMP-9 activity (264). Human bronchial epithelial cell (HBEC) migration is a dynamic, stepwise process in which MMP-9 expression is quickly and specifically regulated. Up-regulation of MMP-9 expression in migrating HBEC might reflect a modification of the ECM in close contact with these cells. Additionally, regulation of MMP-9 expression in migrating HBEC could be via cell-cell and cell-ECM adhesion molecules and changes in cell shape (265). In migrating cells, cell-ECM adhesions are provided by specialized regions of close contact, called primordial contacts (a type of cell-matrix adhesion usually transiently formed at the advancing edge of a migrating cell), distributed at the leading edge of the cell in the direction of migration (87, 266, 267). Primordial contacts of migrating HBEC are characterized by a dense accumulation of actin filaments and the presence of vinculin and type IV collagen (87, 261). These primordial contacts are very short-lived structures. MMP-9 is localized and active at these cell-ECM contacts, blocking MMP-9 activation leads to cells remaining fixed on the previously established primordial contacts and unable to migrate further (87).
2.6 Focal adhesion vs. podosome: what is the difference?

A variety of cell-matrix adhesions have been identified as focal adhesions, podosomes, and invadopodia. These adhesion sites contain integrin clusters able to develop special structures, which are different in their architecture and dynamics although they share almost the same group of proteins (163). Here we compare the organization, dynamics and interplay between focal adhesions and podosome, in order to understand how such subcellular sites - though closely related in their composition - can be structurally and functionally different.

These two cell-ECM interactions both contain specific adhesion receptors named integrins, cytoskeletal elements, and a wide variety of interconnecting adaptor proteins and signaling proteins. Podosomes contain a ring of adhesive molecules centered on an actin column, and their general orientation is perpendicular to the substrate and the plasma membrane. This contrasts with the elongated structure of focal adhesions with a tangential orientation with respect to the ECM. Dynamics and tension of both structures are also different, with podosomes being more dynamic and instable as compared to focal adhesions. In all cases, alteration of their dynamics results in modifications of cell differentiation and migration (268-270). These distinct properties suggest specific functions: the most commonly proposed function is that podosomes could be involved in matrix degradation and invasion, whereas focal adhesions are rather associated with matrix remodeling such as fibronectin fibrillogenesis (271, 272). Podosomes may be initiated from focal adhesions due to changes in the phosphorylation status of proteins and in the composition of phosphoinositides on the plasma membrane (174).

2.7 Podosome vs. invadopodia: what is the difference?
Podosomes and invadopodia both are actin-rich membrane structures that form close contact with the surrounding substrate, with dimensions ranging from 0.5 to several µm (273). The similarities and distinctions between podosomes and invadopodia are under debate (Figure 2-3) (Table 2-1). Podosomes and invadopodia share many common features, including appearance, components and similar proteolytic function (272). Many of the same signals regulate both podosomes and invadopodia formation; for example, Src kinase, PKC, PI3K and Rho GTPases (150). Moreover, the N-WASP-Cortactin-Dyn2-Arp2/3 complex is essential for the formation of both structures and is believed to be the minimal machinery necessary for their formation (141).

The three major differences between podosomes and invadopodia have been proposed as location, depth and lifetime (272, 274). Podosomes most often form at the periphery of the cell, while invadopodia are close to the nucleus and are associated with Golgi complex, allowing for the prolonged secretion of MMPs. It is generally accepted that podosomes are typically 0.5-2 µm (depth) inward invaginations into the cells, while invadopodia are 2-10 µm (depth) outward protrusions into the ECM. Podosome have a shorter lifetime (approximately 2-10 min), while invadopodia have a much longer lifetime (hours). Given the short lifetime of individual podosomes, they are only able to degrade ECM within their immediate vicinity. Comparatively, invadopodia are more persistent and are more likely to be found in cancer cells invading across tissue boundaries. It has been suggested that the short-lived podosomes can probe the substrate for sites of attachment before maturing into invadopodia (159, 274, 275). The morphology of podosomes can be dots, rosettes and belts, which may indicate different stages of maturation.
Table 2-1. Comparison between podosomes and invadopodia in 2D vs. 3D cultures

<table>
<thead>
<tr>
<th></th>
<th>2D-Glass Coverslip Conditions</th>
<th>3D- Matrix Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Podosomes</td>
<td>Invadopodia</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td>Cell periphery, ventral surface</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Protrusion direction and depth</strong></td>
<td>Necessarily inward invaginations; 0.5-2 µm (depth) inward</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Lifetime</strong></td>
<td>2-10 min</td>
<td>N/A</td>
</tr>
</tbody>
</table>
The current debate surrounds the issue of whether these differences are real or if they are due to various culture and assay conditions. Some authors have suggested podosomes to be artificial cellular structures due to cell culture conditions (272). Podosomes are inward invaginations of the ventral surface cell membrane when cells are cultured on glass surfaces in the common 2D culture system. In the 3D culture system with cells being cultured on matrix gel, *invadopodia* are typically seen as *outward* protrusions of the ventral surface cell membrane into the ECM. However, podosomes with both inward and outward protrusions were seen in a recent study in leukocyte cells on tissue slides, imaged by electron microscopy (276-278).

Yamaguchi and Condeelis also described podosomes to be outward protrusions of the ventral surface cell membrane into the ECM when cells are cultured in 3D conditions, and thus may represent an immature form of invadopodia (274, 279). In our view, we consider podosomes to be dynamic, transient precursors to invadopodia formation, on the “lower end” of the continuum of ECM-degrading structures. Podosomes may be responsible for “tasting” the ECM substrates available, and temporarily degrading matrix proteins. Should conditions call for invadopodia formation, these podosomes may transform into full-fledged invadopodia, capable of continuous and stronger activation of MMPs, more intense digestion of the ECM, and cellular invasion. The molecular mechanisms that regulate the switch from podosomes further down the continuum into invadopodia are largely unknown (280), and could play a significant role in the initiation and regulation of tumor invasion and metastasis.

In summary, in this chapter, I reviewed the cellular structures involved in cell motility such as focal adhesion, podosome and invadopodia for cell adhesion, cell migration and cell invasion; I summarized the molecular component proteins and signaling transduction pathways
involved in podosome assembly such as Src, PI3K/Akt and MAPK. The importance of PKC in podosome formation and cell motility is discussed in greater details in the next chapter.
3. Chapter Three. General background - Protein kinase C family in cytoskeleton rearrangement and cell motility

Part of the content of this chapter has been prepared as a review article:

Xiao H, Liu M. PKC in cell motility. (In preparation)
3. Chapter Three. General background - Protein kinase C family in cytoskeleton rearrangement and cell motility

Protein kinases C (PKC) play a vital role in cell migration and invasion in various types of cells. Accumulating evidence show that PKCs are highly involved in the process of podosomes and invadopodia formation. PKCs are also important in human lung physiology and alteration of PKCs is linked to lung disorders. In this section, firstly, I will introduce PKC family and review their subfamily members, molecular structure and the mechanism of PKC activation and inactivation. Secondly, I will summarize the role of each PKC subfamily in cell migration and invasion.

3.1 Introduction of PKC family

PKC is a family of serine/threonine kinases implicated in the transduction of signals coupled to receptor-mediated hydrolysis of membrane phospholipids (281). These kinases transduce signals involved in short-term processes (such as ion fluxes (282), neurotransmitter release (283)), mid-term process (such as receptor modulation (284)), and long-term processes (such as cell proliferation (285), synaptic remodeling (286, 287) and gene expression (288)). Various external signals such as growth factors, neurotransmitter, and hormones, stimulate the hydrolysis of inositol phospholipids and initiate formation of the second messenger diacylglycerol (DAG) to mediate PKC activation (289, 290).

According to sequence homology and sensitivity to activators, at least 10 isoforms encoded by 9 different genes have been described (Figure 3-1 and 3-2) (291). The various PKCs are grouped into three subfamilies: (i) classical or conventional PKCs (α, βI, βII and γ); (ii) novel
PKCs (δ, ε, η and θ); and (iii) atypical PKCs (ζ and λ/i) (292). Except these three subfamilies, there is PKCμ or protein kinase D1 (PKD1) (293).

3.1.1 Molecular structure of PKC family

The general structure of the different PKCs includes conserved domains (C1-C4) separated by variable sequences (V1-V5) (Figure 3-1) (294). C1-C2 represents the regulatory portion of each enzyme, where the specific activators interact with, while C3-C4 form the catalytic region responsible for both the substrate binding and the activity (294). The catalytic domain is characterized by a high degree of homology among the various kinases (295). The C1 domain bears one (in atypical) or two (in conventional and novel) cysteine-rich domains (294, 296), located near the amino-terminals region, which represent the docking-sites for phosphatidylserine (PS) (297) and the physiological activator diacylglycerol (DAG) as well as for the analogous phorbol esters (i.e. PMA, PDBu (phorbol-12,13-dibutyrate)) (298). The atypical isoforms are sensitive and activated by PS and also ceramide and phosphatidylinositol-3,4,5-trisphosphate (295, 299). The C2 region contains the Ca\(^{2+}\) binding site (295, 300) and it is present in the conventional isoenzymes. The nPKCs are not responsive to Ca\(^{2+}\) because of the absence of amino acids residues essential for the Ca\(^{2+}\) binding in the C2-like domain; recruitment to membrane is dependent on a C1 region with higher affinity for phospholipids in comparison with cPKCs (295, 300, 301). C3 bears the ATP binding lobe, while C4 contains the substrate docking sequence (294).
Structure of the PKC isozymes and PKC-related kinases

Classical PKCs
\((\alpha, \beta I, \beta II, \gamma)\)

Novel PKCs
\((\delta, \varepsilon, \eta, \theta)\)

Atypical PKCs
\((\zeta, \iota/\lambda)\)

PKC\(\mu\)/PKD

Figure 3-1. Molecular structure of PKCs.
3.1.2 Mechanism of PKC activation

All known PKCs, at the N-terminal side, are characterized by a pseudosubstrate or autoinhibitory region, adjacent to C1, which keeps each isoform in an inactive conformation (Figure 3-3) (295, 302). It has been proposed that mature and phosphorylated cPKCs can be in the cytosol and kept in an inactive conformation by intramolecular interactions between the N-terminal pseudosubstrate region and the kinase domain. The interaction with the specific activators allows the activation of the enzyme by opening its folded conformation: these cofactors are in fact able to decrease the affinity of the pseudosubstrate domain for the catalytic site that can exert its phosphorylating activity (295). The latent kinase can be recruited to the membrane and activated by lipid ligands (such as phorbol ester), leading to a massive conformational change that releases the pseudosubstrate domain from the substrate-binding site, allowing for substrate binding, phosphorylation and activation of downstream signaling effectors (302, 303). This activation mechanism is related to the translocation of PKCs to different intracellular sites, and the subsequent phosphorylation of specific substrate by the translocated enzyme (292). The translocation and activation of PKC from one to another subcellular compartment was thought to reflect only the interaction between PKC and lipids (292, 304). However, several proteins can interact with inactive and active PKCs, dictating enzyme location in both basal and stimulated conditions, underlining the additional relevance of protein-protein interactions in PKCs homeostasis (304).
**PKC phosphorylation sites in the catalytic domain**

![Diagram showing phosphorylation sites]

<table>
<thead>
<tr>
<th>Type</th>
<th>mPKCα</th>
<th>hPKCβI</th>
<th>hPKCβII</th>
<th>mPKCγ</th>
<th>mPKCδ</th>
<th>mPKCε</th>
<th>hPKCη</th>
<th>mPKCθ</th>
<th>mPKCζ</th>
<th>hPKCη/λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>Thr497 TTRTFCGTPDY</td>
<td>Thr500 TTKTFCGTPDY</td>
<td>Thr500 TTKTFCGTPDY</td>
<td>Thr514 TTRTFCGTPDY</td>
<td>Thr505 PPSTFCGTPDY</td>
<td>Thr566 TTTTFCGTPDY</td>
<td>Thr512 TATTFCGTPDY</td>
<td>Thr538 KTNTFCGTPDY</td>
<td>Thr410 TSTTFCGTPNY</td>
<td>Thr403 TTSTFCGTPNY</td>
</tr>
<tr>
<td></td>
<td>Thr638 PVLTPPDQ</td>
<td>Thr642 VELTPTDK</td>
<td>Thr641 PVLTPPDQ</td>
<td>Thr655 PALTPPD</td>
<td>Ser643 PQLSFSDK</td>
<td>Thr710 P I LTVEA</td>
<td>Thr555 PVLTPID</td>
<td>Ser676 PRLSFADR</td>
<td>Thr560 VQLTPDDE</td>
<td>Thr555 VQLTPDDD</td>
</tr>
<tr>
<td></td>
<td>Ser657 FEGFSYVN</td>
<td>Ser662 FAGFSYTN</td>
<td>Ser660 FEGFSYFN</td>
<td>Thr674 FQGFTYVN</td>
<td>Ser662 FHGFSFVN</td>
<td>Ser729 FKGFYFG</td>
<td>Ser674 FRNFNYVS</td>
<td>Ser695 FSNFSFNI</td>
<td>Ser579 FEGFEYVN</td>
<td>Ser574 FEGFEY I N</td>
</tr>
<tr>
<td>Novel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3-2.** The serine/threonine phosphorylation sites of PKCs in the catalytic domain.
Figure 3-3. Molecular mechanism of PKCs activation.
3.2 The role of PKCs in cell motility

3.2.1 Classical PKCs in cell motility

Classical or conventional subfamilies of PKC contain four members, α, βI, βII, and γ (289, 305). These Ca\(^{2+}\)-dependent cPKC isozymes are activated by Ca\(^{2+}\), DAG, and phosphatidylserine (PS) (289). Additional endogenous activators include cis-unsaturated fatty acid (306) and lysophosphatidyl choline (lysoPC) (307).

In general, classical PKC activation seems to positively affect cell motility, invasion and metastasis (292). Overexpression or stimulation of PKCα generally plays a major role in enhancement of cell migration in multiple cancer cell types. For example, early studies showed that over-expression of PKCα in MCF-7 breast cancer cells leads to increased anchorage-independent growth, tumorigenicity and metastasis in mice (308). Introduction of the ERBB2 receptor into MDA-MB-435 breast carcinoma cells confers increased invasiveness through Src-dependent activation of PKCα (309). Overexpression of PKCβII in rat intestinal epithelial cells increased cell invasiveness, possibly through increased Ras and MEK activation (310). One means through which classical PKC might enhance invasion and metastasis is the regulation of integrins (292). PKCα has been shown to directly interact with β1-integrin and increase migration on β1-integrin substrates, possibly through regulation of internalization and recycling of β1-integrin in human breast cancer MCF-7 and MDA-MB-231 cells (311). Furthermore, disruption of the PKCα-β1-integrin association blocks chemotactic migration in human breast carcinoma cells (MCF-7 and MDA-MB-231) and in the Phoenix amphotropic retroviral packaging cells (312). In human colon adenocarcinoma cell lines, a high level of PKCα expression together with a low level of E-cadherin was strongly related to a high migratory
activity (313). Similarly, The motility and invasion of low-metastatic rat prostate AT2.1 tumor cells were increased by thymelea toxin, a selective activator of PKCα over PKCδ (314).

Besides the above mentioned cancer cells, classical PKCs are also critical for cell migration of other cell types. In GT1 hypothalamic neurons, PKC signaling pathway promoted morphological differentiation via an increase in β-catenin, a cell-cell adhesion molecule (315). PKCα promotes GT1 neuronal migration by activating focal adhesion complex proteins such as p130Cas and FAK (315). In rat embryo fibroblasts (REF52 cells), PKCα redistributed to the leading lamellopodia of cells stimulated to migrate into an artificial wound (316). In human T lymphocyte cell line HUT-78, and freshly isolated normal human T lymphocytes activated by PMA or purified protein derivative prepared from *mycobacterium tuberculosis*, inhibition of classical PKC activity by Gö6976 suppressed lymphocyte polarization and migration following CD44 ligation, which suggested that PKCα was important for lymphocyte motility (317).

However, there are also some evidences that PKCα may negatively regulate cell migration in certain conditions. In bronchial epithelial BEAS2B cells, hog barn dust added to the entire culture medium slows airway epithelial cell migration through a PKCα-dependent mechanism in a wound healing assay (27). In MDA-MB-231 breast cancer cells, PKC activation via PMA treatment inhibits EGF-induced cell spreading, the initial event of motility and chemotaxis (318). Among the five PKC isoforms (α, δ, ε, τ and λ) identified in this cell line, PMA treatment only induced PKCα translocation from the cytosol to the membrane, an event that correlated with the development of the rounded morphology (318). One possible explanation of these inhibitory effects is the asymmetrical stimulation of cells by phorbol ester added to the cell culture medium. Therefore, the directioned cell migration is inhibited by enhanced locomotion.
3.2.2 Novel PKCs in cell motility

Novel PKCs contain δ, ε, η and θ (319). nPKCs also have twin C1 domains and a C2-like domain which in the case of nPKCs, precedes the C1 domain in their N-terminal regulatory regions (320, 321). The C2-like domain sequences of nPKCs lack calcium-co-ordinating acidic residue side chains (320, 321). Hence nPKCs are activated by DAG/phorbol ester, without requiring calcium (320, 321).

Similar to classical PKCs, novel PKCs, especially PKCδ, are also vital for cell migration and invasion in many cancer cells. In renal carcinoma cell line CCF-RC1, PKCδ regulated tumor cell migration by affecting the expression and activity of integrin β1 subunit and FAK (322).

PCPH/ENTPD5 (Pcph proto-oncogene protein or ectonucleoside triphosphate diphosphohydrolase 5) is not expressed in normal prostate, but its expression increases along cancer progression stages, being detectable in benign prostatic hyperplasia, highly expressed in prostatic intraepithelial neoplasia, and remaining at high levels in prostate carcinoma. The expression level and/or mutational status of PCPH/ENTPD5 contribute to the invasiveness of prostate cancer cells through a mechanism involving PKCδ (323). KITENIN (KAI1 COOH-terminal interacting tetraspanin) recruits Dishevelled (Dsh, a family of proteins involved in non-canonical Wnt signaling pathways) and PKCδ to form a functional complex, which acts as an executor in regard to cell motility, and thereby controls colorectal cancer cell invasion to contribute to promoting metastasis (324). Quercetin (QUE; 3,5,7,3′,4′-tetrahydroxyflavone) inhibits tumor invasion via suppressing PKCδ/ERK/AP-1-dependent MMP-9 activation in breast carcinoma cells (325). Sorafenib (Bay43-9006; Nexavar) is a Raf and VEGF receptor inhibitor that blocks receptor phosphorylation and MAPK-mediated signaling (326). Coadministration of Sorafenib with novel PKC inhibitor Rottlerin potently inhibits cell migration in human malignant
glioma cells (326). In EGFR-overexpressing invasive cells such as MDA468 breast cancer cells, inhibition of MAPK activity with MEK inhibitor PD98059 blocks early stages of cell migration (up to 4 h) (327). While inhibition of PKCδ activity with Rottlerin or dominant-negative PKCδ expression blocks sustained cell migration after 4 h and up to 12 h, the combination of MAPK and PKC inhibitors completely blocked TGF-α-induced cell migration (327).

Novel PKCs are also important for cell motility of mesenchymal cells such as smooth muscle cells (SMCs), fibroblasts, astrocytes and other cell types. In rat aortic SMCs, upon treatment of PDGF-BB or TGF-β1, a fraction of PKCδ rapidly translocated from the cytosol to the post-nuclear particulate fraction at 15 sec and reached an apparent maximum at 30 min, which is important in the control of PDGF-BB or TGF-β1 stimulated vascular smooth muscle cell migration (328). In response to mechanical stress, SMCs from PKCδ-/- mice showed an abnormal cytoskeleton structure, which was related to a diminished phosphorylation of paxillin, FAK, and vinculin (329). Mechanical stress enhanced SMC migration was diminished in these PKCδ-/- SMC cells, which indicates that PKCδ is a key signal transducer between mechanical stress and cell migration (329). In SMCs, PKCα, δ and ε were expressed and they were activated upon integrin engagement with different kinetics. PKCε was activated early, whereas PKCα and PKCδ were activated later (330). Activation of PKCε was necessary for cell attachment to fibronectin (330).

In mouse fibroblasts, EGF stimulates myosin light chain phosphorylation, a marker for contractile force, concomitant with PKCδ activation (331). Myosin-based cell contractile force is considered to be a critical process in cell motility. The dominant-negative PKCδ construct or PKCδ RNAi abrogated EGF-induced cell contractile force generation and motility through dephosphorylation of myosin light chain (331). In primary human dermal fibroblasts, during cell
migration PDGF-BB strongly stimulates membrane translocation and leading edge clustering of PKCδ (332).

In rat brain astrocytes, activation of ERK1/2 by a PKCδ-dependent event mediated through Elk-1 pathway is essential for MMP-9 gene up-regulation and cell migration induced by bradykinin (333). Phosphoprotein enriched in astrocytes-15 kDa (PEA-15), enriched in astrocytes, also inhibits glioblastoma organotypic astrocyte cell migration in a PKCδ dependent mechanism (334).

In human eosinophils, PKCδ mediates cell motility, CD11b expression and MMP-9 granule release during migration (335). Engagement of CD44 using immobilized mouse antibodies or hyaluronan-enriched extracellular matrix lattices induces active migration in T lymphocytes accompanied by cytoskeletal rearrangement and cell polarization. PKCδ inhibitor Rottlerin reduced CD44-activated cell migration but did not completely ablate it (317).

In addition to PKCδ, other novel PKCs such as ε, η and θ also play an important role in cell migration. Sphingosine 1-phosphate (S1P) signals through S1P(1) and G(i) to activate PKCε and, subsequently, a PLD2-PKCζ-Rac1 cascade is necessary to stimulate the migration of human lung endothelial cells during the angiogenic process (336). Expression of PSη, a peptide inhibitor based on the PKCη pseudosubstrate sequence, slowed the rate of PMA induced endothelial cell migration (337). In an in vitro three-dimensional assay in which endothelial cells organize into capillary tubules, the endothelial cells that expressed PSη formed fewer such tubules (337).

On the other hand, there are also some reports that PKCδ may inhibit cell migration under certain conditions. PKCδ-mediated increased cell substrate adhesion as a limiting factor for TNF-α stimulated neutrophil transendothelial migration (338). In freshly isolated bovine aorta endothelial cells, lysoPC, a major lipid constituent of oxidized low-density lipoprotein, can
activate PKCδ and inhibit cell migration in wound healing assay (339). Phosphorylated PKCδ associated with and phosphorylated syndecan-4 (339), which increased α-actinin binding to the variable region of syndecan-4 and inhibited the ability of syndecan-4 to bind and activate PKCα (339). The combined effects led to dissociation of the focal adhesion complex and inhibition of endothelial cell migration (339).

3.2.3 Atypical PKCs in cell motility

The atypical PKCs, PKCζ and PKCλ/τ, have four functional domains: a PB1 domain at the N-terminus, a pseudosubstrate (PS) sequence, a C1 domain consisting of a single Cys-rich zinc-finger motif, and a kinase domain in the C-terminus (340). Since the C1 domain of aPKC isotypes lacks the repeat structure found in those of cPKCs and nPKCs, aPKCs do not respond to DAG and phorbol esters directly (341). Thr410 in the activation loop of aPKCs is phosphorylated by PDK1 which binds to the hydrophobic motif (340). aPKCs are associated with Par-3, Par-6 and Cdc42 in a complex that plays an important role in cell polarization (342). PKCτ can be tyrosine phosphorylated by the non-receptor tyrosine kinase c-Src in PC12 cells (343). aPKC τ plays a critical role in human lung cancer cell growth (344) and is an oncogene in human non-small cell lung cancer progression (345).

Interestingly, PKCζ plays a major role in cell motility in hematopoietic cell lineage, stem cells and progenitor cells. Sialomucin endolyn (CD164) is an adhesion receptor that regulates the adhesion of CD34+ cells to bone marrow stroma and the recruitment of CD34+CD38(lo/-) cells into cycle. It modulated CXCL12-mediated migration of umbilical cord blood CD133+ cells through PKCζ and Akt signaling (346). SDF-1 and its receptor (CXCR4) play a major role in migration, retention, and development of hematopoietic progenitors in the bone marrow.
Overexpression of PKCζ in leukemic pre-B acute lymphocytic leukemia G2 cells and U937 cells led to increased directional motility to SDF-1 (347). SDF-1 triggered PKCζ phosphorylation, translocation to the plasma membrane, and kinase activity (347). SDF-1-induced proliferation and MMP-9 secretion also required PKCζ activation. PI3K was identified as an activator of PKCζ, and Pyk-2 and ERK1/2 as downstream targets of PKCζ (347). In vivo engraftment of human CD34+ enriched cells to the bone marrow of NOD/SCID mice was PKCζ dependent. Injection of mice with inhibitory PKCζ pseudosubstrate peptides resulted in mobilization of murine progenitors (347). Knockdown of PKCζ by small interference RNA (siRNA) impaired CSF-1-induced chemotaxis of human acute monocytic leukemia THP-1 cells and impaired migration of mouse peritoneal macrophages (348). In human peripheral monocytes, and murine macrophage-like cell line J774.1, PKCζ is an essential for transducing the motility signal induced by superoxide and a chemotactic peptide, fMLP. In these cells, PKCζ was activated to phosphorylate RhoGDI-1, which liberated RhoGTPases, leading to their activation. These events were inhibited by myristoylated PKCζ peptides in these cells (349).

PKCζ is also involved in cancer cell migration and invasion. In human breast cancer MDA-MB-231, MCF-7 and T47D cells, EGF induced PKCζ translocation from the cytosol to the plasma membrane and activation of PKCζ probably via PI3K. PKCζ is an essential component of EGF-stimulated chemotactic signaling pathway in these human breast cancer cells (350). In human pancreatic adenocarcinoma cells, PKCζ plays a critical role in maintaining a high linear motility score in motile subclones. In motile cells, PKCζ is constitutively associated with the plasma membrane, whereas in nonmotile cells, PKCζ is totally excluded from the plasma membrane (351). In a preclinical astrocytome model, PTEN deficiency resulted in a marked increase in cell invasiveness that was specifically suppressed by inhibitors of PKCζ (352).
PKCζ may also be critical for cell motility in many other cell types. In human pulmonary artery endothelial cells, PKCζ play a critical role in sphingosine 1-phosphate potently stimulated endothelial cell migration through a PLD2-PKCζ-Rac1 cascade (336). Human immunodeficiency virus-1 (HIV-1) envelope glycoprotein gp120 induces toxicity and alters expression of tight junction proteins in human brain microvascular endothelial cells (353). gp120 can also cause dysfunction of blood-brain barrier via PKCζ related signaling pathways and mediated intracellular calcium release leading to cytoskeletal alterations and increased monocyte migration (353). During cell migration of primarily mouse fibroblasts, human water channel aquaporin-9 (AQP9) induced actin polymerization in the filopodia extension of lamellipodia is augmented by activation of PKCζ (354). In MC3T3-E1 osteoblast-like cells, insulin-like growth factor-1 stimulated cell migration in a PKCζ dependent manner (355). In primary human mesangial cells, connective tissue growth factor (CTGF) stimulated cell migration is associated with a PKCζ-GSK3β signaling axis (356). CTGF induced cell migration and cytoskeletal rearrangement through the phosphorylation and translocation of PKCζ to the leading edge of migrating cells (357). Inhibition of CTGF-induced PKCζ activity with a myristilated PKCζ inhibitor or transient transfection of human mesangial cells with a PKCζ kinase inactive mutant (dominant negative) expression vector led to a decrease in CTGF-induced migration (357).

In terms of cell invasion, PKCζ mainly enhances cell invasion into the underneath matrix through up-regulation of MMPs. PKCζ induced phenotypic alterations associated with malignant transformation and tumor progression in mammary cells (358). The stable overexpression of PKCζ in immortalized mammary epithelial NMuMG cells, activated the ERK pathway, enhanced clonal cell growth and exerted profound effects on proteases secretion (358). PKCζ overexpression markedly altered the adhesive, spreading, and migratory abilities of mammary
epithelial NMuMG cells (358). Ursolic acid (UA), a constant constituent of Rosmarinus officinalis extracts, is a triterpenoid compound which has been shown to have antioxidant and anticarcinogenic properties (359). UA was able to reduce IL-1β or TNF-α-induced rat C6 glioma cell invasion through suppressing the association PKCζ with ZIP/p62 and downregulating the MMP-9 expression (359). During glioma cell invasion through the brain extracellular matrix, PKCζ regulated transcription of the MMP-9 gene induced by IL-1 and TNF-α in glioma cells via NF-κB (237).

Powell et al. found that PKCζ mRNA levels were reduced markedly in metastatic Dunning R-3327 rat prostate tumors relative to the nonmetastatic Dunning H tumor and normal rat prostate (360). They established stably transfected PKCζ in Dunning R-3327 MAT-LyLu rat prostate tumor cells. Nine independent clones of PKCζ-expressing cells exhibited a lower tendency to metastasize to lungs relative to vector-transfected cell clones, and the ability of four PKCζ overexpressing MAT-LyLu cell clones to invade through Matrigel in a Boyden chamber assay was greatly reduced (361). These results contradict with most observations of the role of PKCζ in promoting cell motility. Whether this is specific to this type of prostate tumors in rats, or indicating the different function of PKCζ in vivo need to be further investigated.

### 3.2.4 PKCµ in cell motility

PKCµ or PKD1 is a serine/threonine protein kinase, which contains two cysteine-rich domains that bind diacylglycerol or phorbol esters, but it lacks the Ca²⁺ binding domain found in cPKCs (362). PKCµ also has a pleckstrin homology (PH) domain that regulates its kinase activity, but does not harbor the typical PKC autoinhibitory pseudosubstrate motif (362). Accumulating evidences showed that PKCµ plays a very important role in cell cycle, cell
division, and cell proliferation. However, whether PKCµ is involved in cell migration is largely unknown. Cortactin, paxillin and PKCµ were co-immunoprecipitated as a complex from invadopodia-enriched membranes of invasive breast cancer MDA-MB-231 cells (363). In contrast, this complex of proteins was not detected in lysates from non-invasive cells that do not form invadopodia (363).

In summary, in this chapter, I reviewed the molecular structures of PKC family protein kinases and the molecular mechanisms of their activation. I also summarized their distinct roles in the regulation of cytoskeleton reorganization and cell motility. Most studies focused on the role of single PKC isoform. Less evidences showed the interaction and crosstalk between different PKC isoforms in cell motility. My thesis studies focus on coordinated activation of PKC isoforms and relationship with other signaling pathways in podosome formation and functions in normal human bronchial epithelial cells.
4. Chapter Four. Rationale, hypothesis and specific aims
4. Chapter Four. Rationale, hypothesis and specific aims

4.1 Rationale

The airway epithelial cells not only act as a protection from the external environment, but also have a variety of biological functions including fluid and ion transport, mucus secretion and ciliary transport, interaction with and/or recruitment of inflammatory cells, antimicrobial activities, protection against oxidant and proteases, and modulation lung repair after injury (364, 365). Human lung bronchial epithelium is the primary target of lung diseases such as chronic obstructive pulmonary disease, asthma, cystic fibrosis and lung cancer. Lung airway epithelial cells are also important components of host defense (366). Epithelial cell movement over the damaged matrix is a complex phenomenon including adhesion and deadhesion of cells to matrix components and response to “migration-inducing” substances (364). Bronchial epithelial cells express a variety of integrin and non-integrin receptors that can mediate their adhesion to the extracellular matrix (367, 368). Cell migration is the most critical and first event occurring during the epithelial repair process (7, 87).

Podosome is an actin-rich structures involved in cell migration and invasion across the tissue boundaries (369). It was mainly found in mesenchymal cells and hematopoietic linage cells, such as monocytes or macrophages (143-145), dendritic cells (370, 371), natural killer cells (372), and osteoclasts (373, 374). It was also found in certain malignant metastatic invasive cancer cells (148). Whether normal epithelial cells, in particular, normal human bronchial epithelial cells can form podosome was largely unknown.

Phorbol esters such as PMA and PDBu, are often employed in biomedical research to activate PKC family (375). PKC can mediate non-receptor tyrosine kinase c-Src activation and subsequent podosomes formation in aorta endothelial cells (376) and vascular smooth muscle
cells (377). Several signaling proteins including PI3K/Akt and Src are described to serve as downstream PKC effectors (375). However, which PKC isozyme(s) are involved in the process of podosome formation and how do they regulate the proteolytic function to degrade extracellular matrix are largely unknown. Whether Src tyrosine kinase and PI3K/Akt signaling pathways are associated with PDBu-induced podosome assembly and how do these molecules regulate the proteolytic function of podosomes in lung epithelial cells are still unclear. Phorbol ester can induce dramatic cytoskeletal structure changes in lung epithelial cells (378). I speculate that some of these structures are podosomes in lung epithelial cells and may be involved in epithelial cell migration and invasion during lung physiological and pathophysiological conditions.

4.2 Hypothesis

Normal human bronchial epithelial cell migration and invasion may be mediated by podosome, and the formation and proteolytic function of podosomes are regulated by multiple signal transduction pathways (including PKC, PI3K/Akt, Src and MAPKs) in a coordinated fashion.

4.3 Specific aims

In my PhD thesis research work, I acquired primary normal human bronchial epithelial cells and normal (non-cancerous) human lung bronchial epithelial BEAS2B cells and challenged these cells with PDBu to activate PKCs. My hypothesis was tested in the following specific aims:
1. To determine whether phorbol ester can induce podosome formation in primary normal (non-cancerous) human lung bronchial epithelial cells and to characterize these molecular structures (Chapter 6)

2. To identify which PKC isozyme(s) may contribute to podosome assembly and which PKC isoform(s) control the proteolytic function in human lung bronchial epithelial cells (Chapter 7)

3. To determine whether PI3K/Akt/Src/MAPKs signaling pathway(s) are involved in regulating podosome formation and proteolytic function in human lung bronchial epithelial cells (Chapter 8).
5. Chapter Five. Materials and methods
5. Chapter Five. Materials and methods

5.1 Reagents and antibodies

Bisindolylmaleimide I (BIM I), Ro-31-8220, Gö6976, Rottlerin, BAPTA/AM, myristoylated PKCζ pseudosubstrate (PS) and scrambled pseudosubstrate as its negative control (PI), LY294002, Wortmannin, PP2, PP3, Su6656, GM6001, GM6001NC, Genistein, SP600125, PD98059, SB20358 and Akt inhibitor II were from EMD Biosciences (Darmstadt, Germany). Antibodies against different PKC isozymes and phosphorylated PKCs were from Cell Signaling (Danvers, MA). siRNA targeting PKCα, PKCδ, PKCμ, PKCζ, MMP-2, MMP-9, MMP-14, control siRNA and fluorescein conjugated control siRNA, anti-integrin, anti-FAK, and mouse monoclonal MMP-9 neutralization antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-vinculin, anti-actinin, anti-talin and anti-tubulin antibodies were from Sigma. Anti-phospho Src Y416, anti-phospho PI3K p85 T458/p55 T199, anti-phospho PKD1 S241, anti-phospho Akt S473, anti-phospho Akt T308, anti-phospho GSK3β S9, anti-phospho JNK2/3 p54 T183/Y185 and JNK1 p46 T183/Y185, anti-phospho p38 MAPK T180/Y182, anti-phospho p44/p42 MAPK (ERK1/2) T202/Y204, anti-phospho ERK1/2 and anti-phospho PI3K p85α antibodies were from Cell Signaling (Danvers, MA). Anti-phospho-tyrosine (4G10), anti-PI3K p85α and anti-cortactin (p80/85, clone 4F11) antibodies were from Upstate (Billerica, MA). Anti-MT1-MMP, anti-MMP2, and anti-MMP9 antibodies were from Biomol (Plymouth Meeting, PA). Anti-GAPDH antibody was from Upstate (Billerica, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ). Alexa Fluor 594 labeled secondary antibody, FTIC labeled secondary antibody, Oregon green 488 phalloidin, rhodamine phalloidin, tetramethylrhodamine isothiocyanate (TRITC), and Hoechst dye 33342 were from Molecular Probe (Eugene, OR).
pEGFP C2 vector, pEGFP-PKCα and pEGFP-PKCζ plasmid constructs were gifts from Dr. Peter J. Parker (London Research Institute, Cancer Research UK). mCherry-Tks5 plasmid and anti-Tks5 polyclonal antibody were from Dr. Sara Courtneidge (Burnham Institute for Medical Research, California, USA). PDBu, DMSO, fibronectin from bovine plasma, anti-vinculin antibody, mouse IgG1, rabbit IgG and other reagents without further mention were purchased from Sigma (St. Louis, MO).

5.2 Cell culture

Primary normal human bronchial epithelial cells and Bronchial Epithelial Cell Medium Bullet Kit were from Lonza Walkersville Inc. (Walkersville, MD). The Bronchial Epithelial Cell Medium BulletKit (BECM BulletKit) contained Bronchial Epithelial Cell Basal Medium (BECB Medium) and the following supplements, 0.4% BPE, 0.1% Hydrocortisone, 0.1% hEGF, 0.1% Epinephrine, 0.1% Transferrin, 0.1% Insulin, 0.1% Retinoic Acid, 0.1% Triiodothyronine, and 0.1% GA-1000. These primary cells were freshly isolated from human bronchus (non-cancerous and non-disease). They were confirmed by cytokeratin staining as the epithelial cell marker. The primary cells were cultured according to the manufacture’s instruction. These primary cells were only subject to 1-2 passages before use. Human bronchial epithelial BEAS2B cells, human lung adenocarcinoma A549 cells from ATCC (Manassas, VA) and v-Src transformed NIH 3T3 cells from Dr. G. Steven Martin (University of California at Berkeley, Berkeley, CA) were cultured in Dulbecco’s modified eagle’s medium (DMEM, low glucose) (Life Technologies, Rockville, MD) with 10% fetal bovine serum (FBS) (GIBCO, Carlsbad, CA), penicillin (1 mg/ml) and streptomycin (1 mg/ml) (Life Technologies) at 37°C in a 5% CO2 humidified atmosphere following standard procedures (379, 380).
5.3 siRNA transfection

siRNA was transfected into BEAS2B cells by Oligofectamine (Invitrogen, Carlsbad, CA) following manufacturer’s protocol (381, 382). BEAS2B cells (10,000 cells/well) were plated into 6-well plates. siRNA (50 nM) and 10 µl Oligofectamine in OPTI-MEM (Invitrogen) was added to each well at approximately 50% confluence of cells, and 48 h after incubation, cells were stimulated with PDBu 500 nM for 30 min and followed by immunofluorescent staining.

5.4 Immunofluorescence staining

Cells were stained according to a standard staining protocol at room temperature (383). BEAS2B cells (10,000 cells/well) were cultured on glass coverslips (VWR, Mississauga, Canada) in 6-well plates. Cells were pre-incubated with designated pharmacological inhibitors and then challenged with PDBu 500 nM for 30 min. Cells were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature, permeabilized with 0.1% Triton X-100/PBS for 10 min on ice and blocked with 1% BSA/PBS for 60 min for non-specific binding. Cells were incubated with designated primary antibody for 2 h at room temperature and then designated secondary antibody conjugated with Alexa Fluor 594 (Molecular Probe) for 1 h at room temperature in dark. Then the cells were incubated with Oregon Green 488 Phalloidin (Molecular Probe) for 1 h at room temperature and Hoechst dye 33342 for 10 min at room temperature in dark. Between each step, cells were gently washed with PBS for three times. After carefully washed with double distilled water, the coverslips were mounted on glass slides with Dako fluorescence mounting medium (Dako, Mississauga, Canada).

For in situ zymography assay, cells were cultured on coated glass coverslips (VWR, Mississauga, Canada) in 6-well plates. Cells were pre-treated with designated chemical inhibitors
and then stimulated with PDBu 500 nM for 8 h. Cells were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature and permeabilized with 0.1% Triton X-100/PBS for 10 min on ice. Then cells were stained with Rhodamine Phalloidin (Molecular Probe) for 1 h at room temperature and Hoechst dye 33342 for 10 min at room temperature in dark. Cells were gently washed with PBS for three times between each step. The coverslips were mounted on glass slides with Dako fluorescence mounting medium (Dako, Mississauga, Canada).

5.5 Microscopy and image analysis

The inverted laser scanning fluorescence confocal microscope (Olympus FluoView Confocal, FV1000-ASW) equipped with acquisition and analysis software (Version 01.02.01), and a 1 X 81 oil immersion objective (numerical aperture [NA], 1.40) was used. Cell images triple stained with Hoechst dye 33342, Oregon Green 488 Phalloidin, and Alexa Fluor 594 labeled secondary antibody were obtained using selective laser excitation at 405 nm (Diode Laser), 488 nm (Multiline Argon Laser), and 543 nm (Helium Neon Laser), respectively. Z-stack scanning microscopy images were acquired at step of 0.1 µm from the bottom to the top of the cell.

Phase-contrast and fluorescence images were also taken with a Nikon TE2000 inverted fluorescence microscope (40X objective with an optical magnification lens of 10X and oil immersion objectives of 40X [NA, 1.30] and 60X [NA, 1.40]), while digitally acquired images were taken with a Nikon DXM 1200F camera (LUCIA 5.0 acquisition software, Nikon).

Fluorescence images were analyzed and processed with Simple PCI v6.0 (C-Image Inc., Burlington, MA). Quantification of PDBu-induced cytoskeletal structures was done by counting at least 200 cells. Cells with dramatic cytoskeleton re-organization and at least 5 of the
podosome-like structures, either small dot (> 1 µm), rosette ring, or belt in cytoplasm were considered to be podosome-positive cell. Cells with long and thin stress fibers were considered as podosome negative cells.

We quantified the co-localization of the confocal images by Olympus FV10-ASW Version 01.07.02 imaging software for Pearson’s correlation coefficient according to this equation:

\[
R_r = \frac{\sum_{i} (S_{1i} - S_{1\text{aver}}) \cdot (S_{2i} - S_{2\text{aver}})}{\sqrt{\sum_{i} (S_{1i} - S_{1\text{aver}})^2 \cdot \sum_{i} (S_{2i} - S_{2\text{aver}})^2}}
\]

In this equation, \( S_1 \) is the signal intensity of pixels in the first channel and \( S_2 \) is the signal intensity of pixels in the second channel. \( S_{1\text{aver}} \) and \( S_{2\text{aver}} \) indicate the average intensity of pixels in the first and second channel, respectively. We only quantified the cytoplasm area of each cell and excluded the peripheral edge of the cell.

5.6 Live cell imaging

BEAS2B cells (100, 000 cells/well) were seeded on round glass coverslips (diameter 50 mm) (Fisher Scientific, Ottawa, Canada) in 60-mm cell culture dish for live cell imaging. Cells were grown to 20-30% confluence before treated with Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s instructions (382, 384). Plasmids (2 µg) were added to each 60-mm cell culture dish. After 48 h transfected cells were subjected to live cell imaging. The time-lapse series of cells were taken at 37°C using the above mentioned confocal microscope equipped with a computer-driven camera and autofocus system in humidified chamber with 5% CO₂. Differential Interference Contrast (DIC) and fluorescence images were
taken every 5 sec for up to 25 min. Original digital images were converted to movies by Olympus FluoView FV10-ASW Version 01.07.02 software with 330 msec frames.

5.7 Fibronectin based in situ zymography

Fibronectin was conjugated with TRITC succinamidyl ester (Molecular Probes, Eugene, OR) following manufacturer’s protocol with some modifications (385). Glass coverslips (12 mm) were pre-cleaned with 20% nitric acid for 30 min. Ethanol-sterilized coverslips were coated with 50 µg/ml poly-L-lysine for 20 min, and fixed with 0.5% glutaraldehyde for 15 min. The coverslips were inverted on an 80 µl drop of gelatin sucrose matrix for 1 h, and then incubated with fibronectin matrix (0.2% TRITC-conjugated fibronectin) for 1 h in dark. The residual reactive groups in the fibronectin-TRITC matrix were quenched with sodium borohydride at 5 mg/ml for 15 min and sterilized with 75% ethanol. The entire procedure was performed at room temperature. Extensive washing with PBS was performed between steps.

To test the ability of cells to degrade matrix, cells were plated on coated coverslips in 24-well plates and incubated at 37 °C for 4 h. Foci of degraded matrix were observed as dark holes on the red fluorescent fibronectin matrix. A cell with at least one hole under the cell or near the cell edge was considered as a positive cell in degrading matrix. As a positive control for the in situ zymography technique, NIH3T3 cells were grown to 20-30% confluence, and transfected with plasmid expressing constitutively active chicken SrcY527F (a kind gift from Dr. B. Elliot, Queen’s University, Canada) with Lipofectamine PLUS reagent (Invitrogen), according to the manufacturer’s instructions. Transfected cells were analyzed 24 h later using the same protocol (385).
5.8 Gelatin based in situ zymography

Glass coverslips were pre-cleaned with nitric acid and sterilized with ethanol. After treated with 50 µg/ml poly-L-lysine and fixed with 0.5% glutaraldehyde, the coverslips were coated with a base of gelatin sucrose gel at the bottom and then a layer of 0.2% gelatin conjugated with Oregon Green 488 (Molecular Probes, Eugene, OR) gel on top. The residual reactive groups in the gelatin conjugated matrix were quenched with 5 mg/ml sodium borohydride. Cells were plated on coated coverslips in 6-well plates and incubated at 37°C for 8 h. Foci of degraded matrix were observed as dark holes (1-2 µm) on the green fluorescent gelatin matrix. Cells with at least one hole (> 1 µm) under the cell or near the cell edge were counted in a blinded fashion as positive to degrade matrix.

5.9 Cell invasion assay

Fibronectin gels were prepared in Transwell chambers inserted in 24-well culture plates, with 100 µl of 2.5 mg/ml fibronectin in PBS with penicillin (1 mg/ml) and streptomycin (1 mg/ml), incubated at 37°C for 48 h. Subsequently, 10,000 cells were added to each insert for 4 h to allow for cell adhesion. PDBu was added to the Transwell insert and the lower well. After overnight incubation, any cells remaining in the insert were removed using cotton Q-tips. The nuclei of invaded cells were stained with Hoechst dye 33342 (146). Quantification of invaded cells was performed for at least 20 fields per coverslip, by counting the number of nucleus sprouts that had invaded through the fibronectin gels per field (20X objective with magnification lens of 10X). Cells in the Transwell inserts were fixed with 4% formaldehyde, and stained for F-actin using Oregon Green 488 phalloidin. Confocal scanning microscopy was performed to visualize cellular protrusions inside the pores.
5.10 Western blotting

Western blotting was performed according to a standard protocol as previously described (382, 384). Cell lysates were harvested using protein lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 100 µg/ml soybean trypsin inhibitor, 100 µg/ml benzamidine hydrochloride, 1 mM PMSF, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml pepstatin A, 50 µg/ml antipain, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 10 mM sodium orthovanadate). Protein (40 µg protein of each sample) was first separated by SDS-PAGE, and transferred to nitrocellulose membranes by Semi-Dry Transfer Cell (BioRad, Hercules, CA). After blocking non-specific binding by 5% BSA in TBST buffer (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween 20), the membranes were incubated in primary antibodies for overnight at 4 °C and HRP-conjugated secondary antibodies for 1 h at room temperature. The signals were tested with Enhanced Chemiluminescence detection solution (Amersham Pharmacia Biotech, Piscataway, NJ) or SuperSignal Chemiluminescent substrate (PIERCE Chemical, Rockford, IL). The membranes were stripped off with Restore Western Blot Stripping Buffer (PIERCE Chemical, Rockford, IL) for 3h at room temperature and then re-probed with another primary antibody.

5.11 Gelatinase zymography gel

Gelatin zymography was performed according to standard protocol (147). SDS-PAGE gels (10%) containing gelatin 0.1% (1 mg/ml) were prepared according to a standard procedure (385). Cell lysates and culture medium were freshly harvested and prepared with 6X zymography sample buffer (12.5 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS (w/v), 0.5% bromophenol blue). Cell lysates with 30 µg protein were added to each well and gels were run.
with 1X Tris-Glycine SDS running buffer under standard running conditions (~90V, constant voltage) for about 10 h (385). After running, the gels were incubated in renaturing buffer (2.5% triton X-100 in H₂O) and then incubated in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij35) at 37°C overnight. Gels were stained with 0.5% (w/v) Coomassie Blue R-250 staining solution and destained with destaining solution (50% methanol, 10% acetic acid, 40% H₂O).

5.12 Real-Time quantitative RT-PCR

The primers used for qRT-PCR for human MMP-2 were 5’-TTGCTGGAGACAAATTCTGGAG-3’ (hMMP-2F) and 5’-GGGAAGCCAGGATCCATTTT-3’ (hMMP-2R); for human MMP-9 were 5’-ACGCACGACGTCTTCCAGTAC-3’ (hMMP-9F) and 5’-AAGCGGTCCTGGCAGAAATA-3’ (hMMP-9R); for human MMP-14 were 5’-GGCCCAAAGCAGCAGCT-3’ (hMMP-14F) and 5’-AGCCGTAAAACTTCTGCATGG-3’ (hMMP-14R). Total RNA was extracted from BEAS2B cells with TRIZOL Reagent (Invitrogen), according to the manufacturer’s instructions (386). cDNA was synthesized from total RNA (1 µg) using MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA) and random hexamers from Taqman Reverse Transcription Reagent kit (Roche Applied Science, Toronto, Canada) (386). qRT-PCR was performed using 2 × QuantiTect SYBR Green PCR kit (Roche, Mannheim, Germany) on LightCycler480 (Roche, Mannheim, Germany) according to a standard protocol (386). The amplification mixtures (10 µL) contained 2.5 µL of the 1:10 diluted cDNA, 5 µL of SYBR Green I Master mix (Roche, Mannheim, Germany) and 300 nM forward and reverse primers. Conditions for PCR included 95 °C for 5 min, and 40 cycles of 94 °C for 15 s, 60°C for 60 s. Each assay included a standard curve of five serial dilutions and a no-template
negative control. All assays were performed in triplicate. The MMP-2/-9/-14 gene expression levels were normalized to the level of 18S ribosomal RNA (386).

5.13 Statistics

Each experiment was performed independently for at least three times. The quantification of each value represents the mean of at least three independent measures $\pm$ SD. Significance was determined by ANOVA followed by Tukey's post-hoc analysis or by Student's t-test where appropriate.
6. Chapter Six. Phorbol ester-induced podosomes in normal human bronchial epithelial cells

The content of this chapter was published in *Journal of Cellular Physiology*, and the figures were reprinted with the permission of the Wiley InterScience Wiley-Liss Inc. from:

6. Chapter Six. Phorbol ester-induced podosomes in normal human bronchial epithelial cells

6.1 Summary

Spreading and migration of the basal cells neighboring a wound is essential for airway epithelial repair. To gain insight into the molecular mechanisms that govern these cellular processes, we asked whether normal human airway epithelial cells can form podosomes, a cellular structure discovered from cancer and mesenchymal cells that controls migration and invasion. Herein, we report that phorbol-12, 13-dibutyrate (PDBu), a PKC activator, induced reorganization of cytoskeletal structure in primary normal human bronchial epithelial cells, and in normal human airway epithelial BEAS2B cells. Z-stack scanning confocal microscopy showed that PDBu-induced podosome-like structures contain actin-rich columns that arise from the ventral surface of the cell, and also revealed the presence of circular ruffles/waves at the dorsal cell surface. The molecular components of these cytoskeletal structures were determined with immunofluorescent staining. Using in situ zymography, we demonstrated that PDBu-induced podosomes were capable of degrading fibronectin-gelatin-sucrose matrix. PDBu also increased epithelial cell invasion across Transwell chamber. Podosomes and circular dorsal ruffles may be important for epithelial cell migration and invasion, thus contributing to respiratory epithelial repair and regeneration.

6.2 Introduction

The respiratory epithelium is frequently exposed to and damaged by infectious and non-infectious environmental agents. A proper wound repair process is critical to maintain the
integrity of the epithelium. Airway and alveolar epithelial cells are essential components of host defense system (387). Cell survival and repair also play important role in acute lung injury and other lung diseases related to inflammatory responses (388). Regardless of the source of injury, lesions in the airway epithelium can lead to cell death and shedding, and resulting in the loss of surface epithelium (18, 87). After injury, the airway epithelium initiates a wound healing process to restore its barrier integrity. The remaining viable epithelial cells at the edge of the wound dedifferentiate, spread, and migrate over the denuded basement membrane to cover the de-epithelialized zone (16, 389, 390). Cell migration is the most critical and first event occurring during the epithelial repair process (7, 87).

Recently, many studies have implicated a specialized structure called the podosome as a potential mechanism for cell migration and invasion (369). Podosomes are actin-rich cellular structures which protrude into the extracellular matrix (ECM), resulting in localized remodeling activities (141, 142). Accordingly, matrix degradation at podosomes is thought to contribute to cellular invasiveness in physiological and pathological situations. Podosomes were first observed as electron-dense actin-rich structures on the ventral surface of v-Src transformed fibroblasts (369). Later, similar structures were found in mesenchymal cells, especially cells from hematopoietic lineage, such as monocytes or macrophages (143-145), dendritic cells (370, 371), natural killer cells (372), and osteoclasts (373, 374). Podosomes have been found in certain malignant metastatic invasive cancer cells (148). For examples, Spinardi et al. found dynamic podosome-like structures in rat bladder carcinoma epithelial 804G cells stably transfected with human β4 integrin subunit. Interestingly, they also showed similar structures in a few human primary keratinocytes (151). However, whether normal human epithelial cells can form podosomes either spontaneously or under stimulation is largely unknown.
Several stimuli are able to induce podosome formation in cell types that do not spontaneously have podosomes, such as TGF-β in aorta endothelial cells (147), phorbol ester in vascular smooth muscle cells (146) and endothelial cells (376). Phorbol esters, e.g. phorbol 12-myristate 13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu), are often used to activate protein kinase C (PKC) (375). PKC activation regulates a diverse set of cellular processes including cell proliferation, differentiation, migration, and invasion (375). PKC may mediate non-receptor tyrosine kinase c-Src activation and subsequent podosome formation in fibroblasts (377). Several signaling proteins including c-Src and focal adhesion kinase (FAK) often serve as downstream PKC effectors (375). Phorbol ester induced dramatic changes in cytoskeletal structure in murine lung epithelial cells (378).

We hypothesized that during airway repair, normal epithelial cells may form podosomes in order for the basal cells neighboring the wound to digest ECM molecules for cell spreading and transmigration. In the present study, we challenged BEAS2B cells (a cell line derived from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals) with PDBu to determine podosome formation and function of podosome-like structures. Importantly, we also found these structures in primary normal human bronchial epithelial cells with similar functions.

6.3 Results

6.3.1 PDBu induces reorganization of cytoskeleton in normal human bronchial epithelial cells
To determine the effects of PDBu on cytoskeletal structure in normal human bronchial epithelial cells, BEAS2B cells were treated with PDBu, and the changes in cell morphology was recorded with live cell imaging. After PDBu treatment, cell morphology changed immediately with lamellipodia retraction and ruffling (data not shown). The filamentous (F)-actin was stained and captured with confocal scanning microscopy. Untreated cells displayed long and thin stress fibers in the cytoplasm (arrow in Figure 6-1A), and cortical filaments along the periphery (Figure 6-1A, arrowhead). PDBu treatment significantly reduced the number of stress fibers and induced dramatic rearrangement of F-actin structures. The newly formed structures can be characterized as actin-rich small dots (0.5 µm) (Figure 6-1B), rosette rings (Figure 6-1C-c1), and belts in the cytoplasm (Figure 6-1C-c2), and membrane ruffles at the edge of peripheral membrane (Figure 6-1C-c3). The number of cells with cytoskeletal structure changes and the types of newly formed structures are PDBu-dose dependent after 30 min of treatment (Figure 6-1D to 6-1H). We then challenged cells with 500 nM of PDBu for different time periods. These newly formed cytoskeletal structures appeared within 10 min (Figure 6-1I). The numbers of small dots (Figure 6-1J) and rings (Figure 6-1K) remained at the constant levels after 10 min, whereas the numbers of belts (Figure 6-1L) and ruffles (Figure 6-1M) increased gradually. We also treated BEAS2B cells with 500 nM PDBu for 1, 2, 4, 6, 8, 12, or 24 h. Reorganized cytoskeletal structures were observed at all these time points (data not shown), which may be due to the continuous exposure of cells to PDBu.
Figure 6-1. PDBu induces reorganization of cytoskeletal structure in BEAS2B cells.

Normal human lung bronchial epithelial BEAS2B cells were treated with different doses of PDBu for 30 min, or with 500 nM of PDBu for different time periods. F-actin was stained with Oregon Green 488 Phalloidin (Green) and nuclei were counterstained with Hoechst dye 33342 (blue). (A) Long stress fibers and cortical actin fibers were seen in control cells, indicated by an arrow and an arrowhead respectively. PDBu induced reorganization of cytoskeletal structures. The numbers of stress fibers was reduced, and F-actin staining shows newly formed structures: (B) small dots; (C-c1) ring, (C-c2) belt and (C-c3) membrane ruffles. Typical structures were enlarged. Dots, rings and belts are indicated with square boxes and membrane ruffles are shown with circles. Cytoskeletal rearrangement and new structures are quantified after 30 min of treatment with varying concentrations of PDBu, or after different time periods with 500 nM of PDBu. (D) and (I): percentage of cells with dramatic cytoskeletal rearrangement; (E) and (J): small dots; (F) and (K): rings; (G) and (L): belts; and (H) and (M): membrane ruffles.
6.3.2 Identification of ventral podosomes and circular dorsal ruffles

The transient and dynamic nature of these small dots, ring and belt structures indicate that they could be podosomes or podosome-like structures. On the other hand, the ruffles appeared as so called circular dorsal ruffles (or waves) (141). By definition, podosomes are actin-rich columns that arise from the ventral surface (147). We performed immunofluorescent staining for cortactin (an important component of podosomes) and F-actin. In untreated BEAS2B cells, cortactin was evenly distributed in the cytoplasm (Figure 6-2A). After PDBu stimulation, cortactin was redistributed to newly-formed small dots in the cytoplasm and ruffles at the edges of cytoplasm membrane (Figure 6-2A). As shown in a series of digital images of Z-stack scanning confocal microscopy (Figure 6-2B), the small dots in the cytoplasmic region are focal inward invaginations of the ventral cell surface into the cytoplasm. Negative controls for immunofluorescent staining were by using non-specific antibodies mouse IgG1 and rabbit IgG or missing primary antibody (data not shown).

Circular dorsal ruffles/waves are transient actin-based structures formed at the dorsal membrane of cell peripheries (141). To determine the nature of ruffles induced by PDBu in human airway epithelial BEAS2B cells, we performed scanning confocal microscopy along the X-Z axis. The co-localization of cortactin (red) and F-actin (green) at the edge of the peripheral membrane appeared to be swept back along the dorsal side of the cell (arrows in Figure 6-2C). This distinct feature differentiates circular dorsal ruffle (waves) from linear ruffles (141).
Fig 6-2

A

Control

Nucleus | F-actin | Cortactin | Overlay

PDBu

Nucleus | F-actin | Cortactin | Overlay

B

0.0 µm | 0.3 µm | 0.6 µm | 0.9 µm

Bottom

1.2 µm | 1.5 µm | 1.8 µm | 2.1 µm

Nucleus | F-actin | Cortactin

2.4 µm | 2.8 µm | 3.1 µm | 3.4 µm

Top
Figure 6-2. Characterization of ventral podosomes and circular dorsal ruffles in BEAS2B cells.

BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin stained with Oregon Green 488 Phalloidin (Green), cortactin was stained with Alexa Fluor 594 labeled secondary antibody (Red), and nucleus was stained with Hoechst dye 33342 (Blue). (A) PDBu-induced formation of podosomes (small boxes) and membrane ruffles (circles). (B) A series of digital images from Z-stack scanning confocal microscopy demonstrates that small dots are focal inward invaginations at the ventral surface into the cytoplasm. (C) Z-stack scanning confocal microscopy demonstrates that PDBu induced ruffles/waves are focal outward protrusions of the dorsal surface membrane (arrows).
6.3.3 Formation of podosomes in primary normal human bronchial epithelial cells

To further determine the physiological relevance of podosome formation, primary normal human bronchial epithelial cells were stimulated with 500 nM PDBu. These cells showed long and thin stress fibers in the cytoplasm (Figure 6-3A). After PDBu treatment, stress fibers disappeared, and numerous podosomes featured as small dots were found in the cytoplasm, and circular waves/membrane ruffles were found at the periphery of the cells (Figure 6-3A). The podosome structures in primary normal human bronchial epithelial cells were confirmed with Z-stack scanning confocal microscopy. Redistribution of cortactin from the cytoplasm of control cells to the small dots was noted after PDBu stimulation (Figure 6-3B). These dots were clustered at the ventral surface of the cell and co-localized with cortactin as focal inward protrusions (Figure 6-3C square box). The membrane ruffles were identified as circular dorsal ruffles/waves by Z-stack scanning technique as well (Figure 6-3C cricle). We also examined effects of PBDu on A549 cells, a cell line derived from human lung cancer. PDBu also induced podosomes in these cells. However, the doses of PDBu required were much lower (Figure 6-4).
A. No Treatment vs. PDBu

B. F-actin, Cortactin, and Overlay images for Control and PDBu conditions.

C. Depth sections of F-actin, Cortactin, and Nucleus at different depths (0.0 µm, 0.3 µm, 0.6 µm, 0.9 µm, 1.2 µm, 1.5 µm, 1.8 µm, 2.1 µm, 2.4 µm, 2.8 µm, 3.1 µm, 3.4 µm).
Figure 6-3. Characterization of ventral podosomes and circular dorsal ruffles in primary normal human bronchial epithelial cells.

(A) PDBu induced podosome formation and circular dorsal ruffles. Normal human bronchial epithelial cells were treated with or without PDBu 500 nM for 30 min, and F-actin was stained with Rhodamine Phalloidin (Red). Podosomes are indicated within a box and membrane ruffles with a circle. (B) PDBu-induced podosomes (boxes) and membrane ruffles (circles) are co-localized with cortactin. F-actin was stained with Oregon Green 488 Phalloidin (Green), cortactin was stained with Alexa Fluor 594 labeled secondary antibody (Red), and nucleus was stained with Hoechst dye 33342 (Blue). (C) A series of Z-stack scanning microscopy images shows that PDBu induced podosomes are localized at the ventral surface.
Figure 6-4. PDBu induces reorganization of cytoskeletal structure in A549 cells.

(A) A549 cells were treated with different doses of PDBu for 30 min. F-actin was stained with Rhodamine Phalloidin (Red) and nuclei were counter stained with Hoechst dye 33342 (blue). Representative confocal microscopic images are used to show newly formed cytoskeletal structures. Typical structures were enlarged in the right column. Podosomes are indicated with box and membrane ruffles with circle. (B) Quantitative analysis of podosome formation after cells was treated with varying concentrations of PDBu for 30 min.
6.3.4 Accumulation of actin-associate proteins and regulators in podosome-like structures

We then used BEAS2B cells to further identify protein components of PDBu-induced podosomes and circular dorsal ruffles. Immunofluorescent staining was performed using antibodies targeting a number of proteins known to be localized in these structures. Four groups of proteins were studied: (i) actin and actin-associated proteins (e.g., F-actin, cortactin, α-actinin), and regulators (e.g., Arp2/3, WASP, N-WASP); (ii) integrin and integrin-associated proteins (e.g., β1-integrin, vinculin, paxilin, talin); (iii) protein kinases (e.g., Src and FAK), which regulate the actin cytoskeleton and membrane remodeling; and (iv) matrix metaloproteinases (MMPs), which mediate ECM degradation.

In addition to cortactin (Figure 6-2 and Figure 6-3), we also studied α-actinin, which was localized mainly in the cytoplasm and under surface membrane in untreated cells (Figure 6-5A). Upon PDBu stimulation, it was translocated to membrane ruffles, dots, and rings (Figure 6-5A).

Actin regulators such as WASP, N-WASP and Arp2/3 (391) govern actin polymerization and actin network attachment to the moving membranes at the leading edge (392). In BEAS2B cells, Arp3 and WASP are mainly in the cytoplasm and weakly stained along actin filaments or with cortical filaments (Figure 6-5B and 6-5C), whereas N-WASP was found only in the cytoplasm and cell periphery (Figure 6-5D). Upon PDBu stimulation, Arp3 was translocated to both small dots and ruffles (Figure 6-5B); whereas WASP and N-WASP were mainly translocated to membrane ruffles with less distribution in small dots (Figure 6-5C and 6-5D).
Figure 6-5. Localization of actin associated proteins in podosomes and circular dorsal ruffles.

BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green) and nucleus was stained with Hoechst dye 33342 (Blue). α-actinin, Arp3, WASP and N-WASP were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Localization of podosomes was shown with boxes, and membrane ruffles with circles. (A): α-actinin; (B): Arp3; (C): WASP and (D): N-WASP.
6.3.5 Recruitment of integrin and associated proteins in podosome-like structures

The integrin β1 subunit was distributed along the plasma membrane and also in the cytoplasm weakly along the stress fibers in untreated cells. Upon PDBu treatment, β1 integrin was translocated to the membrane ruffles and small dots (Figure 6-6A). In control cells, vinculin (Figure 6-6B) and paxillin (Figure 6-6C) were mainly in the cytoplasm and were enriched in prominent focal adhesions, at the ends of stress fibers. Upon PDBu-stimulation the punctate structure of focal adhesions disappeared, and vinculin was distributed mainly in the cytoplasm but not in podosome-like structures (Figure 6-6B). Paxillin was also mainly in the cytoplasmic region, however, increased staining was also seen at the newly formed ruffles (Figure 6-6C). In control cells, talin was found at the cortical filaments and also at focal adhesion sites; PDBu treatment led to the translocation of talin to the membrane ruffles (Figure 6-6D). The different distribution and translocation of these three integrin-associated proteins suggest that they may play different role in cytoskeletal organization in human airway epithelial cells.
Figure 6-6. PDBu-induced redistribution of integrin and integrin associated proteins.

BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green) and nucleus was stained with Hoechst dye 33342 (Blue). Integrin β1 subunit, vinculin, paxilin and talin were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Localization of podosomes was shown with boxes, and membrane ruffles with circles. (A): Integrin β1 subunit; (B): vinculin; (C): paxilin and (D): talin.
6.3.6 Lack of vinculin in PDBu-induced small dots in normal human lung epithelial cells

Vinculin is known to be an important component of podosome-like structures in many cell types (272, 393). To determine the relationship between vinculin and actin-rich small dots, BEAS2B cells, primary normal human bronchial epithelial cells (NHBE), and human lung cancer A549 cells were treated with PDBu (500 nM for 30 min), followed by immunofluorescent staining. We also stained v-Src transformed NIH3T3 cells for comparison because vinculin has been found in so-called “rosettes” or ring-like structures in these cells (393). The F-actin staining was switched from Oregon Green 488 Phalloidin (green) to Rhodamine-labeled Phalloidin (red). Vinculin was revealed with FITC-labeled secondary antibody. Our results show that vinculin was clearly found in ring-like structures in v-Src transformed NIH3T3 cells (Figure 6-7A), indicating that the antibody and staining conditions we used were comparable to that of others. In PDBu treated A549 cells, vinculin staining was found in many actin-rich dots (Figure 6-7B). The vinculin staining was also found in ring-like structures, surrounded by actin-rich rings (Figure 6-7B). In primary NHBE cells, vinculin remained at the focal adhesion like sites, and was not localized to the actin-rich dots (Figure 6-7C). In BEAS2B cells, again, vinculin was found in belts and ruffles after PDBu stimulation, but not in small dots (Figure 6-7D).
Figure 6-7. Lack of translocation of vinculin to actin-rich dots in normal human airway epithelial cells after PDBu stimulation.

BEAS2B cells, primary NHBE cells, and human lung cancer A549 cells were treated with or without PDBu 500 nM for 30 min. Untreated v-Src transformed NIH3T3 cells were used as staining control. F-actin was stained with Rhodamine Phalloidin (Red) and nucleus was stained with Hoechst dye 33342 (Blue). Vinculin was revealed with FITC labeled secondary antibody (Green). Localization of podosomes was shown with boxes, and membrane ruffles with circles. (A): v-Src Transformed NIH3T3 cells; (B): A549 cells; (C): primary NHBE cells, and (D): BEAS2B cells.
6.3.7 Increased protein tyrosine kinases in podosome-like structures

Increased protein tyrosine phosphorylation is commonly observed in podosomes and circular dorsal ruffles. In control BEAS2B cells, phosphotyrosine staining was localized predominately along the cell periphery and in the cytoplasm at structures that appear to be the focal adhesion sites (Figure 6-8A). PDBu stimulation enhanced phosphotyrosine staining at the ruffles and small dots (Figure 6-8A). Phosphorylation of tyrosine-416 of c-Src is known to be a sign of its activation (382, 384, 394, 395). PDBu treatment resulted in activation (increased staining) and translocation of c-Src into actin-rich structures at the cell periphery and in small dots (Figure 6-8B). In untreated cells, FAK was in cytoplasm and weakly along stress fibers (Figure 6-8C). Upon PDBu treatment, FAK was translocated to membrane ruffles and to a few small dots as well (Figure 6-8C).

6.3.8 Distribution of MMPs in podosome-like structures

We further examined the distribution patterns of MMPs. In untreated cells, MT1-MMP was cytosolic, localized predominantly around the nucleus (Figure 6-9A); MMP-2 and MMP-9 were found both in the cytoplasm and along the cell periphery (Figure 6-9B and 6-9C). After PDBu treatment, a portion of MT1-MMP remained in the peri-nuclear region, while enhanced staining was found along the membrane ruffles and in some small dots (Figure 6-9A). MMP-2 and MMP-9 staining was increased around the nucleus after PDBu stimulation (Figure 6-9B and 6-9C). MMP-2 was found at the membrane ruffles and small dots (Figure 6-9B) whereas MMP-9 was only found along membrane ruffles (Figure 6-9C).
Figure 6-8. Protein tyrosine kinases were recruited to PDBu-induced podosomes and circular dorsal ruffles.

BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green) and nucleus was stained with Hoechst dye 33342 (Blue). Protein tyrosine phosphorylation, phospho-SrcY416 and focal adhesion kinase (FAK) were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Localization of podosomes was shown with boxes, and membrane ruffles with circles. (A): Tyrosine phosphorylated proteins; (B): phospho-SrcY416; (C): FAK.
Figure 6-9. Matrix metalloproteases are associated with podosomes and circular dorsal ruffles.

BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green) and nucleus was stained with Hoechst dye 33342 (Blue). MT1-MMP, MMP-2 and MMP-9 were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). (A): MT1-MMP; (B): MMP-2; and (C): MMP-9. Localization of podosomes was shown with boxes, and membrane ruffles with circles. MMP-9 was co-localized with belt-like actin structures (indicated with arrows).
6.3.9 PDBu-induced podosome-like structures can degrade extracellular matrix

MMP-9 is crucial for human airway epithelial migration (87). MT1-MMP is necessary for distal airway epithelial repair after injury (396). To determine if proteolytic activity is associated with the PDBu-induced podosomes in epithelial cells, in situ zymography were performed. BEAS2B cells were plated on a coverslip pre-coated with a layer of TRITC-conjugated (a red fluorescent dye) fibronectin matrix and a base of gelatin and sucrose gel. ECM substrate degradation was visualized as black areas devoid of red fluorescence. As shown in Figure 6-10A, almost no matrix degradation was found under the control condition. In PDBu-treated cells, the dark holes formed by degradation of the fibronectin-TRITC matrix were generally found in the cytoplasm area with the sizes similar to small dots. We also transfected NIH 3T3 cells with constitutively activated SrcY527F as a technical control to ensure the assay works well, which showed stronger degradation of ECM as small dots (Figure 6-10A lower panel). We also performed in situ zymography with primary normal human bronchial epithelial cells using a similar method; digestion of the matrix was also observed (data not shown). Since the in situ zymography was performed 4 h after PDBu stimulation, these dark holes represent the consequence of ECM degradation. However, due to cell migration, this degradation did not overlap well with actin-rich dots.
Figure 6-10. BEAS2B cells with podosomes can degrade matrix and are invasive.

(A) Digestion of fibronectin-gelatin-sucrose matrix by PDBu-induced podosomes. BEAS2B cells were seeded on coverslips coated with TRITC conjugated fibronectin-gelatin-sucrose matrix. After treatment with 500 nM PDBu for 4 h, degradation of ECM was shown as dark dots and F-actin was stained to show cytoskeletal structures. NIH 3T3 cells co-transfected with β-actin-GFP and constitutively activated chicken SrcY527F constructs were included as a technical control. These experiments performed by Rob Eves (Queen’s University) and me. (B) BEAS2B cells with podosomes are invasive. BEAS2B cells were seeded into a Transwell insert with 8 µm pores coated with fibronectin gel. After 500 nM PDBu treatment, cells remaining in the Transwell insert were fixed and stained with Oregon Green 488 Phalloidin (Green) for F-actin. The cells crossing the insert were visualized by nucleus Hoechst dye 33342 staining (blue). (C) Results were quantified for the invasiveness of BEAS2B cells. *: P<0.05 compared with control group.
To explore the migration properties of BEAS2B cells bearing podosomes, we performed a Transwell fibronectin gel invasion assay. After overnight incubation, Z-stack scanning confocal microscopy was applied to visualize cells inside the fibronectin gel from the top of the Transwell. BEAS2B cells with podosomes degraded fibronectin gel, were able to invade through the gel to the bottom side of the well (Figure 6-10B). The number of cells crossed the Transwell was quantified by counting the stained nuclei (Figure 6-10C). The results indicated that PDBu treatment increased the invasiveness of normal human bronchial epithelial cells.

6.4 Discussion

The respiratory epithelium is known to undergo continuous damage and repair. Rapid re-epithelialization by epithelial cell de-differentiation and migration are important features of epithelial repair in vivo (75). Repair generally involves several steps, including migration and spreading of epithelial cells at the margin of the injury into the damaged region, invading of epithelial cells from the underneath layers to the surface of the epithelium, and proliferation of epithelial cells (7). In the present study, we identified podosome and circular ruffle structures in primary normal human bronchial epithelial cells and in BEAS2B cells stimulated with the phorbol ester, PDBu. In most of our experiments, 500 nM of PDBu was used, which is lower than doses typically used in podosome studies with other cell types. As shown in Figure 6-1, podosome-like structures were found in lower doses of PDBu treated cells. These structures were revealed by Z-stack scanning confocal microscopy with multiple molecular markers. Importantly, we demonstrated digestion of ECM by podosomes and the invasive properties of cells treated with PDBu.
Although podosomes in different cell types showed similar functions in promoting matrix degradation and invasion, their morphologies, numbers and sizes appear to be diverse. For example, podosomes in primary human macrophages look like hundreds of small tiny actin-rich dots (0.2-0.5 µm) distributed at the ventral surface underneath the cytoplasm (143-145). In smooth muscle cells, podosomes appear as small actin-rich dots (0.5-1 µm) which often aggregate to form bands (2 µm) along the cortex of the cell (146). In bovine aorta endothelial cells, podosomes form a single ring (15 µm) beside the nucleus (147). In v-Src transformed NIH 3T3 fibroblasts, podosomes are numerous rosette-like structures (2 µm) (148). In melanoma cells, podosomes are fewer in number, and can be seen as small dots (approximately 1 µm) in the cytoplasm (149). In rat bladder carcinoma 804G cells, podosome-like structures were identified around hemidesmosomes that make connections with intermediate filaments, as small dots surrounded by rings (150, 151). The features of PDBu-induced podosomes in human airway epithelial cells are seen as numerous small dots in the cytoplasm, with occasional rosette rings and belts. Linder described podosomes as dot-like structures attached to substrate, containing actin regulators and plaque proteins, with the number of around 20-100 per cell, with the maximum size of 1 µm X 0.4 µm (272). The sizes of the small dots in BEAS2B cells and normal human bronchial epithelial cells are within this range.

The molecular component of rosettes of podosomes contain several groups of proteins identified in aorta endothelial cells (147). In the present study, we identified some of these proteins in podosome-like structures and some in circular dorsal ruffles. Spinardi et al. showed the presence of vinculin in the small rings around the small dots in rat bladder carcinoma 804G cells that were stably transfected with human β4 integrin subunit (150, 151). However, in the present study, we did not find translocation of vinculin to small dots in BEAS2B cells, nor in
primary NHBE cells. In an early study, Dwyer-Nield et al. (378) reported PMA-induced cytoskeletal reorganization in mouse lung epithelial E10 cells. They found vinculin dissociated from the focal adhesion plaques and distributed throughout the cytoplasm (378). Our observations were similar to what reported in that study. Interestingly, we found vinculin in actin-rich dots in human lung cancer A549 cells, as well as in rosettes in v-Src transformed NIH3T3 cells, which are tumorigenic. Thus, our results implicated that the composition and function of podosome-like structures formed by normal epithelial cells may be different from that of cancer and tumor cells. We could use vinvulin antibody to do immunoprecipitation in these four cell types and identify the special proteins and kinases involved in vinculin recruitment to podosomes. An alternative view is that these cells may form invadopodia rather than podosomes. There is much discussion as to whether these two structures are related, which is beyond the scope of this study. It has been suggested that the short-lived podosomes can probe the substrate for sites of attachment before maturing into invadopodia (159, 274, 279). Gatesman A et al. demonstrated that actin filament-associated protein (AFAP) is required to mediate PKCα activation of the nonreceptor tyrosine kinase c-Src and the subsequent formation of podosomes in fibroblast cells upon PMA stimulation (377). Recently, Dorfleutner A et al. reported that the phosphorylation and/or dephosphorylation of AFAP is involved in the regulation of podosome stability and lifespan in A7r5 smooth muscle cells (171). In a separate study, we over-expressed GFP tagged AFAP in BEAS2B cells and performed live cell imaging after PDBu treatment. Small dots appeared within 2 min, with fast turnover, from 10 sec to 5 min (data not shown). Therefore, we believe that the small dots in the cytoplasm of airway epithelial cells are likely to be podosomes than invadopodia.
We observed translocation of the three MMPs to podosome-like structures in BEAS2B cells after PDBu stimulation. We found MT1-MMP and MMP-2 in actin-rich dots and MMP-9 in belt-like structures. All three MMPs were found in membrane ruffles upon PDBu stimulation in BEAS2B cells. It has been proposed that the protease profiles in lamellipodia and in invadopodia (podosomes) are similar, but the proteolytic activities of these structures are different, determined by the balancing between proteases and their inhibitors (397). Tissue inhibitor of matrix metalloproteinase-2, for example, has been found to regulate the ECM-degrading activities of MT1-MMP and MMP-2 (397). The distribution and interaction between TIMPs and MMPs should be further studied.

Podosomes and membrane ruffles in human airway epithelial cells comprise MMPs to endow proteolytic properties. Interestingly, although PDBu induced formation of both podosomes and circular dorsal ruffles, the digestion of matrix was only seen as small dots. We further demonstrated the invasiveness of PDBu-treated cells using the Transwell system. PDBu-induced formation of podosomes may play a critical role in normal epithelial cell invasion and migration during airway repair. PKC activation could be an underlying mechanism of these cellular processes.

Circular dorsal ruffles/waves, also called actin ribbons, are another transient cytoskeletal structure that form in response to external stimuli. PMA-induced morphological changes of keratinocytes have been reported as far as two decades ago (398), which have since been described as circular dorsal ruffles (141). These ruffles were located at the edges of cells as distortions of plasma membrane, very similar to what we reported in the present study. We used Z-stack scanning confocal microscopy to demonstrate that these structures are indeed swept back along the dorsal side of the cell. Moreover, in our time course study, the ruffles appeared
gradually after PDBu stimuli, which is in contrast to the rapid formation of peripheral ruffles seen in lamellipodia (141). The molecular components of circular dorsal ruffles are similar to podosomes in BEAS2B cells. The function of these circular dorsal ruffles is yet unknown. It has been proposed that they might promote degradation of ECM during three-dimensional migration (141).

Alteration of cytoskeletal structures can be induced by growth factors, cytokines and many other biological mediators. For example, we have found dramatic depolymerization of F-actin in rat lung epithelial cells after LPS stimulation (399-402), which are essential for TNF-α (399) and macrophage inflammatory protein-2 (402) expression in these cells. Whether these stimuli can induce formation of podosomes and/or circular dorsal ruffles merits further investigation.

In summary, PDBu as a PKC activator induced formation of podosomes and circular dorsal ruffles in primary normal human bronchial epithelial cells and in BEAS2B cells. The molecular components of these structures are similar but only podosomes showed evidence of being able to digest matrix. These transient cytoskeletal structures may play important roles in mediating airway epithelial cell migration and invasion, which could be important for tissue repair, airway branching and lung development. Dysfunction of this system may contribute to acute lung injury and chronic obstructive pulmonary disease, as well as other disorders of the airway and the lung.
7. Chapter Seven. PKCζ regulates recruitment of MMP-9 to podosomes, and its release and activation

The content of this chapter was prepared as an original research article in *Molecular Biology of the Cell*:

Chapter Seven. PKCζ regulates recruitment of MMP-9 to podosomes, and its release and activation

7.1 Summary

Podosomes are transient cell surface structures essential for degradation of extracellular matrix during cell migration and invasion. Protein kinase C (PKC) is involved in the regulation of podosome formation; however the roles of PKC isoforms in podosome formation and function are largely unknown. In Chapter 6, we reported that phorbol-12, 13-dibutyrate (PDBu), a PKC activator, induced the formation of podosome-like structures in normal human bronchial epithelial cells. Here, we demonstrate that PDBu-induced podosome formation is mainly mediated through redistribution of conventional PKCs, especially PKCα, from the cytosol to the podosomes. Interestingly, although blocking atypical PKC (PKCζ) did not affect PDBu-induced podosome formation, it significantly reduced gelatin matrix degradation at podosomes. Inhibition of PKCζ reduced PDBu-induced gelatin matrix degradation at podosomes by reducing recruitment of MMP-9 to podosomes, and its release and activation. Down-regulation of MMP-9 by siRNA and neutralization antibody also significantly reduced gelatin matrix degradation. PDBu-induced recruitment of PKCζ and MMP-9 to podosomes was blocked by inhibition of novel PKC with Rottlerin or PKCδ siRNA. Our data suggest that while the conventional PKCα controls podosome formation and dynamics, however, it is the atypical PKCζ that plays a dominant role in the recruitment of MMP-9 to podosomes for gelatin matrix degradation.
7.2 Introduction

Podosomes, first described by Marchisio and colleagues (403), are short protrusions of the ventral cell surface in direct contact with the substrate matrix. Recently, it has been discovered that these cellular structures are responsible for cell invasion by degrading the extracellular matrix (ECM) barriers (272, 369). These structures are composed of an F-actin core, surrounded by a ring structure that is consisted of actin associated proteins, signal transduction proteins, and matrix metalloproteases (MMPs), which include membrane-bounded MMPs (such as MMP-14) and secreted MMPs (such as MMP-2 and MMP-9) (272). Several exogenous signals can trigger the formation of podosomes, including phorbol ester, bombesin, bradykinin, cytokines, growth factors, and growth hormones (404). These agonists act by activating tyrosine and serine/threonine kinases, among which protein kinase C (PKC) appears to be a key mediator of podosome formation (376, 405). PKC may lead to the activation of non-receptor tyrosine kinase, c-Src, which in turn facilitates podosome formation (377).

PKC is a family consisting of 11 isozymes, which are classified into three subfamilies, conventional/classical, novel, and atypical PKCs, based on their second messenger requirements (375). The conventional PKCs (cPKCs) are α, βI, βII, and γ, which require Ca\(^{2+}\), diacylglycerol (DAG), and phospholipid for activation (375). The novel PKCs (nPKCs) include δ, ε, η, and θ, which require DAG but not Ca\(^{2+}\) for activation (406). On the other hand, atypical PKCs (aPKCs), including ζ and η, require neither Ca\(^{2+}\) nor DAG for activation (375, 407). Due to their structural similarity to DAG, phorbol esters, e.g. phorbol 12-myristate 13-acetate (PMA), phorbol-12,13-dibutyrate (PDBu), are often employed to elicit PKC related signals (375, 407). PKC\(_{\mu}\), also called protein kinase D1, is another serine/threonine kinase, which has two C1 domains and a pleckstrin homology domain but no Ca\(^{2+}\) binding domain (292). It shares similar
functions with PKCs. Each PKC isozyme may have distinct roles in various cellular processes (408).

PKC activation has been shown to remodel actin stress fibers into F-actin-enriched podosome columns in cultured cells (213). Short-term exposure to PMA or PDBu induced appearance of podosomes and rosettes in endothelial cells (376), vascular smooth muscle cells (405, 409), and osteoclasts (410). Actin filament-associated protein AFAP-110 was required to mediate PKC\(\alpha\) related activation of c-Src and subsequent formation of podosomes (377). PKCs have been implicated in podosome formation in human umbilical vein endothelial cells (376), and vascular smooth muscle cells (411). However, the contribution of different PKC isozymes in these cellular processes is largely unknown.

In Chapter 6, we reported that PDBu-induced formation of podosome-like structures in normal human bronchial epithelial cells. These structures are enriched in MMPs and endowed with proteolytic activity to degrade fibronectin-gelatin-sucrose matrix (122). In the present study, we asked whether distinct PKC isoenzymes are involved in podosome formation, and recruitment of MMPs for ECM digestion in PDBu-stimulated human bronchial epithelial cells. We found that cPKCs, especially PKC\(\alpha\), is crucial for the formation of the podosomes, PKC\(\zeta\) plays an important role to control the recruitment, release, and activation of MMP-9 at podosomes, and PKC\(\delta\) controls the recruitment of PKC\(\zeta\) to podosomes.

7.3 Results

7.3.1 PDBu-induced PKC phosphorylation and translocation to podosomes and circular ruffles
Recently we have reported that phorbol ester (PDBu) could dramatically change cell morphology and cytoskeletal structures in a dose- and time-dependent manner in primary normal human bronchial epithelial cells and in human bronchial epithelial BEAS2B cells. Ventral podosome-like structures and dorsal circular dorsal ruffles were identified with Z-stack scanning confocal microscopy, together with immunostaining of a number of known marker proteins (122). To determine which PKC isozymes are activated upon PDBu stimulation, we examined their phosphorylation status after challenging BEAS2B cells with 500 nM PDBu from 0 to 240 min. The phosphorylation status of cPKCs was determined by western blotting with two antibodies, one targets the phosphorylation sites of PKCα at T638 and PKCβII at T641 (Figure 7-1Aa1), and another pan-PKC antibody that targets amino acid sequence similar to the PKCβII S660 region (Figure 7-1Aa2). PDBu treatment did not induce significant alteration of phosphorylation levels of cPKCs. On the other hand, after PDBu treatment, phosphorylation of PKCδ T505 significantly increased after 1 min, peaked at 20-30 min and declined gradually (Figure 7-1Aa4). The phosphorylation of PKCδ S643/676 increased gradually within the first 10-20 min followed by a sharp increase at 30 min and plateaued for an hour, and then decreased (Figure 7-1Aa5). The phosphorylation of PKC0 T538 also increased upon PDBu stimulation within the first 10 min (Figure 7-1Aa6). PDBu also induced phosphorylation of aPKC within 1 min as detected by an antibody for PKCζ/λ T410/403 (Figure 7-1Aa8). The phosphorylation of PKCμ S744/748 (Figure 7-1Aa10), and PKCμ S916 (Figure 7-1Aa11) dramatically increased within 5 min of PDBu treatment. Total protein levels of PKCα, PKCδ, PKCζ, PKCμ and GAPDH remained unchanged during the eprimental period (Figure 7-1). These results suggest a rapid activation of nPKC, aPKC, and PKCμ induced by PDBu in BEAS2B cells.
### Figure 7-1. PDBu-induced PKC phosphorylation.

(A) PDBu-induced PKC phosphorylation. BEAS2B cells were treated with PDBu 500 nM for 0 min to 240 min. The total or phosphorylation of PKC isoforms was revealed by designated antibodies.
We then examined PDBu-induced distribution of PKC isoforms and phosphorylated species. Under control conditions, PKCα, δ, and ζ, representative of cPKC, nPKC, and aPKC subfamilies, respectively, as well as PKCμ were localized in the cytoplasm and perinuclear region, but none co-localized with prominent actin stress fibers. PDBu treatment induced disassembly of actin stress fibers and translocation of PKC isoforms and their phosphorylated forms to small dots, rings, and circular ruffles (Figure 7-2A and 7-2B). The colocalization coefficient of F-actin staining with PKC isoforms or their phosphorylated forms was measured with multiple cells and expressed as Pearson’s correlation coefficient values in Figure 7-2C and 7-2D. We also did immunofluorescent staining for PKCα, phosphorylated PKCα/βII T638/641, and phosphorylated PKC pan βII S660 in primary normal human bronchial epithelial cells with similar results (data not shown). Therefore, although the phosphorylation status of cPKC isoforms did not change drastically, they were clustered at the sites of podosomes and circular ruffles in human bronchial epithelial cells upon PDBu stimulation.
Figure 7-2. PDBu-induced PKC translocation to podosomes.

PDBu induced translocation of PKC isoforms (A) and their phosphorylated forms (B) to podosomes. Cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green). PKC isoforms and their phosphorylated forms were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Co-localization of PKC isoform with F-actin in small dots and ring structures was shown with small boxes and enlarged in the underneath panel. Circular ruffles were indicated with arrowheads. The overlapping of F-actin staining with PKC isoforms (C) and their phosphorylated forms (D) were quantified and expressed as Pearson's correlation coefficients. *: P<0.05 compared with “no treatment” group.
7.3.2 PDBu-induced podosome assembly depends on cPKCs

To determine the proteolytic activity of PDBu-induced podosomes, we examined gelatin matrix degradation by *in situ* zymography. After 60 min of stimulation with PDBu, enriched podosome like small dots were revealed with F-actin staining (red) in the cytoplasm, which were co-localized with or surrounded by digested area (shown as black holes) on gelatin pre-coated matrix (green). The depth of the podosomes and digested matrix can be seen through X-Z and Y-Z sections (Figure 7-3A).

To determine roles of PKC isoenzymes in PDBu-induced podosome formation and gelatin matrix degradation, cells were pre-treated with chemical inhibitors for 1 h and then challenged with PDBu (500 nM for 8 h). As shown in Figure 7-3B, pretreatment of cells with BIM I and Ro-31-8220, which are blockers of both cPKCs and nPKCs (412, 413), effectively inhibited PDBu-induced disassembly of stress fibers and formation of podosomes. Similarly, Gö6976, a specific pharmacological inhibitor for Ca²⁺-dependent PKCα and PKCβI (413), and BAPTA/AM, a membrane-permeable Ca²⁺ chelator effectively reduced PDBu-induced podosomes. In contrast, Rottlerin, a cell-permeable PKC inhibitor that exhibits greater specificity for PKCδ and PKCθ (414), had little effect (Figure 7-3B). Quantification of podosome number per cell and the percentage of cells with podosomes confirmed the inhibitory effects of these inhibitors (Figure 7-3C and 7-3D).
Figure 7-3. PDBu-induced podosome formation is mainly mediated through classical PKC, but matrix degradation can be blocked by multiple PKC inhibitors.

(A) PDBu stimulation (500 nM, 60 min) induced podosomes (actin rich small dots, Red, indicated by arrows) were closely surrounded by degraded (Black) gelatin matrix (Green). (B) BIM I, Gö6976, but not Rottlerin, prevented PDBu-induced podosome formation; however, all three inhibitors reduced matrix degradation. Cells cultured on gelatin matrix (Green) were pre-incubated with each inhibitor 1 µM for 1 h, and then stimulated with PDBu (500 nM for 8 h). F-actin was stained with Rhodamine Phalloidin (Red). Digestion of gelatin matrix was revealed as black dots. (C) Quantitative analysis of podosome number per cell. (D) Quantitative analysis of number of cells with podosomes. (E) Quantitative analysis of matrix degradation. *: p < 0.05 in comparison with PDBu-treated control and DMSO (vehicle control) groups.
In control and DMSO (vehicle only) pre-treated groups, cells aggressively degraded the gelatin matrix upon PDBu treatment (Figure 7-3B). Surprisingly, all the inhibitors tested significantly prevented PDBu-induced matrix degradation compared to control and DMSO groups (Figure 7-3B). Quantification of cells with gelatin degradation is shown in Figure 7-3E. This observation is intriguing because it suggests that podosome formation is controlled by cPKCs, but ECM degradation by the formed podosomes can be further regulated by nPKCs and perhaps other PKC isoymes.

7.3.3 PDBu-induced matrix degradation can be blocked by siRNAs of multiple PKC isoforms

Since chemical inhibitors may have non-specific effects (415), we then employed siRNAs to knock down PKC isoforms to determine the roles of selected PKCs in podosome formation and gelatin matrix degradation. As a negative control, a fluorescein conjugated control siRNA that has no known homology to mammalian genes was used. The efficiency of siRNA transfection was >90% as determined by the % of green fluorescent cells (data not shown). PKCα, δ, ζ, and μ were chosen as representative of cPKC, nPKC, aPKC and PKCμ, respectively. siRNAs reduced the expression of the targeted PKC protein without significant effects on other PKCs, as shown by western blotting (Figure 7-4B). Only PKCα siRNA significantly inhibited PDBu-induced podosomes (Figure 7-4A and 7-4C and 7-4D), which supports the specific role of cPKCs in controlling PDBu-induced podosome formation. Interestingly, siRNA-mediated knockdown of PKCα, PKCδ, PKCζ, or PKCμ significantly prevented PDBu-induced gelatin degradation (Figure 7-4A and 7-4E) revealed by in situ zymography.
Figure 7-4. PDBu-induced podosome formation is reduced by PKCα siRNA but matrix degradation can be blocked by multiple PKC siRNA.

BEAS2B cells cultured on gelatin matrix (Green) were transfected with siRNA for 48 h and then stimulated with PDBu (500 nM for 8 h). (A) Representative staining of control, PKCα, and PKCζ siRNA-treated cells. (B) Western blots to show specificity of siRNAs. (C) Quantitative analysis showing that only PKCα siRNA significantly reduced the number of podosomes per cell. (D) Quantitative analysis showing that PKCα siRNA also significantly reduced the number of cells with podosomes. (E) Quantitative analysis showing that siRNA for PKCα, δ, ζ, and μ reduced PDBu-induced matrix degradation. *: p < 0.05 in comparison with PDBu-treated control and control siRNA groups.
These results further suggest that the presence of podosome like structures may not necessarily lead to ECM degradation. The latter could be a process regulated by multiple PKC isoforms.

7.3.4 PKCζ mediates PDBu-induced proteolytic activity of podosomes, MMP-9 recruitment to podosome, and MMP-9 release and activation

We were particularly intrigued by the fact that siRNA targeting PKCζ reduced PDBu-induced gelatin matrix degradation. This atypical PKC does not have lipid-binding region, hence, it must be a down-stream target of cPKC and/or nPKC isozymes. Thus, we investigated the role of PKCζ kinase activity in PDBu-induced podosome formation and ECM degradation.

BEAS2B cells were pre-incubated with myristoylated PKCζ pseudosubstrate PS (which is cell permeable) to inhibit its activity, or the negative control PI (which is also myristoylated and cell permeable), followed by treatment with 500 nM PDBu for 8 h. We confirmed the specific inhibitory effect of PS but not PI on the phosphorylation of PKCζ by western blotting (data not shown). These pre-treatment did not block PDBu-induced podosome formation. However, inhibition of PKCζ kinase activity by its pseudosubstrate (PS) (416) significantly prevented the matrix degradation (Figure 7-5A and 7-5B). The negative control peptide (PI) had no such effect.
Figure 7-5. PKCζ regulates proteolytic activity of PDBu-induced podosomes and MMP-9 translocation to podosomes.

(A) PS, a specific pseudosubstrate for PKCζ (but not PI, the scrambled negative control peptide for PS) prevented the proteolytic activity of PDBu-induced podosomes. Cells cultured on gelatin matrix (Green) were pre-incubated with peptide (1 µM for 1 h), and then stimulated with PDBu (500 nM for 8 h). F-actin was stained with Rhodamine Phalloidin (Red). Digestion of gelatin matrix was revealed as black areas. (B) Quantitative analysis of percentage of cells showing matrix degradation. *: p < 0.05 in comparison with the PDBu-treated control group. (C) PS but not PI blocked MMP-9 translocation to podosomes. Note the MMP-9 (lower panel) was not clustered as actin rich small dots (higher panel). (D) PS but not PI reduced translocation of MMP-9 and MMP-14 to podosomes. *: p < 0.05 in comparison with the PDBu-treated PI control group.
To identify MMP isozymes regulated by PKCζ, whole cell lysates and culture medium were collected for gelatin gel zymography assay. In the whole cell lysates, the expression and activity of MMP-9 were higher than MMP-2, whereas in the cell culture medium, MMP-2 was the major form revealed by the zymography (Figure 7-6A and 7-6B and 7-6C). The level of both pro- and active-MMP-2 had no dramatic changes upon PDBu stimulation, while the level of pro- and active-MMP-9 in culture medium were increased after PDBu challenge in a time-dependent manner (Figure 7-6A). PKCζ pseudosubstrate PS, but not the negative control PI, inhibited PDBu-induced secretion and activation of MMP-9 (Figure 7-6B). Similarly, compared to control siRNA, PKCζ siRNA also inhibited PDBu-induced MMP-9 secretion and activation (Figure 7-6C). Results from repeated experiments are quantified by densitometry (Figure 7-6D and 7-6E).

To investigate whether PKCζ pseudosubstrate affects on MMP gene expression, BEAS2B cells were pre-incubated with PKCζ pseudosubstrate PS or the negative control PI. Total RNA was extracted for qRT-PCR assay after PDBu stimulation (500 nM for 4 h). PDBu stimulation increased MMP-9 mRNA approximately 30-35 folds, increased MMP-14 mRNA around 2-3 folds, but had no effect on MMP-2 gene expression; however, PKCζ pseudosubstrate PS did not inhibit PDBu-induced gene expression of MMP-9 or MMP-14 (Figure 7-6F). PKCζ siRNA also did not inhibit PDBu-induced gene expression of these MMPs (data not shown).

We have previously shown recruitment of MMP-2, MMP-9, and MMP-14 to podosomes after PDBu stimulation (122). To determine the role of PKCζ on these processes, immunostaining together with F-actin staining was performed. The co-localization of MMPs in actin-rich small dots was quantified. PKCζ pseudosubstrate PS but not PI significantly reduced PDBu-induced translocation of MMP-9 and MMP-14 (but not MMP-2) to podosomes (Figure 7-5C and 7-5D).
Figure 7-6. PKCζ regulates MMP-9 release and activation.

(A) PDBu induced MMP-9 secretion and enzymatic activity time-dependently. Cells were challenged with PDBu 500 nM from 0 to 8 h. The culture medium was examined with gelatin gel zymography. (B) PS reduced PDBu-induced MMP-9 secretion and enzymatic activity. Cells were pretreated with PS or PI (1 µM for 1 h) and challenged with PDBu 500 nM for 8 h. The culture medium and whole cell lysates (WCL) were examined with gelatin gel zymography. The MMP-2 and -9 bands were as indicated. (C) PKCζ siRNA reduced PDBu-induced MMP-9 secretion and enzymatic activity. Cells were transfected with control or PKCζ siRNA and challenged with PDBu 500 nM for 8 h. Whole cell lysates and culture medium were examined with gelatin gel zymography. (D) Quantitative analysis of MMP-9 bands. *: p < 0.05 in comparison with the PDBu-treated control and PI groups. (E) Quantitative analysis of MMP-9 bands. *: p < 0.05 in comparison with the PDBu-treated control and control siRNA groups. (F) PS or PI did not affect PDBu-induced MMP gene expression. Cells were pretreated with PS or PI and challenged with PDBu for 4 h. RNA was extracted and qRT-PCR was performed. qRT-PCR was performed by Xiao-Hui Bai.
7.3.5 PDBu-induced MMP-9 is involved in degradation of gelatin matrix

To determine which MMP is responsible for the PDBu-induced proteolytic activity of podosomes, we transfected cells with siRNAs for MMP-2, MMP-9, or MMP-14, and examined the gelatin matrix degradation by in situ zymography. Cells were transfected with siRNA for 48 h and then challenged with 500 nM PDBu for 8 h. Each siRNA reduced the protein level of targeted MMP and had no effects on other two MMPs examined (Figure 7-7B). In MMP-9 or MMP-14 siRNA transfected group, although cells still formed podosomes upon PDBu stimulation, the proteolytic activity was significantly inhibited compared to control siRNA and MMP-2 siRNA groups. In contrast, the inhibitory effect of MMP-2 siRNA on gelatin matrix degradation was not statistically significant (Figure 7-7A and 7-7C). To further confirm the role of secreted MMP-9 in digesting gelatin matrix, we used neutralization antibody for MMP-9. In comparison with mouse IgG as the negative control, MMP-9 antibody pre-incubation significantly reduced the enzymatic activity without affecting podosome formation (Figure 7-7D and 7-7E).
A

Control siRNA | MMP-2 siRNA | MMP-9 siRNA | MMP-14 siRNA

F-actin Overlay

B

Control siRNA | MMP-2 siRNA | MMP-9 siRNA | MMP-14 siRNA

b1 | b2 | b3 | b4

MMP-2 | MMP-9 | MMP-14 | GAPDH

C

% Cells Degrading Matrix

Control siRNA | MMP-2 siRNA | MMP-9 siRNA | MMP-14 siRNA

No Treat | PDBu

D

mlg G | MMP-9 AB

F-actin Overlay

E

% Cells Degrading Matrix

mlgG | MMP-9 AB

No Treat | PDBu
Figure 7-7. Role of MMP-9 and MMP-14 in PDBu-induced proteolytic activity of podosomes.

(A) siRNA for MMP-9 and MMP-14 (but not for MMP-2) prevented matrix degradation of PDBu-induced podosomes. BEAS2B cells cultured on gelatin matrix (Green) were transfected with siRNA for 48 h, and then stimulated with PDBu 500 nM for 8 h. F-actin was stained with Rhodamine Phalloidin (Red). Digestion of gelatin matrix was revealed as black areas. (B) The specificity of siRNAs for MMPs was determined with western blotting. (C) Quantitative analysis of percentage of cells showing matrix degradation. *: p < 0.05 in comparison with the PDBu-treated control siRNA group. (D) Neutralization antibody for MMP-9 prevented PDBu-induced matrix degradation of podosomes. Cells cultured on gelatin matrix (Green) were pre-treated with either MMP-9 neutralization antibody or mouse IgG (10 ng/ml for 8 h) and then stimulated with PDBu 500 nM for 8 h. (E) Quantitative analysis of percentage of cells showing matrix degradation. *: p < 0.05 in comparison with the PDBu-treated mIgG group.
7.3.6 Recruitment of PKCζ to PDBu-induced posodosomes depended on nPKC

To determine how PKCζ was recruited to podosomes in response to PDBu, we expressed PKCζ-GFP in BEAS2B cells and performed live cell imaging. PKCζ-GFP appeared as small dots in the cytoplasm and at the peripheral ruffles after PDBu stimulation, and similar as podosomes these small dots showed fast turnover (Figure 7-9A upper panel). Tks5/Fish has been shown to an important component of podosomes in invasive cancer cells (148). Indeed, we also found Tks5/Fish in PDBu-induced small dots and rings in BEAS2B cells (Figure 7-8A). When PKCζ-GFP and Tks5-mCherry were co-expressed in BEAS2B cells, they were well co-localized in the cytoplasm as small dots and rings (Figure 7-8B). Interestingly, nPKC inhibitor Rottlerin pre-treatment prevented PDBu-induced translocation of PKCζ-GFP to podosomes (Figure 7-8B and 7-9A lower panel). Data are quantified in Figure 7-9D. In PKCδ siRNA (but not in control siRNA) transfected cells, PDBu-induced translocation of PKCζ (Figure 7-9B and 7-9E) or MMP-9 (Figure 7-9C and 7-9F) to actin-rich small dots was significantly reduced. Rottlerin and PKCδ siRNA also prevented PDBu-induced PKCζ phosphorylation (Figure 7-9G and 7-9H). Rottlerin and PKCδ siRNA did not significantly inhibited MMP-9 mRNA expression (Figure 7-9I and 7-9J). These results suggest that nPKC, especially PKCδ, is involved in the recruitment of PKCζ to podosomes, and its subsequent regulatory effects on MMP-9 recruitment and gene expression.
A. F-actin and Tks5 images showing overlap and Pearson's correlation coefficient.

B. PKCζ-GFP, Tks5-mCherry, Overlay, and DIC images under different conditions: Medium + PDBu and Rottlerin + PDBu.

C. Graph showing Pearson's correlation coefficient for Tks5 with color bars indicating significance (*).

D. Graph showing Pearson's correlation coefficient for Tks5-mCherry vs PKCζ-GFP with color bars indicating significance (*) for different conditions.
Figure 7-8. Rottlerin reduced PDBu-induced translocation of PKCζ to podosomes.

(A) PDBu induced translocation of Tks5 to podosomes. BEAS2B cells were treated with or without PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green). Tks5 was stained with specific antibody and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Co-localization of Tks5 with F-actin in small dots was shown with small boxes, which were enlarged as single channel images in lower panel. The 3D structure of podosomes was also shown as Z-stacks of scanning confocal images on the right. (B) Rottlerin, an nPKC inhibitor reduced PDBu-induced colocalization between PKCζ-GFP and Tks5-mCherry (as a marker of podosomes). Cells were co-transfected with vectors expressing PKCζ-GFP and Tks5-mCherry, pre-treated with or without Rottlerin (1 µM for 1 h) and then stimulated with PDBu (500 nM for 30 min). (C) The overlap of F-actin staining with Tks5 was quantified and expressed as Pearson’s correlation coefficients. *: p < 0.05 in comparison with untreated control group. (D) Co-localization of PKCζ-GFP with Tks5-mCherry was quantified and expressed as Pearson’s correlation coefficient values. *: p < 0.05 in comparison with only PDBu-treated group.
**Fig 7-9**

A. Imaging of PKCζ-GFP expression under different treatments.

B. Fluorescence images of F-actin and PKCζ in control and PKCζ siRNA conditions.

C. Fluorescence images of F-actin and MMP-9 in different treatments.

D. Graph showing the Pearson's Coefficient over time for PKCζ-GFP small dots under different conditions.

E. Bar graph comparing the Pearson's Coefficient for PKCζ and MMP-9 under control and PKCζ siRNA conditions.

F. Bar graph showing MMP-9 mRNA expression under No Treat and PDBu conditions.

G. Western blot analysis for PKCζ and MMP-9 under control and siRNA conditions.

H. Western blot analysis for GAPDH under control and siRNA conditions.

I. Bar graph showing MMP-9 mRNA expression under Control, Rottlerin, and PKCζ siRNA conditions.

J. Bar graph showing MMP-9 mRNA expression under Control and PKCζ siRNA conditions.
Figure 7-9. PKCδ controls recruitment of PKCζ and MMP-9 to podosomes.

(A) Rottlerin (1 μM), an nPKC inhibitor, prevented PDBu-induced PKCζ-GFP to form small dots in cytoplasm. BEAS2B cells transfected with pEGFP-PKCζ were subjected to PDBu (500 nM) stimulation. Images from time-lapse confocal microscopy show that PKCζ-GFP is clustered as small dots in cytoplasm and circular ruffles at the peripheral membrane. (B) PKCδ siRNA inhibited PKCζ translocation to PDBu-induced podosomes. Note the PKCζ (right panel) was not clustered at actin rich small dots (left panel). (C) PKCδ siRNA abolished MMP-9 translocation to PDBu-induced podosomes. Note the MMP-9 (right panel) was not clustered at actin rich small dots (left panel). (D) Quantitative analysis of the number of PKCζ-GFP small dots in BEAS2B cells from live cell imaging. Co-localization of F-actin with PKCζ (E) or with MMP-9 (F) was measured and expressed as Pearson’s correlation coefficients. *: p < 0.05 in comparison with the PDBu-treated control siRNA group. (G) Rottlerin prevented PDBu-induced phosphorylation of PKCζ. Cells were pre-incubated with Rottlerin (1, 5, 10 μM) for 1 h before challenged with PDBu 500 nM for 30 min. (H) PKCδ siRNA reduced phosphorylation of PKCζ. BAES2B cells were transfected with control siRNA or PKCδ siRNA for 48 h and then challenged with PDBu 500 nM for 30 min. (I) Rottlerin did not significantly reduced PDBu-induced MMP-9 mRNA expression. Cells were pretreated with Rottlerin 1 μM for 1 h and challenged with 500 nM PDBu for 4 h. (J) PKCδ siRNA did not significantly reduced PDBu-induced MMP-9 mRNA expression. Cells were transfected with control or PKCδ siRNA for 48 h and challenged with 500 nM PDBu for 4 h.
7.4 Discussion

The PKC family of serine-threonine kinases is critical signal transducers participating in many agonist-induced signaling cascades (292), as well as in mechanotransduction in lung cells (417). In the present study, we found that PDBu-induced formation of podosomes and the degradation of ECM are two separate steps, and different PKC isoforms play distinct but coordinated roles in these processes in normal human bronchial epithelial cells.

Our results demonstrated that inhibition of cPKCs, prevented podosome formation and subsequently gelatin matrix degradation. It has been shown that both PKCα and PKCδ were necessary for podosome assembly in human endothelial cells; however only constitutively active PKCα25E but not PKCδA147E could mimic the effect of PMA to induce podosome formation (376). It is possible that in phorbol ester-induced podosomes, cPKC subfamily is the most direct effector of these lipid ligands. The phosphorylation status of cPKCs was not significantly changed during PDBu stimulation; however, cPKCs were found in podosomes and circular ruffles detected by antibodies against PKCα, and phosphorylated cPKCs. It has been proposed that mature and phosphorylated cPKCs can be kept in the cytosol in an inactive conformation by intramolecular interactions between the N-terminal pseudosubstrate region and the kinase domain. The latent kinase can be recruited to the membrane and activated by lipid ligands (such as phorbol ester), leading to a massive conformational change that releases the pseudosubstrate domain from the substrate-binding site, allowing for substrate binding, phosphorylation and activation of downstream signaling effectors (302, 303). Therefore, although the phosphorylation status of cPKCs is not significantly altered upon PDBu stimulation, redistribution of latent phosphorylated cPKC may lead to their activation and subsequent initiation of podosome
formation and function. Although we do not have direct evidence to support the existence of latent cPKCs, our results can be explained by this concept well.

It has been proposed that the distribution of PKC isoforms in different cellular compartments determines their functions by presenting them to different substrates and interaction partners (418). We were surprised to see that the phosphorylation status of novel and atypical subfamilies of PKCs and PKCμ increased dramatically upon PDBu stimulation, and multiple phosphorylated PKCs were recruited to the sites of podosomes and circular ruffles, instead of to different compartments in the cell. Down-regulation of PKCα, δ, ζ, and μ with siRNA prevented PDBu-induced gelatin matrix degradation. Clearly, these PKC family members were not only clustered together but also involved in the function of podosomes. Indeed, Rottlerin (nPKC inhibitor) and PKCδ siRNA prevented PKCζ and MMP-9 recruitment to podosomes. These results suggest that the function of podosomes requires the synergy of multiple PKC family members.

One of the interesting findings of the present study is that the formation of podosomes and the proteolytic degradation function of podosomes can be uncoupled. Rottlerin, siRNAs for PKCδ, ζ, and μ, siRNAs for MMP-9 and MMP-14, and PKCζ pseudosubstrate, reduced PDBu-induced gelatin matrix degradation without affecting podosome formation. Our data are in agreement with the recent report that MMP-14 deficient mouse bone marrow cells and spleen dendritic cells produced podosomes but lost the ability to degrade extracellular matrix after TLR signaling (419). It is possible that actin-rich podosome structures only provide a platform to recruit other proteins, and the activities of MMPs and other enzymes are further regulated locally at the sites of these structures.
Interestingly, we found that PKCζ is involved in controlling the proteolytic function of PDBu-induced podosomes, mainly through MMP-9. Both PKCζ siRNA and PKCζ pseudosubstrate reduced PDBu-induced secretion of MMP-9 to the culture medium and its conversion from pro- to active form. In rat C6 glioma cells, IL-1 and TNFα induced gene expression of MMP-9 via PKCζ through NFκB (237). In contrast, we found that in BEAS2B cells PKCζ pseudosubstrate did not block PDBu-induced MMP-9 gene expression. On the other hand, PKCζ pseudosubstrate blocked PDBu-induced translocation of MMP-9 and to less extend of MMP-14 to podosomes. This is a novel finding in podosome studies.

Our results suggest that MMP-9 is the major MMP in PDBu-induced gelatin matrix degradation in BEAS2B cells. The expression of MMP-14 is also up-regulated by PDBu, and MMP-14 siRNA partially reduced PDBu-induced gelatin matrix degradation. The MMP-2 level in the cell lysates is relatively lower than that of MMP-9, but higher in the culture medium, suggesting that it is constitutively released. Our data also show that MMP-2 is not significantly involved in PDBu-induced gelatin matrix degradation. Of course, we cannot exclude the possibility that MMP-2 may be important for the degradation of other types of matrix molecules. IL-1 induced an MMP activation cascade (MMP-14→MMP-13→MMP-9) in hepatic stellate cells in 3D matrix (420). In non-malignant monkey kidney epithelial cells, pro-MMP-9 activation was accomplished via a cascade of zymogen activation initiated by MMP-14 and mediated by MMP-2 (421). The proteolytic activity of PMA-induced podosomes in endothelial cells depended on MMP-14 mediated activation of MMP-2 but not MMP-9 (422). The interaction among MMP molecules is another interesting area of research.

Taken together, our results suggest that cPKC (particularly PKCα) can regulate rapid podosome formation in normal human bronchial epithelial cells as an upstream signal; atypical
PKCζ may be the direct effector at the site of podosomes that modulates the recruitment, release, and activation of MMP-9. The coordinated actions of PKC isozymes, of course with different regimen, may also exist in podosomes formed by other cell types under different conditions.
8. Chapter Eight. Coordinated regulation of PKC activation-induced podosome formation and proteolytic activities of PI3K, Src and MAPK pathways in human bronchial epithelial cells

The content of this chapter was prepared for an original research article in *Journal of Cell Science*:

8. Chapter Eight. Coordinated regulation of PKC activation-induced podosome formation and proteolytic activities through PI3K, Src and MAPK pathways in human bronchial epithelial cells

8.1 Summary

Podosomes are cellular structures, which migratory and actively invading cells rely on to break through the extracellular matrix. In chapter 7, we reported that phorbol 12, 13-dibutyrate (PDBu), a protein kinase C (PKC) activator, induced podosome formation in normal human bronchial epithelial cells, through PKCζ to recruit and activate MMP-9 at the podosomes. The objective of this chapter of study was to explore signaling pathways that are involved in PKC activation-induced podosome formation and matrix degradation. Herein, we reported that PDBu increased phosphorylation of PI3K p85, PDK1, Akt, GSK3β, Src, ERK1/2 and JNK. PI3K p85, phosphorylated Akt, Src, ERK and JNK were recruited to podosomes. Inhibitors for PI3K, Akt and Src suppressed PDBu-induced podosome formation and matrix degradation. In contrast, blockers for MEK/ERK or JNK did not inhibit podosome formation but reduced proteolytic activity of podosomes, decreased MMP-9 recruitment to podosomes, and its release and activation. Further inhibition studies demonstrated that PI3K/Akt and Src are upstream of ERK1/2 and JNK. These results indicate that podosome assembly and podosomal proteolytic activity are regulated by multiple signal transduction pathways at different levels in a coordinated fashion.
8.2 Introduction

Cell migration plays important role in lung development, growth, and repair after injuries. Podosomes are actin-rich structures located at the ventral surface of the cell membrane that migratory and actively invading cells rely on to break through the extracellular matrix (163). Podosomes have been mainly reported in mesenchymal cells (such as fibroblasts, osteoclasts, smooth muscles cells and endothelial cells) (150) and cancer cells (148, 172). Recently, we reported that phorbol 12,13-dibutyrate (PDBu), a protein kinase C (PKC) activator, induced podosome formation in normal human bronchial epithelial cells, which may be important for airway development and repair (122). We further found that while the conventional PKCs (especially PKCα) control PDBu-induced podosome assembly, it is the atypical PKCζ that regulates the gelatin matrix degradation by influencing the recruitment of MMP-9 at the podosomes and its release and activation (423). PKC activation also induced podosomes in smooth muscle cells (424), endothelial cells (147, 425), and fibroblasts (426).

It has been shown that Src, PI3K and MAPK (mitogen-activated protein kinases) pathways can be activated by PKC and involved in podosome formation (214, 376). The class Ia PI3K has a regulatory subunit (e.g. p85) and a catalytic subunit (e.g. p110). Phosphorylation of the p85 subunit can lead to the activation of the catalytic subunit for PI(3,4,5)P3 formation (200, 427), which can bind PH domain containing proteins, such as Akt (190, 191). Activated Akt can modulate cell motility, cell polarity and cortical actin structures (102). PDK1 can phosphorylate Akt for its activation (192-194). Non-receptor Src family tyrosine kinases play a central role in podosome regulation (154). It has been shown in fibroblasts phorbol ester PMA can recruit AFAP (actin filament associated protein) to PKCα, and AFAP can then recruit and activate c-Src for podosome formation (201, 377). PI3K activation is required for this PKC-directed activation
of c-Src by AFAP (201). MAPK signaling pathways are also important for gene expression, cell polarity (428), and cell invasion (429). In vascular smooth muscle cells (213, 214, 430), murine bone marrow-derived dendritic cells (215), or bone marrow-derived neutrophils (216), ERK1/2 have been reported to mediate podosome formation. Big MAPK Erk5 promoted Src-induced podosome formation in fibroblasts (217).

Most of these previous studies focused on podosome formation. Recent studies suggest that the proteolytic activity of podosomes can be further regulated by multiple mechanisms after podosome formation (419, 431). The objective of the present study was to determine the involvement of PI3K/Akt, Src and MAPK pathways in PKC-induced proteolytic activity, as well as podosome formation, in normal human bronchial epithelial cells.

8.3 Results

8.3.1 PDBu-induced protein phosphorylation and translocation of PI3K/Akt related signaling molecules

To determine whether PI3K/Akt pathway is activated upon PDBu stimulation, we examined the phosphorylation status of signaling proteins after challenging human bronchial epithelial BEAS2B cells with 500 nM PDBu from 0 to 120 min. The phosphorylation of PI3K p85 subunit was increased and reached the maximum at 60 min. The phosphorylation of PDK1 was also increased gradually. The phosphorylation of AktS473 increased to the plateau at 30 min. The phosphorylation of GSK3β, a down-stream signal of Akt, was also gradually enhanced after PDBu treatment (Figure 8-1A).
Figure 8-1. PDBu-induced phosphorylation and translocation of PI3K/Akt related proteins.

(A) PDBu-induced protein phosphorylation. BEAS2B cells were treated with PDBu 500 nM from 0 min to 120 min. The phosphorylation of PI3K p85, PDK1, Akt and GSkβ was examined with western blotting. (B) PDBu-induced translocation of PI3K/Akt related proteins to podosomes and circular ruffles. Cells were treated without or with PDBu 500 nM for 30 min. F-actin was stained with Oregon Green 488 Phalloidin (Green). The target proteins were stained with specific antibodies and revealed with Alexa Fluor 594 labeled secondary antibody (Red). Co-localization of proteins with F-actin in small dots and ring structures is shown with small boxes and enlarged in the underneath panel. Circular ruffles were indicated with arrowheads. (C) Quantitative analysis of target proteins overlapping with F-actin staining by Pearson’s correlation coefficients. *: P<0.05 compared with “no treatment” group.
Next, we investigated the effects of PDBu-stimulation on distribution of PI3K/Akt related signaling proteins. Under control condition, PI3K p85α, phosphorylated PDK1, Akt and GSK3β were localized in the cytoplasm and peri-nucleus region, but not overlapped with actin stress fibers (Figure 8-1B). PDBu stimulation resulted in dramatic cytoskeleton reorganization, that is, disassembly of actin stress fibers and formation of actin-rich small dots, rings and belts in the cytoplasm and circular membrane ruffles at the peripheral (Figure 8-1B). The molecular components and the 3-D structure of these podosomes and circular ruffles have been characterized previously (122). PDBu treatment induced translocation of PI3K p85, and phosphorylated Akt to the newly formed podosomes and circular ruffles (Figure 8-1B). The co-localization of F-actin staining with these proteins were quantified and expressed as Pearson’s correlation coefficient values (Figure 8-1C).

### 8.3.2 Effects of PDBu-stimulation on phosphorylation and distribution of Src and MAPKs

To determine whether c-Src and MAPKs are activated upon PDBu treatment, we examined the phosphorylation of c-Src, ERK1/2, JNK and p38 after challenging the BEAS2B cells with 500 nM from 0 to 120 min. The phosphorylation of SrcY416, a sign of c-Src activation, was increased dramatically at 30 min. The phosphorylation of ERK1/2 was increased during the first 10 minutes and slightly decreased thereafter. The phosphorylation of JNK was increased dramatically within the first 30 min followed by a rapid decrease to the basal level at the end of 120 min. The phosphorylation of p38 was not dramatically altered by PDBu treatment, in comparison with other MAPK molecules examined (Figure 8-2A).
Figure 8-2. PDBu-induced phosphorylation and translocation of Src and MAPK signaling proteins.

(A) PDBu-induced phosphorylation of Src and MAPK signaling proteins. (B) PDBu induced translocation of phosphorylated Src and MAPK molecules to podosomes. (C) Quantitative analysis of these proteins overlapping with podosomes by Pearson’s correlation coefficients. *: \(P<0.05\) compared with “no treatment” group. See Figure 8-1 legend for experimental design.
Then, we examined the PDBu-stimulated re-distribution of c-Src and MAPKs. Phosphorylated SrcY416, ERK1/2, JNK and p38 were mainly distributed in the cytoplasm and peri-nucleus area under control condition. After PDBu stimulation, phosphorylated SrcY416 was co-localized with podosomes and circular ruffles. Phosphorylated ERK1/2 and JNK (but not p38) were also accumulated in PDBu-induced podosomes (Figure 8-2B). Quantification of the co-localization of F-actin and these proteins showed significant increased phosphorylated ERK and JNK in podosome after PDBu stimulation (Figure 8-2C).

8.3.3 PI3K/Akt and Src regulates podosome assembly and proteolytic activity

To determine the role of PI3K/Akt signaling in PDBu-induced podosome, we pre-incubated cells with inhibitors (1 µM for 1 h) for PI3K (LY294002 or Wortmannin) or Akt (Akt inhibitor II) before challenging them with PDBu (500 nM for 8 h). The effects of these inhibitors on podosome formation and gelatin matrix degradation were determined with in situ zymography assay with experimental conditions optimized (423). These inhibitors prevented PDBu-induced disruption of actin stress fiber, reduced podosome formation (Figure 8-3A and 8-3B), and blocked gelatin matrix degradation of PDBu-induced podosomes examined with in situ zymography (Figure 8-3A and 8-3C).
Figure 8-3. PI3K and Akt regulate podosome assembly and its proteolytic activity through MMP-9 expression, release and activation.

(A) LY294002 and Wortmannin, inhibitors for PI3K, and Akt inhibitor II (Akt II), prevented PDBu-induced podosome formation and proteolytic activity of podosomes. Cells cultured on gelatin matrix (Green) were pre-incubated with each inhibitor (1 µM for 1 h), and then stimulated with PDBu (500 nM for 8 h). F-actin was stained with Rhodamine Phalloidin (Red). Digestion of gelatin matrix was revealed as black areas. (B) Quantitative analysis of percentage of cells showing podosomes. (C) Quantitative analysis of percentage of cells showing matrix degradation. (D) LY294002, Akt II and PP2 (inhibitor for Src) attenuated PDBu-induced MMP-9 mRNA gene expression. Cells were pretreated with each inhibitor (1 µM for 1 h) and challenged with PDBu for 4 h. RNA was extracted and qRT-PCR was performed. *: p < 0.05 in comparison with PDBu-stimulated control group. (E and F) LY294002, Akt II and PP2 reduced PDBu-induced MMP-9 protein expression and enzymatic activity. Cells were pretreated with each inhibitor (1 µM for 1 h) and challenged with PDBu 500 nM for 8 h. The culture medium (F) and whole cell lysates (WCL) (E) were examined with gelatin gel zymography.
Figure 8-4. Src is involved in podosome formation and proteolytic activity.

(A) PP2, inhibitor for Src, prevented PDBu-induced podosome formation and proteolytic activity of podosomes. See Figure 8-3 legend for experimental design. Similarly, Genistein (general inhibitor for tyrosine kinases), Su6656 (Src inhibitor), and PP3 (negative control for PP2) (1 μM 1 h each) were studied. (B) Quantitative analysis of percentage of cells showing podosomes. (C) Quantitative analysis of percentage of cells showing matrix degradation. *: p < 0.05 in comparison with PDBu-stimulated control group.
We have shown that MMP-9 expression, recruitment to podosomes, release and activation are crucial for PDBu-induced proteolytic activities of podosomes (423). To examine whether PI3K/Akt pathway affects MMP-9 gene expression, cells were pre-incubated with the inhibitors (1 µM for 1 h), and total RNA was extracted for qRT-PCR assay after PDBu treatment (500 nM for 4 h) (423). PDBu stimulation increased MMP-9 mRNA approximately 35-40 folds. LY294002, Wortmannin, or Akt II almost completely blocked PDBu-induced MMP-9 mRNA expression (Figure 8-3D). To investigate MMP isozymes regulated by PI3K/Akt pathway, whole cell lysates and cell culture medium were collected for gelatin gel zymography assay. In cell lysates, the expression and activity of MMP-9 were higher than MMP-2, while MMP-2 was more prominent in the cell culture medium (Figure 8-3E and 8-3F). The expression and activation of MMP-2 were not significantly altered upon PDBu treatment both in the cell lysates and cell culture medium (Figure 8-3E and 8-3F). The latent and active forms of MMP-9 were increased after PDBu challenge in both cell lysates and medium. LY294002 or Akt II dramatically prevented the protein expression, activation and secretion of MMP-9 (Figure 8-3E and 8-3F). The inhibitory effects are statistically significant (P<0.05) as quantified by densitometry (data not shown).

To identify the role of Src in PDBu-induced podosome, we pre-incubated cells with Genistein (a general inhibitor for tyrosine kinases), PP2 or Su6656 (specific inhibitors for Src) (1 µM for 1 h). Each of these inhibitors prevented PDBu-induced podosome assembly and gelatin matrix degradation, whereas PP3, a non-functional analogue of PP2, did not show such inhibitory effects (Figure 8-4). PP2 also significantly reduced PDBu-induced MMP-9 gene expression (Figure 8-3D), protein synthesis, activation and release (Figure 8-3E and 8-3F). The
inhibitory effects are statistically significant (P<0.05) as quantified by densitometry (data not shown).

8.3.4 ERK and JNK mediate proteolytic activity of PDBu-induced podosomes without affecting podosome formation

To investigate whether MAPKs are involved in PDBu-induced podosomes, cells were pre-treated with PD98059 (inhibitor for MEK, up-stream kinase of ERK), SP600125 (inhibitor for JNK) and SB20358 (inhibitor for p38) (1 μM for 1 h) prior to the PDBu-stimulation. None of these inhibitors prevented podosome formation upon PDBu challenge (Figure 8-5A and 8-5B). Interestingly, PD98059 and SP600125 (but not SB20358) blocked the gelatin matrix degradation (Figure 8-5A and 8-5C). Pre-treatment of cells with PD98059, SP600125 and SB20358 significantly inhibited PDBu-stimulated MMP-9 mRNA expression (Figure 8-5D). Pretreatment of cells with PD98059 or SP600125 (to less extend with SB20358) also reduced PDBu-induced MMP-9 protein expression and activation (Figure 8-5E and 8-5F).

To determine whether MAPKs play a role in MMP-9 recruitment to PDBu-induced podosomes, we pre-incubated cells with these inhibitors and performed immunofluorescent staining for MMP-9. The co-localization of MMP-9 in actin-rich small dots was quantified. PD98059 and SP600125 but not SB20358 significantly reduced PDBu-induced translocation of MMP-9 to podosomes (Figure 8-6).
Figure 8-5

A. F-actin Overlay

Control

PD98059

SP600125

SB20358

B. % Cells With Podosomes

Control

PD98059

SP600125

SB20358

C. % Cells Degrad Matrix

Control

DMSO

PD98059

SP600125

SB20358

D. MMP-9 mRNA

Control

DMSO

PD98059

SP600125

SB20358

E. WCL

Pro-MMP-9

Active MMP-9

Pro-MMP-2

Active MMP-2

F. Medium

Pro-MMP-9

Active MMP-9

Pro-MMP-2

Active MMP-2

161
Figure 8-5. ERK and JNK mediate proteolytic activity of PDBu-induced podosomes.

(A) PD98059 (inhibitor for MEK, upstream kinase of ERK) and SP600125 (inhibitor for JNK) but not SB20358 (inhibitor for p38) (1 µM each for 1h), prevented proteolytic activity of PDBu-induced podosomes, but not affect podosome formation. See Figure 8-3 legend for experimental design. (B) Quantitative analysis of percentage of cells showing podosome assembly. (C) Quantitative analysis of percentage of cells showing matrix degradation. (D) PD98059, SP600125 and SB20358 attenuated PDBu-induced MMP-9 mRNA gene expression. See Figure 8-3 legend for experimental design. *: p < 0.05 in comparison with PDBu-stimulated control group. (E) PD98059, SP600125 and SB20358 (to less extent) reduced PDBu-induced MMP-9 protein expression and enzymatic activity.
Figure 8-6. Inhibitors for ERK and JNK pathways prevented MMP-9 translocation to PDBu-induced podosomes.

(A) Cells were pre-incubated with PD98059, SP600125, or SB20358 (1 µM for 1 h), and then stimulated with PDBu (500 nM for 30 min). F-actin was stained with Oregon Green 488 Phalloidin (Green). MMP-9 was revealed by Alexa Fluor 549 labeled secondary antibody (Red). (B) Quantitative analysis of percentage of MMP-9 overlapping F-actin staining by Pearson’s correlation coefficients. *: p < 0.05 in comparison with PDBu-stimulated control group.
It has been shown that U0126, a specific inhibitor for MEK, blocked PMA-induced podosome formation in smooth muscle cells (214), and in fibroblasts (217). In contrast, we found that PD98059 only blocked proteolytic activity without affecting podosome formation in human bronchial epithelial cells (Figure 8-5). Thus, we also pre-incubated BEAS2B cells with U0126 or its negative control U0124 (1 µM for 1 h). Compared to U0124, U0126 greatly attenuated gelatin matrix degradation of PDBu-stimulated podosomes without affecting podosome assembly (Figure 8-7A, 8-7B and 8-7C). U0126, but not U0124, also dramatically limited the translocation of MMP-9 to the sites of PDBu-induced podosomes (Figure 8-7D and 8-7E). These interesting data indicated that podosome assembly and matrix degradation are regulated differently in different cell types. It may be due to the distinct expression level and activation status of certain unknown proteins or kinases in these models.

8.3.5 Crosstalk among PI3K/Akt, Src and MAPK pathways

Since the major effect of PDBu is to activate PKCs, we investigated whether PDBu-induced phosphorylations of signal molecules examined in the present studies are mediated via PKCs. As expected, pre-treatment of BEAS2B cells with BIM I (a pan PKC blocker) suppressed PDBu-induced phosphorylation of PI3K p85, Akt, Src, ERK and JNK in a dose-dependent manner (Figure 8-8A). Inhibitor for PI3K (LY294002) (Figure 8-8B) and Akt (Akt II) (Figure 8-8C) attenuated not only the phosphorylation of p85 and Akt but also the phosphorylation of SrcY416 and JNK. Src inhibitor (PP2) reduced phosphorylation of SrcY416, p85, ERK and JNK (Figure 8-8D). Blocker for MEK (PD98059) and JNK (SP600125) prevented the PDBu-induced phosphorylation of ERK1/2 and JNK, respectively (data not shown). Taken together, these data
suggest that PI3K/Akt pathway is upstream of Src, and Src may affect the phosphorylation (activation of ERK1/2 and JNK).
Figure 8-7. Confirmation of ERK pathway in PDBu-induced gelatin matrix degradation, MMP-9 translocation and gene expression with another MEK inhibitor.

(A) Cells cultured on gelatin matrix (Green) were pre-incubated with U0126 (inhibitor for MEK) or its analogue control U0124 (1 µM for 1 h), and then stimulated with PDBu (500 nM for 8 h). F-actin was stained with Rhodamine Phalloidin (Red). Digestion of gelatin matrix was revealed as black areas. (B) Quantitative analysis of percentage of cells showing podosomes. (C) Quantitative analysis of percentage of cells showing matrix degradation. (D) U0126 prevented MMP-9 translocation to PDBu-induced podosomes. F-actin was stained with Oregon Green 488 Phalloidin (Green). MMP-9 was revealed by Alexa Fluor 549 labeled secondary antibody (Red). (E) Quantitative analysis of percentage of MMP-9 overlapping F-actin staining by Pearson’s correlation coefficients. *: p < 0.05 in comparison with PDBu-stimulated control group.
Figure 8-8. Effects of multiple inhibitors on phosphorylation status of PI3K/Akt, Src and MAPKs.

Cells were pretreated with different doses of (A) BIM I (inhibitor for PKCs), (B) LY294002 (PI3K inhibitor), (C) Akt inhibitor II (Akt inhibitor) and (D) PP2 (Src inhibitor) (1, 5, 10 µM for 1 h), and then stimulated with PDBu (500 nM, 30 min). Phosphorylation status of multiple proteins was examined with western blots.
8.4 Discussion

In the present study, we demonstrated that in human bronchial epithelial cells, multiple signal transduction pathways (PI3K/Akt, Src and MAPKs) are involved in PKC activation-induced podosome formation and gelatin matrix degradation in a coordinated fashion. PI3K/Akt and Src appear to be the upstream signals to participate in the regulation of podosome formation, whereas ERK and JNK are the downstream signals to participate in the regulation of gelatin matrix degradation. Interestingly, p38 MAPK was neither associated with PDBu-stimulated podosome formation nor matrix degradation.

In the present study, we showed increased phosphorylation of SrcY416 and multiple molecules in PI3K/Akt pathway. PI3K p85α, phosphorylated Akt, and c-Src were clustered at the PDBu-induced F-actin rich small dots, rings, and belts in the cytoplasm and membrane ruffles. Blocking PI3K or Akt activity with chemical inhibitors significantly prevented podosome formation and proteolytic activity of PDBu-induced podosomes. These inhibitors also reduced PDBu-induced phosphorylation of SrcY416, a sign of activation of c-Src (432). It has been shown that PI3K activity was required for PMA-induced activation of c-Src through AFAP for podosome formation and cell migration (201). Our results support that PI3K/Akt signaling cascade and c-Src are involved in PKC activation-related podosome formation in normal human bronchial epithelial cells as upstream event.

In contrast, our results also suggest that JNK and ERK1/2 are down-stream signaling molecules, acting as direct effectors to regulate gelatin matrix degradation of podosomes in human bronchial epithelial cells. Inhibitors for JNK and ERK pathways did not block podosome assembly, but inhibited gelatin matrix degradation. This finding is very interesting, because ERK1/2 has been implemented in PKC-mediated podosome formation in vascular smooth
muscle A7r5 cells (213, 214), and in primary aorta smooth muscle cells (430). In murine bone
marrow-derived dendritic cells (215) and bone marrow-derived neutrophils ERK1/2 is also
involved in cell migration and invasion, associated with podosome-like structures at the cell
leading edge (216). Big MAPK Erk5 promoted Src-induced podosome formation in fibroblasts
(217). We cannot explain why ERK1/2 and JNK are only involved in gelatin matrix degradation
in human bronchial epithelial cells. However, it has been recently shown that TGF-β induced
formation of F-actin cores (podosomes) requires signaling through PI3K and Src, whereas TGF-
β-induced matrix degradation requires ERK in human breast cancer cells (433). The formation
and proteolytic activity of podosomes should be considered as two highly related by distinct
cellular events. It should point out that our results could be the first report to indicate the
involvement of JNK in podosome related functions. Interestingly, phosphorylated ERK and JNK
were not significantly co-localized with podosomes but inhibition of these pathways reduced
translocation of MMP-9 to podosomes. The intermediate mechanisms of these cellular processes
merit further investigation.

In our recent (122, 423) and the present study, we found that MMP-9 is the most
dominant MMP isozyme in PDBu-induced gelatin matrix degradation. In human lung
adenocarcinoma A549 cells, EGF stimulated Src and JNK/ERK signaling pathway through FAK
at the cell membrane to increase MMP-9 expression and secretion (220). In human melanoma
cells, the homophilic interactions of CD151 (PETA-3/SFA-1), a member of the tetraspanins,
stimulated integrin-dependent signaling to c-Jun through FAK-Src-MAPKs pathways to
enhance cell migration and MMP-9 expression (434). In human breast adenocarcinoma MCF-7
and MDA-MB-435 cells, CD99, a cell surface glycoprotein, enhanced cell migration and MMP-
9 expression through Akt-, ERK-, and JNK-dependent AP-1 activation (435). In the present
study, inhibitors for PI3K, Akt, Src, MEK and JNK also reduced MMP-9 gene expression. The relationships of these pathways in podosome formation, matrix degradation and MMP-9 gene expression should be further determined.

Taken together, our results suggested that PKC-activation induced podosome formation and gelatin matrix degradation requires PI3K/Akt, Src and MAPKs in a coordinated fashion. Since human bronchial epithelial cells are important for maintenance of airway epithelial integrity and repair after tissue injury, these results may contribute to molecular mechanisms related to these physiological and pathophysiological processes.
9. Chapter Nine. Summary, general discussion and perspectives
9. Chapter Nine. Summary, general discussion and perspective

9.1 Summary

Lung airway and bronchial epithelial cells are the major player in host defense and are the primary target of lung diseases such as chronic obstructive pulmonary disease, asthma, cystic fibrosis (364, 365). Epithelial cell migration plays an important role in the lung physiology, lung repair after injury, and lung homeostasis. The serine/threonine PKC family is highly involved in cell proliferation, differentiation, cytoskeleton reorganization, cell migration and invasion, and the progression of tumorigenesis (292). Phorbol ester can induce dramatic cytoskeletal structure changes in lung epithelial cells (378). I hypothesized that some of these structures are podosomes in lung epithelial cells and may be involved in epithelial cell migration and invasion under physiological and/or pathophysiological conditions. In my PhD thesis studies, I acquired primary normal human bronchial epithelial cells and normal (non-cancerous) human lung bronchial epithelial BEAS2B cells and challenged them with phorbol ester PDBu to activate PKCs. My PhD thesis has three major original findings:

1. I discovered that phorbol ester may induce podosome formation in human normal (non-cancerous) bronchial epithelial cells. I characterized these cellular structures and revealed the potential function of these podosomes in normal bronchial epithelial cells

2. I demonstrated that PDBu-induced podosome assembly was mainly mediated through redistribution of conventional PKCs, especially PKCα, from the cytosol to the podosomes, while atypical PKCζ locally played a dominant role in the regulation of proteolytic activity of PDBu-induced podosomes through recruitment, release and activation of MMP-9 as summarized in Figure 9-1
Figure 9-1. Role of PKCs in podosome formation and podosomal proteolytic function.

PDBu-induced podosome formation is mainly mediated through classical PKCs especially PKCα. The recruitment, secretion and activation of MMP-9 and proteolytic activity of podosomes mainly depend on atypical PKCs particularly PKCζ. Novel PKCδ is involved in recruitment of PKCζ to the site of podosomes.
3. I investigated that PI3K/Akt/Src were involved in regulating PDBu-induced podosome formation as the upstream signals; whereas ERK1/2 and JNK MAPKs regulated the proteolytic activity of PDBu-induced podosomes through recruitment of MMP-9 to podosome and MMP-9 expression, secretion and activation.

These results will contribute to the understanding of the molecular mechanism of normal epithelial cell migration and invasion in lung physiology and lung diseases.

9.2 Discussion

Several new findings were elucidated in my thesis. In this section, I will focus on the topics never mentioned in previous chapters.

9.2.1 The role of podosomes in normal bronchial epithelial cells in lung physiology and pathophysiology

Podosome is a newly discovered cellular structure that plays a critical role in cell migration and invasion. Carman et al. found that lymphocytes used podosomes and extended “invasive podosomes” to palpate the surface of, and ultimately form transcellular pores through, the endothelium in diapedesis at ex vivo tissue slides by electronic microscopy. These podosome-like structures were dependent on Src kinase and the actin regulatory protein WASP (276). I speculate that similar cellular structures may exist when airway epithelial cells are trying to across tissue boundaries. When epithelium is damaged, the surviving epithelial cells may migrate towards the wounded area, or invade from the underneath to cover the denuded epithelium as showed in Figure 1-2. Alternatively, when cells migrate over a two-dimensional
surface, matrix degradation of podosomes may also promote deadhesion of the cell. My work that primary normal bronchial epithelial cells can form podosomes and membrane ruffles/waves upon PKC activation, suggests that normal cells have the potential to gain an aggressive motility under stimulation.

9.2.2 PKCs and cell motility in lung physiology and pathophysiology

PKCs are important in many cellular responses in the lung physiology and pathophysiology. Dempsey et al. summarized the potential exogenous stimuli for PKC isozymes in the lung, such as oxygen tension (hypoxia and hyperoxia), mechanical forces (pressure and shear stress), inhaled irritants (cold air, oxidants and other noxious chemicals), inhaled dust (asbestos, silica and beryllium) and certain inhaled allergens causing hypersensitivity pneumonitis (436). These diverse agents are known to induce many different cellular responses such as vessel contraction, increased vascular permeability, cell migration, cell invasion and cell proliferation. In human lung cancer epithelial H460 and H1299 cells, nicotine induced the activation of PKC\(\tau\) and increased cell migration and cell invasion through phosphorylation of \(\mu\)- and m-Calpains (437). In human lung cancer NCI-H69 cells, tobacco nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) enhanced cell migration and invasion through induction of the influx of \(\text{Ca}^{2+}\) and activation of PKC/Raf/MEK/ERK1/2 signaling cascade through \(\alpha_7\text{nAChR}\), which induced phosphorylation of both \(\mu\)- and m-calpain leading to calpain activation and secretion (438). In normal human lung bronchial epithelial BEAS2B cells, hog barn dust slowed airway epithelial cell migration in a wound healing assay in vitro through a PKC\(\alpha\)-dependent mechanism (439). Mechanisms that regulate PKC activity, including signaling
cascades, phosphorylation and interaction with isozyme-specific binding proteins, are potential therapeutic targets for lung diseases.

### 9.2.3 PKC isoforms and isoform redundancy

For each PKC subfamily, I only focused on one representative isoform to study in my thesis. Thus negative results in any one assay may simply reflect the fact that other isoforms of the same class may compensate the deficiency. For example, Rodriguez et al found that \( \nu/\lambda \) isoform aPKC was the major contributor to podosome assembly and invasion, with \( \zeta \) playing a subsidiary role in NIH 3T3 cells transformed by v-Src and by activated by c-Src (SrcY527F) (440). Inhibition of both isoforms had a greater effect than inhibiting either one alone. In Chapter 8, I used a myristoylated pseudosubstrate inhibitor PS for PKC\( \zeta \). The sequence of PS is Myr-SIYRRGARRWRKL-OH. It not only targets human PKC\( \zeta \), but also PKC \( \nu \). Therefore, there may be an alternative interpretation of the data.

### 9.2.4 Cell migration vs. cell invasion

From my point of view, cell migration is a process that cells move along the surface, whereas cell invasion is a situation that cells transmigrate to across different cell layers and/or extracellular matrix. To explore the migration properties of BEAS2B cells bearing podosomes, we performed a wound healing cell migration assay. In control group, cells formed a broad lamellipodia at the leading edge toward the wound. In PDBu treated group, cells appeared membrane ruffles all around. Cells were not polarized toward the wound and PDBu reduced cell migration. Similarly, Slager et al. found that agricultural dust exposure activated PKC\( \alpha \) to slow down BEAS2B cell migration during wound repair (439). In contrast, in Boyden chamber
invasion assay, BEAS2B cells with podosomes degraded the coated fibronectin gel in the Transwell and invaded through the gel to the bottom side of the well. PDBu stimulation enhanced the invasiveness of normal epithelial cells \textit{in vitro}. I suspect that podosomes may have similar function for cell motility in vivo. To prove this concept is difficult, however, with advanced technologies and awareness of cell movement in 3D, it may be explored in the future.

9.2.5 Podosome formation vs. podosomal proteolytic function

With \textit{in situ} zymography assay, I found that Rottlerin, PKCδ/µ/ζ siRNA, PKCζ pseudosubstrate inhibitor, MMP-9/-14 siRNA, PD98059, U0126, SP600125 can inhibit matrix degradation of PDBu-stimulated podosomes without affecting podosome assembly. Several groups have also demonstrated that podosome assembly and podosomal matrix degradation can be uncoupled and regulated by distinct signaling pathways. West et al. found that MMP-14 deficient mouse bone marrow cells and spleen dendritic cells produced podosomes but lost the ability to degrade extracellular matrix after TLR signaling (419). In CA1D cells, a Ras transformed invasive variant of the MCF10A human breast cell line, activation of PI3K and Src kinases upon TGF-β treatment is required for the formation of the actin core of podosomes, whereas ERK activation through Smad2/3 signaling is for activating the protease MMP-9 and degradation of extracellular matrix (433). In v-Src transformed NIH3T3 cells, Tks5 was required for podosome formation, while Tks4 is required for MT1-MMP recruitment and extracellular matrix degradation (431). Therefore, both data from us and other groups signify that podosome assembly and proteolytic function are qualitatively distinct, separately regulated processes. These studies are important because understanding the biology of podosomes and regulation of
their proteolytic function will aid in identification of novel targets for anti-invasive or anti-
metastasis therapy.

In addition, my data could also be explained in an alternative way. The distinction
between podosome formation and proteolytic function is quantitative rather than qualitative.
Accumulation of MMPs and matrix degradation increases as podosome assembly processes, but
the assays for MMP recruitment and matrix degradation are in general less sensitive than those
for detection of abundant cytoskeletal proteins such as actin or cortactin. Thus matrix
degradation can only be observed when podosome assembly and MMP recruitment has
processed beyond a certain threshold size. In this quantitative model, weak inhibitors of podosome
formation could appear to specifically block MMPs recruitment and matrix degradation, because
the recruitment of cytoskeletal components such as actin or cortactin could still be detected when
podosome assembly is only partially inhibited. In Chapter 7 and 8, one interesting phenomenon
is we found that certain inhibitors of MMP recruitment and matrix degradation in this system are
inhibitors of podosome formation in other systems. This might be due to cell type specific. It
could be also interpreted by this “quantitative model” mentioned above. Certain inhibitor may be
more or less effective in different cell types.

9.2.6 MMP-9 gene expression vs. MMP-9 recruitment

In BEAS2B cells, the expression and activity of MMP-9 are inducible by PDBu. MMP-9
is dominant in gelatin matrix degradation in our model. I found that PKCζ pseudosubstrate
inhibitor attenuated MMP-9 enzymatic activity and translocation without interrupting the mRNA
level, but PD98059 and SP600125 inhibited mRNA expression, as well as translocation and
enzymatic activity of MMP-9. It indicated that PKCζ might locally regulate MMP-9 secretion
and activity, and ERK1/2 and JNK signaling can manipulate both MMP-9 gene expression and activity.

The activity of MMPs is generally regulated by several steps, gene transcription (249); mRNA stability (441); secretion and activation of the latent pro-enzymes (442-445); and inhibition by their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), which are able to inhibit metalloproteinase activity by forming non-covalent complexes with active MMPs (8, 226). Recently, it is found that MMP intracellular trafficking is an indispensable step for MMP regulation (446). In N2a neuroblastoma and primary neuronal cells, MMP-2 and MMP-9 and their inhibitor TIMP-1 were secreted in 160–200 nm vesicles in a Golgi-dependent pathway. These vesicles distributed along microtubules and microfilaments, co-localized differentially with the molecular motors kinesin and myosin Va and undergo both anterograde and retrograde trafficking. MMP-9 retrograde transport involved in the dynein/dynactin molecular motor (446). In hippocampal neurons, MMP-2 and MMP-9 vesicles were preferentially distributed in the somato-dendritic compartment and were found in dendritic spines. Non-transfected hippocampal neurons also demonstrated vesicular secretion of MMP-2 in both its pro- and active forms and gelatinolytic activity localized within dendritic spines (446).

MAPK pathway signaling molecules are highly involved in both direct and indirect regulation of MMP-9, through gene expression and protein-protein interaction through integrin β1 as shown in Figure 9-2. Accumulated data indicated that JNK can regulate MMP-9. In mouse fibroblast cells, JNK1 increased the expression of MMP-9 protein (447). In human osteosarcoma U-2 OS cells, JNK1 is necessary for expression of MMP-9 mRNA (448). I found that in addition to decrease MMP-9 mRNA level, JNK pan inhibitor SP600125 also reduced MMP-9 protein expression and activation and secretion in BEAS2B cells.
Figure 9-2. MAPK signaling pathways regulate MMP-9.

Network of how MAPK signaling pathways regulate MMP-9 generated by Ingenuity Pathway Analysis software.
Increasing evidence showed that MAPKs can regulate MMP-9 through integrin β1. Thrombin is a known procoagulatory serine protease to induced invasion and metastasis in various cancers. Thrombin-induced invasion of human osteosarcoma US-OS cells through Matrigel was mediated by PI3K and ERK signaling pathways through the induction and association of MMP-9 and integrin β1 on the cell surface (449). Accumulation and depletion of GM3 gangliosides in epithelial cells cultured on fibronectin correlated inversely with MMP-9 expression and activation, EGFR and MAPK phosphorylation and Jun expression (450). Ganglioside depletion facilitated the interaction of MMP-9 and integrin α5β1 (450), suggesting that dissociation of integrin and MMP-9 as the potential mechanisms for the GM3-induced effects on MMP-9 function and cell migration (450).

α3β1 integrin played a critical role in MMP-9 gene expression, activation and production. In immortalized mouse keratinocytes, α3β1 integrin not only regulated MMP-9 mRNA stability in response to activation of MEK/ERK pathway (441), but also was directly essential for MMP-9 secretion (442). In human ovarian cancer cell line MDAH 2774, α3β1 integrin mediated MMP-9 activity through transcriptional regulation of its endogenous inhibitor TIMP-1 via c-fos binding to the AP-1 up region of the TIMP-1 promoter (451). We also found that β1 integrin was well colocalized with PDBu-induced podosomes in BEAS2B cells. It would be interesting to investigate whether integrin is involved in MMP-9 expression and production through interaction with PKCs.

9.2.7 Study approaches and their limitations

9.2.7.1 Cell lines and primary cells
In my PhD thesis work, I used primary normal human bronchial epithelial cells and normal human bronchial epithelial cell line BEAS2B. Different from primary cells that die in a few weeks in culture, cell lines are modified cells, with the immortal growing property. The modification could cause changes that may affect the nature of the original cells. In my thesis, although most studies were using cell lines, I did confirm my results with primary cells. The primary cells even form better podosomes than cell lines. I also confirmed that PKC play a dominant role in podosome assembly and the proteolytic function in primary cells by multiple approaches. Due to the limited availability and the difficulties to transfect the primary cells, cell lines are still important tools for biochemical studies.

9.2.7.2 Chemical inhibitor and siRNA

I used variety of chemical inhibitors. One potential limitation for pharmaceutical inhibitor is the specificity of these chemicals. I also used specific siRNA to knockdown targeted proteins in my work. siRNA is more specific than chemical inhibitor. However, the potential off-target effects should be kept in mind. Combining multiple methods is necessary for conclusive studies.

9.2.7.3 In vitro vs. in vivo

I mainly used in vitro cell culture model to study the role of epithelial cell migration and invasion with the advanced cell culture and microscopy techniques. There are various limitations of employing cell culture to study the molecular mechanism of epithelial cell migration and invasion in lung repair and regeneration (74). Now, armed with the new technology available such as intravital confocal microscopy, two-photon and multi-photon microscopy, real-time confocal microscopy and so on (452, 453), it is time to return to in vivo model once again. For
example, we could generate rat lung epithelial injury model by naphthalene (81) and study the process of surviving epithelial cell migration and invasion to recover the denude area by immunofluorescent staining. To get high resolution of bronchus epithelium, we could also use GFP-mouse and the “stick objective”, a new product from Olympus (453), to investigate the process of surviving epithelial cell migration and invasion to the injured area by live cell imaging.

9.3 Conclusions

In my PhD thesis, studies provide evidence for the molecular mechanism of podosome formation and their proteolytic function in normal human bronchial epithelial cells. PKC, PI3K, Akt, Src and MAPKs play a coordinated role in phorbol ester-induced podosome formation and their proteolytic function as summarized in Figure 9-3. The upstream signaling pathways classical PKCα, PI3K, Akt and Src control podosome formation. Novel PKCδ facilitated the translocation of atypical PKCζ and MMP-9 to the sites of podosomes. Atypical PKCζ, ERK and JNK work as the downstream signals to locally control podosomal proteolytic function. All these findings may be important for normal epithelial cell migration and invasion during physiological or pathophysiological conditions.
Figure 9-3. PKC, PI3K, Akt, Src and MAPK in podosome formation and podosomal proteolytic function.

Signaling cascades PKC, PI3K, Akt, Src, and MAPK especially ERK and JNK, play a central role in podosome formation and the podosomal proteolytic function. The upstream signaling pathways classical PKCs, PI3K, Akt and Src control podosome formation. Novel PKC facilitated the translocation of atypical PKCs to the sites of podosomes. Atypical PKCζ, ERK, and JNK work as the downstream signals to locally control podosomal proteolytic function. These may be important for normal bronchial epithelial cell migration and invasion during physiological and pathophysiological conditions.
9.4 Future directions

In my PhD thesis, I have used a cellular model of normal human bronchial epithelial cells to study the molecular mechanism of podosome formation and podosomal proteolytic function in vitro. To further continue my work, we design the following potential research topics to carry on. These future studies will aid in elucidation of the mechanism of cell migration and invasion in physiological and pathophysiological conditions.

- To identify the potential interaction between PKCα and ζ and MMP-9, we may perform immunoprecipitation and co-immunoprecipitation. This will reveal whether PKCα, ζ and MMP-9 can bind to each other directly or indirectly.
- To further investigate the signaling cascade of PKCs, PI3K, Src, MAPKs and MMP-9, we need to identify the binding partners of each molecule, upstream activators and downstream substrates.
- To examine whether PKCζ need kinase activity to activate MMP-9 and facilitate MMP-9 secretion, we need to make dominant negative and dominant active constructs to test our idea.
- To investigate whether these cellular structures- podosome, invadopodia, and membrane ruffles- really exist in vivo, we may optimize the staining protocol to stain podosomal markers, cytoskeleton proteins and MMPd, using frozen section of human lung bronchus tissues. We can also use electronic microscope to visualize the structure of lung bronchus epithelium tissue sample to identify the membrane invaginations and protrusions of cells across tissue boundaries.
To determine the role of PKCs in podosomes and cell migration and invasion in vivo, we can stain the native and phosphorylated species of PKCs especially α and ζ on the above mentioned tissue slides to reveal whether they are co-localized with podosomes.

To investigate the role of PKCs in lung repair and regeneration after injury, we may inject PKC chemical inhibitors (BIM I, Gö6976, Rottlerin, PKCζ pseudosubstrate inhibitor) or deliver PKC siRNA to the lungs in the rat naphthalene epithelium regeneration model (81). We can test whether these PKC blockers or PKC siRNA prevent the surviving epithelium repair after denudation by naphthalene.

Similarly, we can also use PI3K, Src and MAPKs inhibitors to test the role of these molecules in epithelial repair and regeneration after injury in the naphthalene model.
10. References
References


191


impairs alveolar epithelial repair by binding to vitronectin. *Am J Respir Cell Mol Biol* 31: 672-678.


198


Src-focal adhesion kinase and receptor association to the cytoskeleton. *Cancer Res* 69: 475-482.


