The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

NHS National Institute for Health Research

cAMP-mediated Regulation of Fibroblast to Myofibroblast Differentiation in Idiopathic Pulmonary Fibrosis

Rebecca L Wright

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

School of Medicine and Health Sciences

The University of Nottingham

April 2016

Abstract

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease with no effective treatment. Myofibroblasts contribute to the pathology of IPF by secreting large amounts of extracellular matrix proteins such as alpha smooth muscle actin (α-SMA) and Collagen I (Col 1). Myofibroblasts have reduced Prostaglandin E2 (PGE_2), a key anti-fibrotic mediator, due to diminished cyclooxygenase-2 (COX-2) expression.

Primary fibroblasts isolated from lungs of IPF patients (F-IPF) expressed significantly less COX-2 in response to IL-1β and increased α-SMA and Col I compared with fibroblasts isolated from lungs of non-fibrotic patients (F-NL). COX-2 was gradually lost in F-NL treated with transforming growth factor-β (TGF-β1), a pro-fibrotic cytokine, whereas PGE_2, and cAMP elevating agents increased IL-1β-induced COX-2 expression in F-IPF. Ras, a small G protein, has been shown to have a role in several fibrotic conditions. Farnesylthiosalicylic acid (FTS), a Ras inhibitor, increased IL-1β-induced COX-2 and prevented TGF-β1-induced reduction of COX-2. Previous studies suggest that COX-2 is epigenetically repressed. LBH589, a HDAC inhibitor, prevented TGF-β1-induced repressed COX-2 whereas BIX01294, a DNA lysine methyltransferase inhibitor, and RG108, a G9a histone methyltransferase inhibitor, both increased IL-1β-induced COX-2 in F-IPF.

In conclusion, the gradual loss of PGE_2/COX-2 anti-fibrotic mechanism during myofibroblast differentiation may contribute to the pathophysiology of pulmonary fibrosis and agents that increase cAMP levels, inhibit Ras or inhibit epigenetic repression of COX-2, may compensate for the lack of endogenous PGE_2.

Publications

John, M., S. Hussain, A. Prayle, **R. Simms**, J. R. Cockcroft, and C. E. Bolton, 2013, Target renal damage: the microvascular associations of increased aortic stiffness in patients with COPD: Respir Res, v. 14, p. 31

Amberbir, A., G. Medhin, W. Erku, A. Alem, **R. Simms**, K. Robinson, A. Fogarty, J. Britton, A. Venn, and G. Davey, 2011, Effects of Helicobacter pylori, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children: Clin Exp Allergy, v. 41, p. 1422-30.

Forrester, D., A. Knox, A. Smyth, H. Barr, **R. Simms,** S. Pacey, I. Pavord and D. Honeybourne, 2015, A Gluthamine Supplementationo for Cystic Fibrosis – A Randomised Placebo controlled trial. Pediatr. Pulmonol. doi: 10.1002/ppul.23370.

Abstracts

Simms R, Coward WR, Knox A and Pang L. Prostaglandin E2 promotes an anti-fibrotic phenotype in pulmonary fibroblasts via the E Prostanoid 2 receptor and cAMP signalling in Idiopathic Pulmonary Fibrosis. Accepted for poster presentation at the American Thoracic Society International Conference, San Francisco, 2012

Simms R, Coward WR, Knox A and Pang L. Prostaglandin E2 promotes an anti-fibrotic phenotype in pulmonary fibroblasts via the E Prostanoid 2 receptor and cAMP signalling in Idiopathic Pulmonary Fibrosis. Accepted for oral presentation at the Annual Institute for Lung Health Respiratory Science Research Meeting, Charnwood, UK, 2012.

Simms R, Coward WR, Knox A and Pang L. cAMP-mediated regulation of fibroblast to myofibroblast differentiation in Idiopathic Pulmonary Fibrosis. Accepted for poster presentation at the American Thoracic Society International Conference, Denver, 2011.

Simms R, Coward WR, Knox A and Pang L. cAMP-mediated regulation of fibroblast to myofibroblast differentiation in Idiopathic Pulmonary Fibrosis. Accepted for poster presentation at the European Respiratory Lung Science Conference, Estoril, Portugal, 2011.

Simms R, Coward WR, Knox A and Pang L. Identification of the sources of lung myofibroblasts using FSP-1 and α-SMA as markers in Idiopathic Pulmonary Fibrosis Accepted for poster discussion at the American Thoracic Society International Conference, New Orleans, 2010.

Acknowledgements

Firstly, I am extremely grateful to The National Institute of Health Research for funding this project. My sincere thanks go to my primary supervisor, Professor Linhua Pang for accepting me into his group. He continuously offered help, support and encouragement in all stages of my PhD and in writing this thesis, even after I had left my position at the University. I would also like to thank him for being open to my ideas and for encouraging and helping me to shape my interests and thoughts. I would like to thank Professor Alan Knox, my second supervisor for his encouragement, support and insightful knowledge.

I would also like to express my deep gratitude and respect to Dr William Coward whose advice and insight was invaluable to me. His knowledge and attitude to research inspired me to embark on my PhD and his patience, motivation, enthusiasm and immense knowledge has helped me constantly throughout. I thank him for his company during endless hours of tissue culture and for keeping a sense of humour when I had lost mine.

Every result described in this thesis was accomplished with the help and support of my fellow lab workers. Thank you to all my friends and colleagues at the Respiratory Research Unit who not only offered a great wealth of knowledge and advice, support and reassurance, but made my five years at the research unit so much fun. A heartfelt thank you goes to, Helen Bailey and Garry Meakin whose encouragement, friendship and smiles were sometimes all that kept me going.

Finally, I would like to thank my friends and family who supported me during my PhD. First and foremost I would like to thank my Mum and Dad for always believing in me and for their

continuous love and support. Both have instilled many admirable qualities in me; they've taught me about hard work and respect, perseverance, strength and character, each of which was needed to complete my PhD. A special thank you goes to my husband, Phil. I cannot begin to express my gratitude and feelings for this amazing man. Through his love, patience, support and unwavering belief in me I've been able to complete my PhD. His kindness and understanding during my PhD has been greatly appreciated especially during times when my temper was particularly trying.

I have laughed, cried and cursed throughout the course of my PhD and without all of my wonderful supporters by my side, especially the select few that I have mentioned, I would have not gotten to where I am today, at least not sanely.

Contents

1 INTRODUCTION 2

1.1 Idiopathic Lung Fibrosis 2

1.1.1 Epidemiology 2

1.1.2 Clinical Features 3

1.1.3 Pathology 5

1.1.4 Treatment 7

1.1.5 Pathogenesis 8

1.1.5.1 Epithelial Injury 10

1.1.5.2 Wound Healing 11

1.1.5.3 Dysregulated Wound Healing 12

1.1.5.3.1 Role of cytokines and growth factors in the pathogenesis of IPF 15

1.1.5.3.1.1 Interleukin-1β 15

1.1.5.3.1.2 Tumour necrosis factor-α 16

1.1.5.3.1.3 Platelet-derived growth factor 17

1.1.5.4 Defective coagulation in the pathogenesis of IPF 17

1.2 Myofibroblasts and IPF 20

1.2.1 Features and Functions of Myofibroblasts 21

1.2.2 Origins of Myofibroblasts 23

1.2.3 Differences between F-IPF and F-NL 28

1.2.4 Inducers and Inhibitors of Fibroblast to Myofibroblast Differentiation 28

1.2.4.1 Myofibroblast Inducing Factors 30

1.2.4.2 Myofibroblast Suppressing factors 32

1.3 TGF-β1 and Myofibroblast Differentiation 33

1.3.1 TGF-β1 Expression and Activation 34

1.3.1.1 TGF-β1 activation in Idiopathic Pulmonary Fibrosis 37

1.3.2 TGF-β1 Signalling 39

1.3.2.1 Canonical TGF-β1 Signalling 39

1.3.2.2 Non-canonical TGF-β1 Signalling 41

1.3.2.3 Canonical and Non-canonical TGF-β1 Cross Talk 45

1.3.3 Effect of TGF-β1 on Myofibroblast Differentiation 46

1.3.4 Importance of TGF-β1 in IPF 47

1.4 PGE_2 in IPF 50

1.4.1 PGE_2 Effect on Myofibroblast Differentiation 54

1.4.2 PGE_2 Production 54

1.4.2.1 COX-2 Expression in F-IPF 57

1.4.3 PGE_2 Receptors 58

1.4.4 PGE_2 Signalling 58

1.4.4.1 cAMP 59

1.4.4.2 cAMP Signalling 63

1.4.4.2.1 PKA 63

1.4.4.2.2 Epac 66

1.4.4.3 cAMP and TGF-β1 signalling 67

1.5 Effect of cAMP Stimulants on Myofibroblast Differentiation 68

1.5.1 Potential Therapeutic effects of cAMP Stimulants in IPF 70

1.6 Ras 72

1.6.1 Ras protein cycle: activation/inactivation 74

1.6.2 Ras Signalling Pathways 78

1.6.3 Ras/MAPK, TGF-β1 and cAMP Crosstalk 81

1.6.4 The importance of Ras in Fibrosis 85

1.7 Epigenetic regulation of gene transcription 88

1.7.1 Histone Modifications 92

1.7.1.1 Histone Acetylation/Deacetylation 92

1.7.1.2 Histone Methylation 93

1.7.2 DNA Methylation 94

1.8 Evidence of Epigenetic Regulation in Myofibroblast Differentiation 95

1.8.1 Epigenetic regulation of TGF-β1-induced Fibroblast to Myofibroblast Differentiation 95

1.8.2 Epigenetic regulation of COX-2 Expression 96

1.8.3 Epigenetic Regulation by cAMP 97

1.9 Summary 98

1.10 Hypothesis and Aims 99

2 METHODS 103

2.1 Introduction 103

2.2 Cell Culture 103

2.2.1 Primary Human Lung Fibroblasts 103

2.2.2 Freezing Cells 107
2.2.3 Cell Counting 107
2.2.4 Materials 107
2.3 Bicinchoninic Acid (BCA) Protein Assay 108
2.4 Western Blot 108
2.4.1 Principle of Assay 108
2.4.2 Cell Lysis 108
2.4.3 Protein Sample Preparation 109
2.4.4 Gel Electrophoresis 109
2.4.5 Protein Transfer 109
2.4.6 Protein Detection 110
2.5 cAMP Assay 111
2.5.1 Principle of Assay 111
2.5.2 cAMP Extraction 111
2.5.3 cAMP Radiation Assay 112
2.6 Immunocytochemistry 112
2.6.1 Principle of Assay 112
2.6.2 Cell Staining 112
2.7 Reverse Transcriptase Polymerase Chain Reaction (RT- PCR) 113
2.7.1 Principle of Assay 113
2.7.2 RNA Isolation 119
2.7.3 Reverse Transcription 120
2.7.4 Quantitative PCR 120

2.8 Active Ras Pull Down Assay 121
2.8.1 Principle of Assay 121
2.8.2 Active Ras Pull Down Method 121
2.8.3 Active Ras Detection 122
2.9 PGE_2 EIA 123
2.9.1 Principle of Assay 123
2.9.2 PGE_2 EIA Method 123
2.10 Cell Viability 124
2.11 Statistical Analysis 125
3 THE EFFECT OF PGE_2 ON FIBROBLAST TO MYOFIBROBLAST DIFFERENITATION 127
3.1 Introduction 127
3.2 Aims 130
3.3 Experimental Protocol 131
3.4 Results 135
3.4.1 F-IPF have a pro-fibrotic phenotype compared with F-NL 135
3.4.2 PGE_2 reverses the fibrotic phenotype in F-IPF 143
3.4.3 TGF-β1 promotes a pro-fibrotic phenotype in F-NL 153
3.4.4 PGE_2 prevents the pro-fibrotic effects of TGF-β1 164
3.4.5 The anti-fibrotic effects of PGE_2 are via the EP2 receptor 172
3.5 Discussion 181
4 THE EFFECT OF THE CAMP ELEVATING AGENTS ON FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION 186

4.1 Introduction 186

4.2 Aims 188

4.3 Experimental Protocol 189

4.4 Results 191

4.4.1 cAMP Production is comparable in F-NL and F-IPF 191

4.4.2 cAMP Elevating Agents Reverse the Fibrotic Phenotype in F-IPF 195

4.4.3 cAMP Elevating Agents Prevent the Pro-fibrotic Effects of TGF-β1 208

4.4.4 The Anti-fibrotic Effects of PGE_2 are via the EP2 Receptor 214

4.4.5 EP2 Receptor expression is greater than EP4 expression in both F-NL and F-IPF 216

4.4.6 The Anti-fibrotic Effects of PGE_2 are Mediated by PKA but not Epac Activation 220

4.4.7 Effect of Roflumilast on PGE_2-induced Fibroblast to Myofibroblast Differentiation 225

4.5 Discussion 229

5 ROLE OF RAS SIGNALLING PATHWAY IN COX-2 EXPRESSION AND FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION 236

5.1 Introduction 236

5.2 Aims 239

5.3 Experimental Protocol 240

5.4 Results 242

5.4.1 FTS induces COX-2 in F-IPF and promotes an anti-fibrotic phenotype 242

_Toc446929689

5.4.2 FTS Prevents TGF-β1-induced repression of COX-2 and fibroblast to myofibroblast differentiation in F-NL 250

5.4.3 FTS does not directly effect cAMP production in F-NL or F-IPF 257

5.4.4 Ras, Raf-1 and B-Raf activity is similar in F-NL and F-IPF but ERK-1/2 expression is reduced in F-IPF 260

5.4.5 TGF-β1 and PGE_2 treatment rapidly activates the Ras/Raf/ERK signalling pathway 263

5.4.6 Chronic TGF-β1 and PGE_2 Treatment has no effect on Ras activity but chronic PGE_2 treatment reduces ERK-1/2 activity 269

5.5 Discussion 275

6 EFFECT OF EPIGENETIC INHIBITORS ON COX-2 EXPRESSION AND FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION 279

6.1 Introduction 279

6.2 Aims 281

6.3 Experimental Protocol 282

6.4 Results 284

6.4.1 Histone acetylation is responsible for promoting a pro-fibrotic phenotype in TGF-β1-induced fibroblast to myofibroblast differentiation 284

6.4.2 Histone acetylation, H3K9 methylation and DNA methylation are involved in maintaining a pro-fibrotic phenotype in F-IPF 288

6.5 Discussion 292

7 CONCLUSIONS AND FUTURE STUDIES 297

7.1 Conclusions 297

7.2 Future Directions 301

8 APPENDIX 305
8.1 Materials 305
8.2 Reagents 306
8.3 Antibodies 309
8.4 Kits 310
8.5 Western Blot Buffers 311
8.5.1 RIPA Buffer 311
8.5.2 Buffer 1 311
8.5.3 Buffer 2 311
8.5.4 Resolving Gel 312
8.5.5 Stacking Gel 312
8.5.6 Lamellae Buffer 312
8.5.7 10X Running Buffer 313
8.5.8 10X Transfer Buffer 313
8.5.9 1X Transfer Buffer 313
8.5.10 10X Tris Buffered Saline with Tween (TBST) 313
8.6 Immunocytochemistry Buffers 314
8.6.1 Blocking Buffer 314
8.7 PCR Primers and RT-PCR cycling conditions 315
9 REFERENCES 317

List of Figures

Figure 1-1 Schematic representation of potential clinical courses of IPF 4

Figure 1-2 Histopathological Features of IPF 6

Figure 1-3 Fibroblastic Foci 6

Figure 1-4 Key Events in the Pathogenesis of IPF 14

Figure 1-5 Pro-fibrotic Microenvironment in IPF Lung 19

Figure 1-6 Origins of Myofibroblasts in IPF 27

Figure 1-7 The Structure of Latent TGF-β1 36

Figure 1-8 Schematic diagram of TGF-β1 signalling pathways 44

Figure 1-9 Pro-fibrotic effects of TGF-β1 49

Figure 1-10 Anti-fibrotic effects of PGE_2 53

Figure 1-11 Biosynthetic Pathways and Receptors for Prostaglandins and Leukotrienes 56

Figure 1-12 Overview of the Phosphodiesterase Families 62

Figure 1-13 cAMP signalling via PKA and Epac 65

Figure 1-14 The GDP/GTP cycle of Ras 75

Figure 1-15 The Activation of Ras 77

Figure 1-16 Signalling cascades downstream of Ras 79

Figure 1-17 Rap1 activation by cAMP regulates ERK 84

Figure 1-18 The Structure of a nucleosome, euchromatin and heterochromatin 90

Figure 1-19 Schematic Diagram of Hypothesis 100

Figure 2-1. Conversion of mRNA to cDNA by Reverse Transcriptase 115

Figure 2-2. Schematic diagram of PCR Thermal Profile 118

Figure 3-1 Experimental Protocol for time course experiments 131

Figure 3-2 Experimental Protocol for TGF-β Removal Experiments 132

Figure 3-3 Experimental Protocol for PGE_2 and TGF-β experiments 133

Figure 3-4 Experimental Protocol for measuring PGE_2 production following treatment with PGE_2 134

Figure 3-5 COX-2, α-SMA and Col 1 Protein Expression in F-NL and F-IPF 137

Figure 3-6 COX-2 mRNA Expression in F-NL and F-IPF 139

Figure 3-7 α-SMA and Col 1 mRNA Expression in F-NL and F-IPF 140

Figure 3-8 PGE_2 Production in F-NL and F-IPF 142

Figure 3-9 Effect of PGE_2 on COX-2 and α-SMA Protein Expression in F-IPF (time course) 145

Figure 3-10 Effect of PGE_2 on COX-2 and α-SMA Protein Expression in F-IPF (concentration-response) 146

Figure 3-11 Effect of PGE_2 on Collagen 1 Protein Expression in F-IPF 147

Figure 3-12 Effect of PGE_2 treatment on endogenous PGE_2 production in F-IPF 149

Figure 3-13 Effect of PGE_2 on COX-2 mRNA in F-IPF 151

Figure 3-14 Effect of PGE_2 on α-SMA and Col 1 mRNA in F-IPF 152

Figure 3-15 Effect of TGF-β1 on COX-2, α-SMA and Col 1 Protein in F-NL 155

Figure 3-16 Effect of TGF-β1 on endogenous PGE_2 production in F-NL 157

Figure 3-17 Long Term Effects of TGF-β1 in F-NL 159

Figure 3-18 Effect of TGF-β1 on COX-2 mRNA in F-NL 162

Figure 3-19 Effect of TGF-β1 time course on α-SMA and Col 1 mRNA in F-NL 163

Figure 3-20 Effect of PGE_2 on TGF-β1-induced Fibroblast to Myofibroblast Differentiation 167

Figure 3-21 Effect of PGE_2 on endogenous PGE_2 production in TGF-β1-treated F-NL..... 168

Figure 3-22 Effect of PGE_2 on COX-2 mRNA in TGF-β1-treated F-NL 170

Figure 3-23 Effect of PGE_2 on α-SMA and Col 1 mRNA in TGF-β1 treated F-NL 171

Figure 3-24 Effect of EP2 Agonist (ONO-AE1-259) Time Course on COX-2 and α-SMA Protein Expression in F-IPF 173

Figure 3-25 Effect of EP2 Agonist (ONO-AE1-259) Dose Response on COX-2 and α-SMA Protein Expression in F-IPF 175

Figure 3-26 Effect of EP4 Agonist (ONO-AE1-329) Time course on COX-2 and α-SMA Protein Expression in F-IPF 177

Figure 3-27 Effect of EP4 Agonist (ONO-AE1-329) Dose Response on COX-2 and -SMA Protein Expression in F-IPF 179

Figure 4-1 Experimental Protocol to test if cAMP elevating agents (PGE_2, Form, Salme and FSK) can prevent pro-fibrotic effects of TGF-β. 189

Figure 4-2 Experimental Protocol to test the effect of PGE_2, Form, Salme and FSK on cAMP Production..... 190

Figure 4-3 Effect of PGE_2 on cAMP Production in F-NL and F-IPF (Time Course). 193

Figure 4-4 Effect of PGE_2, Form and FSK on cAMP Production in F-NL and F-IPF..... 194

Figure 4-5 Effect of Form on COX-2 and α-SMA Protein Expression in F-IPF..... 196

Figure 4-6 Effect of Salme on COX-2 and α-SMA Protein Expression F-IPF..... 198

Figure 4-7 Effect of FSK on COX-2 and α-SMA Expression in F-IPF 200

Figure 4-8 Effect of PGE_2, Form and FSK on Col 1 Protein Expression in F-IPF..... 202

Figure 4-9 Effect of PGE_2, Form and FSK on Endogenous PGE_2 Production in F-IPF..... 204

Figure 4-10 Effect of PGE_2 and FSK on COX-2 mRNA Expression in F-IPF 206

Figure 4-11 Effect of PGE_2 and FSK on α-SMA and Col 1 mRNA in F-IPF 207

Figure 4-12 Effect of FSK on TGF-β1-induced Fibroblast to Myofibroblast Differentiation. 210

Figure 4-13 Effect of FSK on COX-2 mRNA Expression in TGF-β1-treated F-NL 212

Figure 4-14 Effect of FSK on α-SMA and Col 1 mRNA in TGF-β1-treated F-NL..... 213

Figure 4-15 Effect of AH6809 and ONO-AE2-227 on PGE_2-induced cAMP Production in F-NL and F-IPF..... 215

Figure 4-16 EP2 and EP4 Reception Expression and Mean Fluorescence Intensity in F-NL and F-IPF..... 217

Figure 4-17 EP2 and EP4 Receptor Protein Expression in F-NL and F-IPF 219

Figure 4-18 Effect of PKA Agonist, 6-Bnz-cAMP, on COX-2 and α-SMA Protein Expression in F-IPF..... 221

Figure 4-19 Effect of Epac agonist, 8-pCT-2'O-Me-cAMP, on COX-2 and -SMA Protein Expression in F-IPF 223

Figure 4-20 Effect of Roflumilast on COX-2 and α-SMA Protein Expression in F-IPF..... 226

Figure 4-21 Effect of Roflumilast on cAMP Production in F-IPF 228

Figure 5-1 Experimental Protocol for effect of PGE_2 and TGF-β on Ras Signalling Pathways 240

Figure 5-2 Detection of Ras-GTP and Effect of FTS on Ras-GTP in F-IPF 244

Figure 5-3 Effect of FTS on COX-2 and α-SMA in F-IPF 245

Figure 5-4 Effect of FTS on Col 1 Protein Expression in F-IPF 246

Figure 5-5 Effect of FTS on COX-2 mRNA Expression in F-IPF 248

Figure 5-6 Effect of FTS on α-SMA and Col 1 mRNA Expression in F-IPF 249

Figure 5-7 Effect of FTS on COX-2, α-SMA and Col 1 Protein in TGF-β1-treated F-NL 252

Figure 5-8 Effect of FTS on COX-2 mRNA Expression in TGF-β1-treated F-NL 255

Figure 5-9 Effect of FTS on α-SMA and Col 1 mRNA Expression in TGF-β1 treated F-NL 256

Figure 5-10 Effect of FTS on cAMP Production in F-NL and F-IPF 258

Figure 5-11 Effect of FTS Treatment on PGE_2, Form and FSK-induced cAMP Production in F-NL and F-IPF 259

Figure 5-12 Ras, Raf-1, B-Raf and ERK-1/2 Protein Expression in F-NL and F-IPF 261

Figure 5-13 Effect of TGF-β1 on Ras Activity in F-NL 265

Figure 5-14 Effect of PGE_2 on Ras Activity in F-IPF 266

Figure 5-15 Effect of TGF-β1 on Phosphorylated ERK1/2 in F-NL 267

Figure 5-16 Effect of PGE_2 on Phosphorylated ERK1/2 in F-IPF 268

Figure 5-17 Effect of Chronic TGF-β1 and PGE_2 Treatment on Ras Activity in F-NL and F-IPF 271

Figure 5-18 Effect of Chronic TGF-β1 and PGE_2 Treatment on ERK Phosphorylation in F-NL and F-IPF 274

Figure 6-1 Experimental Protocol to assess the effect of epigenetic inhibitors in F-IPF 282

Figure 6-2 Experimental protocol of F-NL treated with epigenetic inhibitors 283

Figure 6-3 The Effect of LBH589, BIX01294 and RG108 on COX-2 and α-SMA Protein Expression in TGF-β1-treated F-NL 286

Figure 6-4 The Effect of LBH589, BIX01294 and RG108 on Col 1 Protein Expression in TGF-β1-treated F-NL 287

Figure 6-5 The Effect of BIX01294, LBH589 and RG108 on COX-2 and α-SMA Protein Expression in F-IPF 289

Figure 6-6 The Effect of BIX01294, LBH589 and RG108 on Col 1 Protein Expression in F-IPF 291

List of Tables

Table 1-1 Myofibroblast Modulating Factors in IPF 29

Table 2-1 Demographic data of F-NL and F-IPF 105

Abbreviations

α-SMA	Alpha-Smooth Muscle Actin
AA	Arachidonic Acid
AC	Adenylyl Cyclase
AEC	Airway Epithelial Cell
Akt	Protein Kinase B
ALK-5	TGF-β1 Receptor Inhibitor-1
AMP	Adenosine Monophosphate
AT1	Type I Airway Epithelial Cell
AT2	Type II Airway Epithelial Cell
ATP	Adenosine Triphosphate
B2M	Beta-2-Microglobulin
BLT	Leukotriene B Receptor
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
cGMP	Cyclic Guanosine Monophosphate
Col 1	Collagen Type I
COPD	Chronic Obstructive Pulmonary Disease

Co-Smad	Common Partner Smad Protein
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphoguanine Dinucleotide
CRE	cAMP Response Element
CREB	cAMP-response-element Binding Protein
CTGF	Connective Tissue Growth Factor
CXCL12	C-X-C Chemokine Ligand Type 12
CXCR4	C-X-C Chemokine Receptor Type 4
CysLT	Cysteinyl-leukotriene
DAPI	4’,6-diamidino-2-phenylindole
DLCO	Diffusing capacity of the lung for carbon monoxide
DMEM	Dulbecco’s Modified Eagle’s Medium
Dnmt	DNA Methyltransferases
dNTPS	Deoxynucleoside Triphosphates
EBV	Epstein Barr Virus
ECM	Extracellular Matrix
ED-A Fibronectin	Extra Type III Domain A
EGF	Epidermal Growth Factor
ELK-1	ETS Domain Containing Protein

Endo-MT	Endothelial to Mesenchymal Transition
EMT	Epithelial to Mesenchymal Transition
EP	E Prostanoid Receptor
Epac	Exchange Protein Activated by cAMP
ERK	Extracellular Signal Regulated Kinase
ET-1	Endothelial
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
F-IPF	Fibroblasts isolated from patients with Idiopathic Pulmonary Fibrosis
FLAP	5-LO Activating Protein
F-NL	Fibroblasts from patients without Idiopathic Pulmonary Fibrosis
Form	Formoterol
FSK	Forskolin
FSP1	Fibroblast Specific Protein-1
FTS	Farnesylthiosalicylic Acid
FVC	Forced Vital Capacity
G Protein	GTP-Binding Protein
GAP	GTP-Activating Protein

GAPDH	Glyceraldehyde-3-Phosphate
GDP	Guanine Tyrosine Diphosphate
GEF	Guanine Nucleotide Exchange Factors
GTP	Guanine Tyrosine Triphosphate
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylases
HDMase	Histone Demethylases
HGF	Hepatocyte Growth Factor
HMTase	Histone Methyltransferases
HP1	Heterochromatin Protein-1
IBMX	3-isobutyl-1-methylxanthine
IFN-γ	Interferon-γ
IGF-1	Insulin Like Growth Factor-1
IL-13	Interleukin 13
IL-14	Interleukin 14
IL-1β	Interleukin -1β
ILD	Interstitial Lung Disease
IPF	Idiopathic Pulmonary Fibrosis
IP-10	Interferon Gamma Induced Protein 10
I-Smad	Inhibitory Smad Protein

JNK	c-Jun N Terminal Kinase
LAP	Latency-associated Peptide
LLC	Large Latent Complex
LPS	Lipopolysaccharide
LT	Leukotriene
LTBP	Latent TGF-β1 Binding Protein
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase Pathway
MCP-1	Monocyte Chemotactic Protein-1
MeCP2	Methyl-CpG-Binding Protein
MEK	Mitogen Activated Protein Kinase Kinase
MET	Mesenchymal to Epithelial Transition
MHC-II	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
NFκB	Nuclear Factor Kappa B
NK	Natural Killer Cells
PAI-1	Plasminogen Activator Inhibitor-1
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase

PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
PGE_2	Prostaglandin E2
PGH_2	Prostaglandin H2
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3 Kinase-Akt-mTor
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PVDF	Polyvinylidene Fluoride
RIPA	Radioimmunoprecipitation Assay Buffer
RALGDS	Ral Guanine Nucleotide Dissociation Stimulator
Rof	Roflumilast
RTK	Receptor Tyrosine Kinase
R-Smad	Receptor mediated Smad Protein
Salme	Salmeterol
Sapks	Stress activated protein kinases
SDS-PAGE Electrophoresis	Sodium Dodecyl Sulphate Polyacrylamide Gel

siRNA	Small Interfering RNA
SLC	Small Latent Complex
TBST	Tris Buffered Saline plus Tween
TF	Transcription Factor
TGF-α	Transforming Growth Factor-α
TGF-β1	Transforming Growth Factor-β
TH1	Type 1 T Helper Cells
TH2	Type 2 T Helper cells
TLR	Toll like Receptors
TNF-α	Tumour Necrosis Factor-α
TRAF6	(TNF)-Receptor-associated Factor 6
TSP-1	Thrombospondin-1
5-LO	5-lipoxygenase

INTRODUCTION

1 INTRODUCTION

1.1 Idiopathic Lung Fibrosis

Idiopathic lung fibrosis (IPF) is a progressive and fibroliferative lung disease of unknown aetiology (Wynn, 2008). The disease is characterised by epithelial cell activation and injury, abnormal tissue repair, accumulation of fibroblasts and myofibroblasts ("fibroblast foci") and excessive extracellular matrix accumulation within the pulmonary interstitium. These key pathological processes lead to hardening and scarring of the lung resulting in irreversible disruption of the lung architect, progressive worsening of pulmonary function and ultimately respiratory failure (Gross and Hunninghake, 2001). Despite extensive research efforts no currently available therapy has been shown to either reverse or even halt the progression of this disorder. Therefore, the identification of novel therapeutic targets is urgently needed (Wells et al., 2008).

1.1.1 Epidemiology

IPF is one lung disease out of a diverse group of lung disorders known as interstitial lung disease (ILD). Although ILDs are different in a variety of features they are grouped together because they share many clinical and physiological features (Pardo and Selman, 2002). Out of the 150 types of ILD IPF is the most common accounting for 50-60% of all cases, and the most fatal (Wang, 2009). IPF is more prevalent in middle aged and elderly males, median age at diagnosis is 66 years old (2000; Hubbard et al., 1996) who are current or former smokers (Baumgartner et al., 1997). In the UK, IPF has an estimated incident rate of 4.6 cases per 100,000 people and there is evidence to suggest this is increasing (Hubbard et al., 1996). With a 5 year survival rate of 43% and a median survival of 2.4 years after diagnosis (Gribbin et al., 2006) it is not surprising that IPF has a prognosis poorer than some cancers.

1.1.2 Clinical Features

Initial signs of IPF include the gradual onset of a non-productive cough, shortness of breath (dyspnoea) and fine basal inspiratory crackles on the chest which progressively worsen over months to years (Wells et al., 2008). Lung abnormalities include: thickening of the bronchioles, honeycomb cysts (fibrotic air spaces) and fibroblast foci. Fibroblast foci are a key feature of actively ongoing fibrosis and a major prognostic factor for IPF patients (Epler et al., 1978).

IPF has a variable clinical course which makes prognosis difficult to evaluate. The classic clinical phenotype of IPF is one of slowly progressive decline in lung function leading to death within several years of diagnosis. However, it has been demonstrated that a subgroup of IPF patients have a rapidly progressive course with shortened survival compared with the patients following the progressive clinical course (Selman et al., 2007). The different clinical phenotypes and distinct patterns of comorbidities and survival are currently being defined (Cottin, 2013). So far, clinical predictors of increased mortality in IPF have been identified and include age (over 70 years of age), smoking history, low body-mass index, pulmonary hypertension or a clinical exacerbation, a period of acute deterioration in respiratory function either due to known complications, such as infection, or of unknown cause (Selman et al., 2011). To improve prognosis, a multidisciplinary staging system has recently been developed for IPF using commonly measured clinical and physiological variables. Four variables were included in the final model: sex, age, forced vital capacity (FVC), a common, spirometry measurement and diffusing capacity of the lung for carbon monoxide (DLCO), a test that measures the extent of oxygen transfer from the alveoli into the blood (Ley et al., 2012).

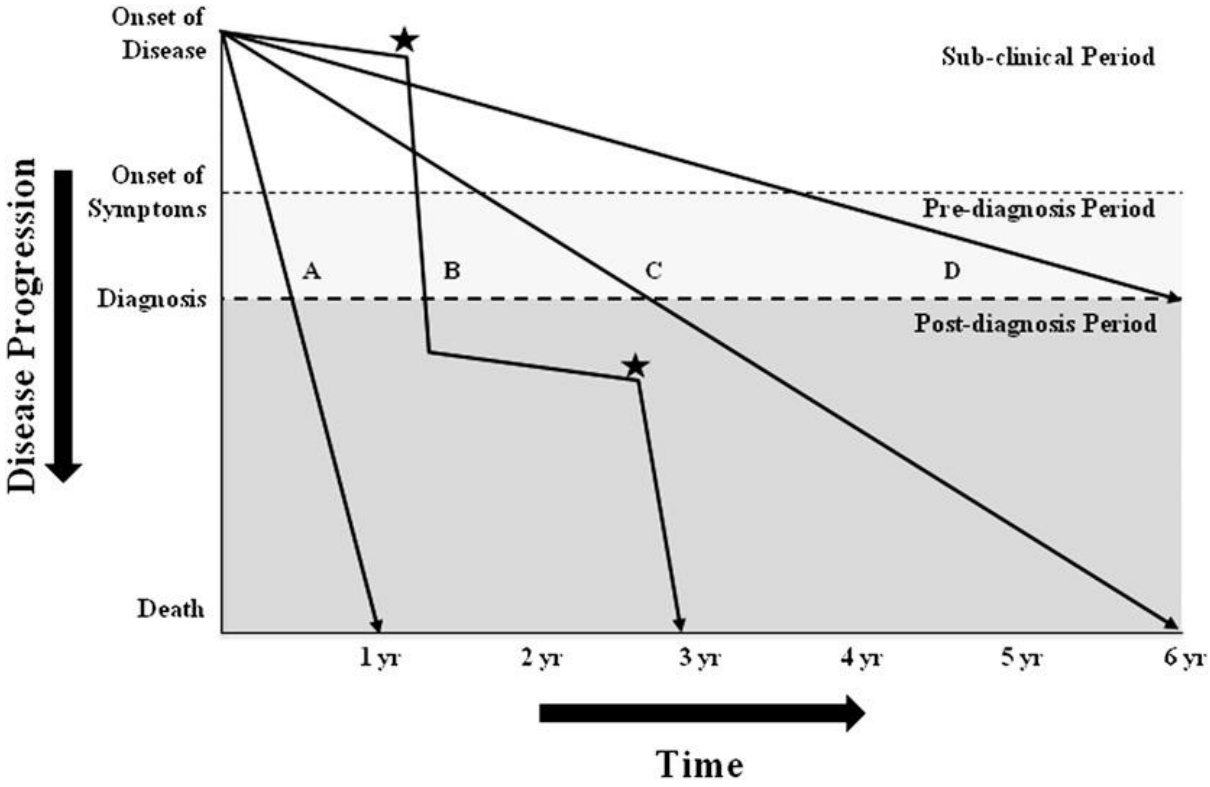


Figure 1-1 Schematic representation of potential clinical courses of IPF

The rate of decline and progression to death in IPF patients may take several clinical forms as demonstrated by this schematic diagram. As IPF progresses there is a subclinical period which can only be identified by radiographic finds. Following this is a symptomatic period consisting of both pre-diagnosis and post-diagnosis clinical phases. The rate of respiratory decline may be rapid (line A), slow (lines C and D) or mixed (line B). In addition, there may be periods of relatively stable disease progression and periods of acute decline known as exacerbations (star) (Ley et al., 2011).

1.1.3 Pathology

Pathological examination allows IPF to be distinguished from other interstitial lung diseases that have similar histological features. Histological features include heterogeneous appearance, with alternating areas of inflammation, honeycombing (cystic spaces due to destruction of the lung architecture), fibroblast foci (aggregates of proliferated fibroblasts and myofibroblasts observed within the honeycomb lesions) and normal lung architecture (Katzenstein and Myers, 1998) (Figure 1-2). In addition, overproduction and disorganised deposition of collagen and patchy epithelial damage are also observed (Figure 1-3).

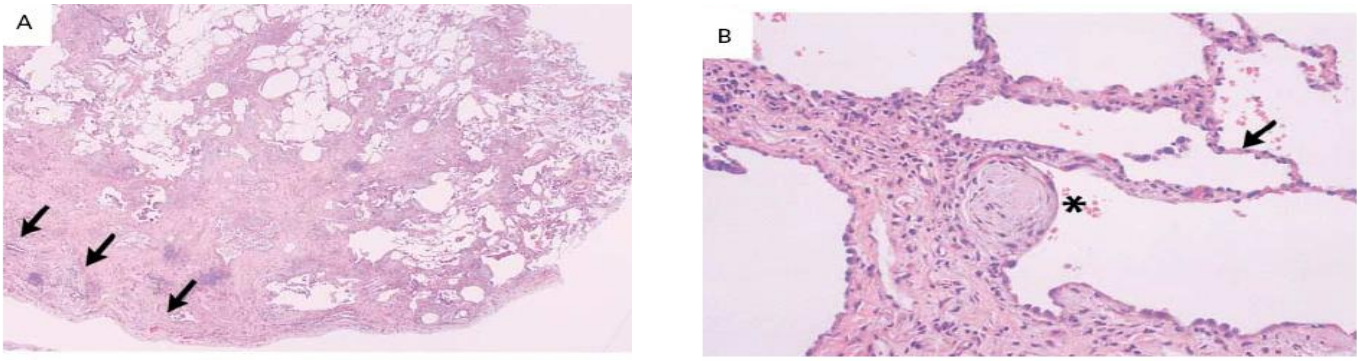


Figure 1-2 Histopathological Features of IPF

A haematoxylin and eosin preparation of an open lung biopsy specimen. A: Dense fibrosis and collapsed air spaces (arrows). B: Pathological heterogeneity is exemplified by dense scarring and a fibroblastic focus (asterisk) adjacent to the relatively normal alveolar septa (arrow) (Gross and Hunninghake, 2001)

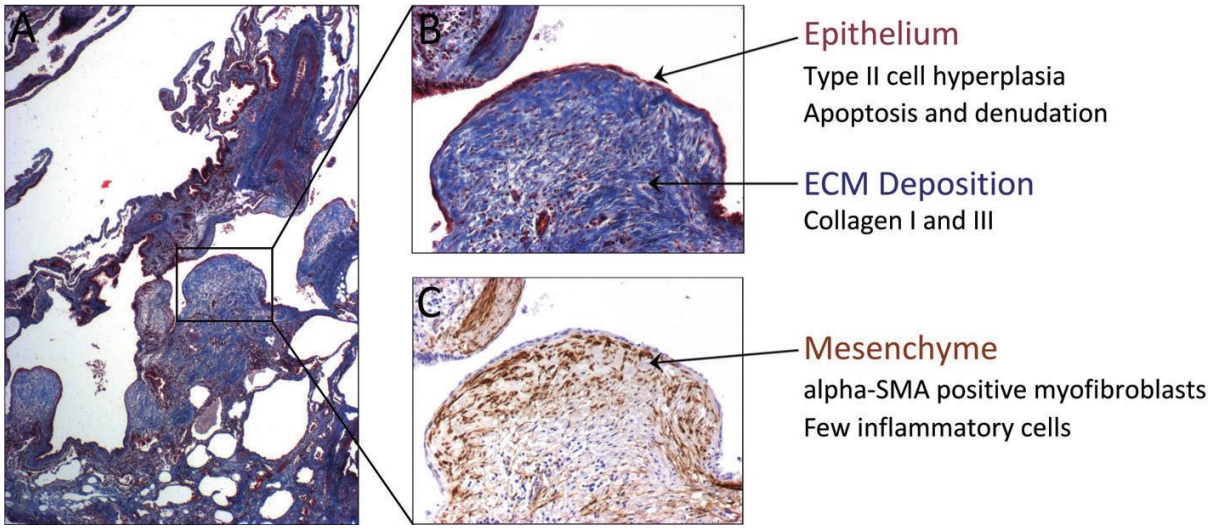


Figure 1-3 Fibroblastic Foci

Fibroblastic foci are the histological hallmark of IPF. A) The fibroblastic focus is the accumulation of fibroblasts and myofibroblasts which are highly synthetic for collagen and have a contractile phenotype B) Histological analysis of a human IPF lung section shows epithelial damage and dense collagen deposition (Blue staining, x10 magnification). C) Immunohistochemistry showing α-SMA-positive myofibroblasts (x20 magnification) (Datta et al., 2011).

1.1.4 Treatment

Historically, IPF was believed to result from chronic inflammation. Therefore, established treatment is based on suppressing the inflammatory response through the use of anti-inflammatory or immunosuppressive drugs. From retrospective studies it is now clear that therapies using anti-inflammatory treatment have little or no clinical benefit and have serious side effects such as indigestion, stomach ulcers. (Lynch and McCune, 1997; Wells et al., 2008). More recently, a clinical trial known as the PANTHER trial investigated the safety and efficacy of a combination of prednisone, azathioprine and N-acetylcystein (Raghu et al., 2012). The study concluded that there was an increased risk of death and hospitalisation in IPF patients treated with a combination of prednisone, azathioprine and N-acetylcystein, as compared with placebo. These findings provide further evidence against the use of combined immunosuppressive therapy in IPF patients.

Accordingly, more recent clinical trials have shifted their focus from anti-inflammatory and immunosuppressant compounds to molecules targeting growth factors, the wound healing cascade and fibrogenesis and have demonstrated that slowing disease progression is possible. Pirfenidone is a compound with anti-fibrotic, anti-inflammatory and anti-oxidant properties and is a recommended treatment for some IPF patients (Landells et al., 2013). Although its precise mechanism of action remains incompletely understood it is likely that Pirfenidone exerts its affects by suppressing fibroblast proliferation, reducing the production of fibroblast-associated pro-fibrotic cytokines and reducing the response to growth factors such as TGF-β1 (Landells et al., 2013). The ASCEND study investigated the safety and efficacy of Pirfenidone in IPF patients (King et al., 2014). The study concluded that Pirfenidone, as compared with placebo, reduced disease progression as reflected by lung function, exercise tolerance, and progression-free survival, in patients with IPF. Patients treated with Pirfenidone had acceptable side effects and fewer deaths (King et al., 2014). The IMPULSIS study evaluated the safety and efficacy of Nintedanib in IPF patients (Richeldi et

al., 2014). Nintedanib is an intracellular inhibitor that targets multiple tyrosine kinases including VEGF, FGF and PDGF receptors (Hilberg et al., 2008). Data from the IMPULSIS trial showed that in patients with IPF, Nintedanib reduced the decline in FVC and showed an acceptable safety profile (Hilberg et al., 2008).

The observations from both the ASCEND and IMPULSIS study suggest that drugs with treatment effects are pleotropic in their mechanisms and multiple mediators and signalling pathways are involved in disease pathogenesis and as such effective therapies will need to target pro-fibrotic signalling pathways at multiple levels.

Lung transplantation is the only treatment for IPF with proven beneficial effects, however, this has several contraindications and most patients are not eligible due to old age, complicating medical conditions and a shortage of organ donators (Wells et al., 2008). The limited treatment options available for IPF emphasises the demand for novel therapeutic strategies. There are many potential targets being evaluated in on-going clinical trials including agents that inhibit epithelial cell damage, prevent fibroblast proliferation and differentiation, and agents that down regulate collagen synthesis (Datta et al., 2011; Gharaee-Kermani et al., 2007). It is important to note that probably no single agent will be sufficient for this complex disease and a combination of drugs acting synergistically to inhibit fibroblast proliferation/differentiation and enhance re-epithelialisation will be necessary to improve clinical outcome.

1.1.5 Pathogenesis

The pathogenesis of IPF is currently unknown although a number of risk factors have been identified. These include cigarette smoking (Baumgartner et al., 1997), viral infections such as

Epstein-Barr virus and Herpes virus (Baumgartner et al., 1997; Stewart et al., 1999), gastroesophageal reflux (Raghu et al., 2006), age and male predominance (Hubbard et al., 1996). In addition, there is evidence to suggest a genetic predisposition to IPF with up to 4% of patients with IPF suffering from a familial form known as familial pulmonary fibrosis (FPF) (Coward et al., 2010a; Kottmann et al., 2009). Although the nature of any genetic component is at present unknown, polymorphic genes for a number of fibrogenic growth factors have been identified (Awad et al., 1998; Blom et al., 2001; Whyte et al., 2000) However, as only a small number of individuals exposed to known risk factors develop IPF, the pathogenesis is likely due to multiple factors. Significant advances in research have been made over the last decade and the pathogenic mechanisms underlying the development of IPF are starting to be distinguished (Strieter and Mehrad, 2009).

The initial hypothesis assumed that fibrosis was a result of chronic inflammation (alveolitis) due to the production of fibrogenic mediators from recruited inflammatory cells (Crystal et al., 1976; Keogh and Crystal, 1982). It was this view that led to the belief that fibrosis could be prevented through inhibition of the inflammatory response. This hypothesis was called into question based on two clinical observations: 1) tissue inflammation does not correlate with the severity or outcome of fibrosis and 2) anti-inflammatory drugs and cytotoxic treatment have no beneficial effects on IPF prognosis (Raghu et al., 2012; Strieter and Mehrad, 2009). Furthermore, experimental evidence also questioned the inflammatory hypothesis as over expression of TGF-β1, a potent pro-fibrotic mediator, leads to progressive fibrosis in mice without any significant inflammation (Sime et al., 1997). This theory of “inflammatory fibrosis” might represent the pathogenesis of the majority of interstitial lung diseases whereby inflammation precedes and provokes fibrosis but inflammation does not seem to be the driving mechanism in the pathogenesis of IPF.

The above observations lead to the hypothesis that IPF proceeds independently of inflammation. Instead, it is suggested that fibrosis occurs as a result of repeated subclinical epithelial injury that triggering a series of repair pathways that are in some way aberrant (Strieter and Mehrad, 2009). It is also argued that the disease becomes more extensive due to repeated injury at different sites within the lung, such that at any one time there are multifocal areas of pathology, each at a different stage of development which could explain the temporal heterogeneity of IPF (du Bois, 2010). The aberrant repair process in IPF patients is likely to be mediated by inadequate repair of the epithelial membrane accompanied by impaired regulation of the myofibroblast allowing fibrosis to proceed without restraint.

1.1.5.1 Epithelial Injury

The events that initially cause epithelial cell damage remain largely unknown. However, taking into account the long pre-clinical phase of the disease it is probably not due to one single insult but a combination of different injuries acting on a more susceptible individual to trigger the disease (Selman and Pardo, 2006). Recent studies have suggested that viral infections (Dworniczak et al., 2004; Tang et al., 2003; Tsukamoto et al., 2000), auto-antibodies (Fischer et al., 2006), gastroesophageal reflux (Raghu et al., 2006), exposure to environmental pollutants and tobacco smoke (Taskar and Coultas, 2006) are all potential sources of repetitive injury to the alveolar epithelium and are associated with an increased risk of IPF. In addition, it is hypothesised that reconstitution of a damaged epithelial barrier may be less efficient compared with younger subjects which could explain why ageing is associated with disease initiation (Selman et al., 2004).

1.1.5.2 Wound Healing

Following injury to the lung it is paramount that tissue architecture is restored in order to regain normal organ function. Damaged epithelial cells therefore need to be replaced to maintain barrier function and integrity. This requires coordinated, spatially and temporally regulated responses, including inflammatory responses, activation of local coagulation pathways and the formation of a provisional matrix which myofibroblasts migrate to in order to promote wound contraction (Coward et al., 2010a).

The alveolus is composed of two types of epithelial cells, type 1 (AT1) and type 2 (AT2), which adhere to the alveolar capillary basement membrane. AT1 cells cover more than 90% of the alveolar surface area and provide a permeable surface for gas exchange. Under homeostatic conditions AT1 cells regulate fibroblasts through the secretion of various mediators and cell-cell contact. On the other hand, AT2 cells are multifunctional cells which act as progenitor cells for AT1 cells (Adamson et al., 1988). Following lung injury and epithelial damage, AT2 cells proliferate, migrate and re-differentiate into both AT1 and AT2 cells and regenerate the damaged area of the lung. Once the epithelium is repaired hyperplastic AT2 cells will undergo regulated apoptosis (Griffiths et al., 2005). In this regenerative phase of the repair process, damaged cells are replaced by cells of the same type, leaving no lasting evidence of damage. Meanwhile, myofibroblasts are recruited and activated at the site of tissue injury. Myofibroblasts deposit extracellular matrix (ECM) proteins, such as collagen, to provide a temporary scaffold for normal tissue repair. Subsequent contraction of myofibroblasts within this matrix closes the epithelial margins and allows re-epithelialisation (Selman and Pardo, 2006). This regenerative phase of repair resolves via apoptosis of fibroblasts and myofibroblasts after restoration of normal, functional pulmonary architecture (Coward et al., 2010a). Although this repair process is initially beneficial, it becomes pathogenic when it is not

controlled appropriately and leads to excessive scar tissue and organ dysfunction (Kumar, 2005).

1.1.5.3 Dysregulated Wound Healing

In IPF, wound healing becomes highly dysregulated. The epithelium is markedly abnormal, showing evidence of persistent apoptosis and dysregulated proliferation of epithelial cells causing disruption of the basement membrane in combination with excessive deposition of ECM proteins. Consequently, normal alveolar structure cannot be restored (Figure 1-4) (Basset et al., 1986; du Bois, 2010).

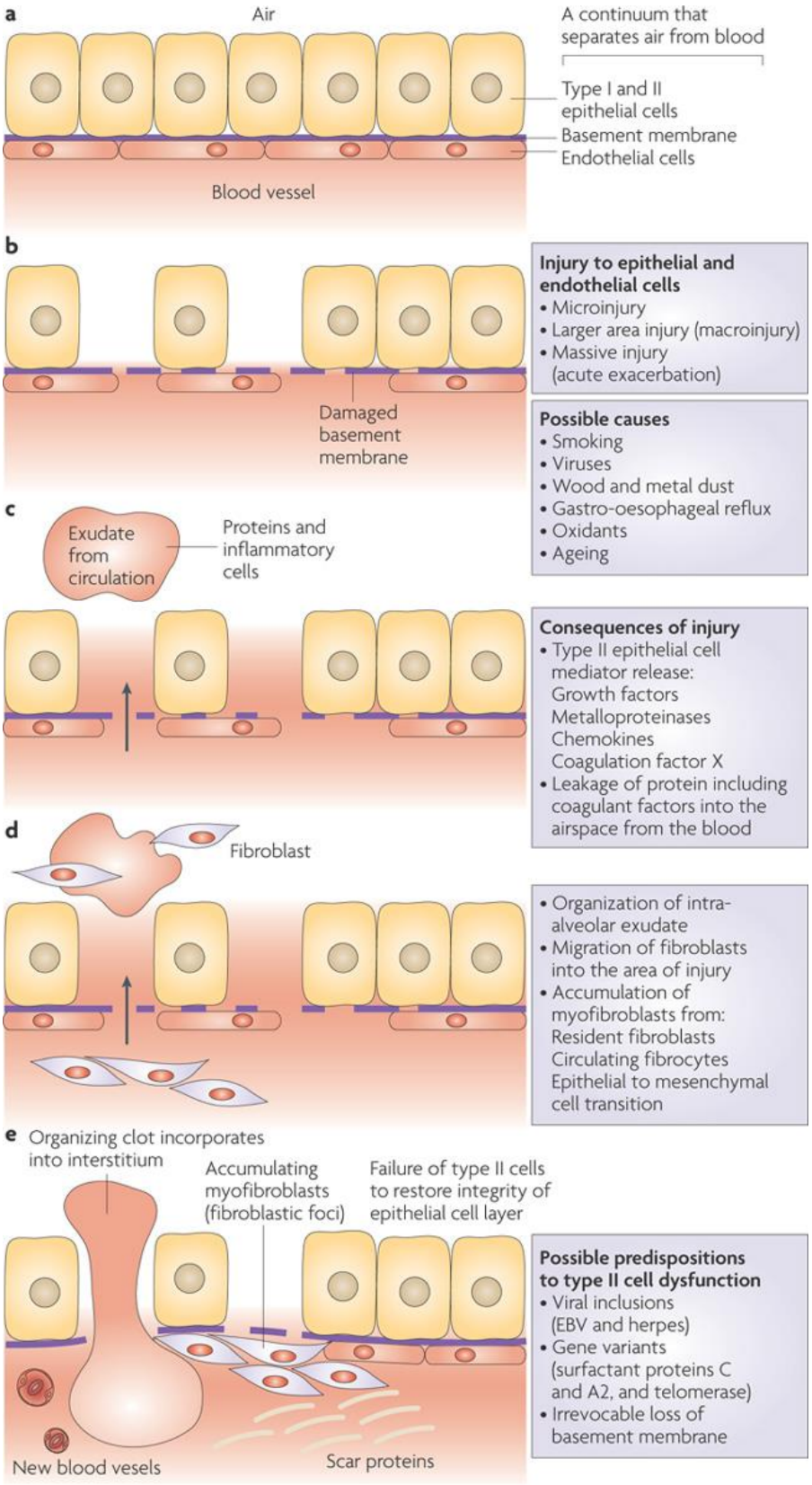

a
Air
A continuum that separates air from blood
Type I and II epithelial cells
Basement membrane
Endothelial cells
Blood vessel
b
Injury to epithelial and endothelial cells
• Microinjury
• Larger area injury (macroinjury)
• Massive injury (acute exacerbation)
Damaged basement membrane
Possible causes
• Smoking
• Viruses
• Wood and metal dust
• Gastro-oesophageal reflux
• Oxidants
• Ageing
c
Exudate from circulation
Proteins and inflammatory cells
Consequences of injury
• Type II epithelial cell mediator release:
Growth factors
Metalloproteinases
Chemokines
Coagulation factor X
• Leakage of protein including coagulant factors into the airspace from the blood
d
Fibroblast
• Organization of intra-alveolar exudate
• Migration of fibroblasts into the area of injury
• Accumulation of myofibroblasts from:
Resident fibroblasts
Circulating fibrocytes
Epithelial to mesenchymal cell transition
e
Organizing clot incorporates into interstitium
Accumulating myofibroblasts (fibroblastic foci)
Failure of type II cells to restore integrity of epithelial cell layer
Possible predispositions to type II cell dysfunction
• Viral inclusions (EBV and herpes)
• Gene variants (surfactant proteins C and A2, and telomerase)
• Irrevocable loss of basement membrane
New blood vesels
Scar proteins

Figure 1-4 Key Events in the Pathogenesis of IPF

a) The alveolar-capillary basement membrane prior to damage. b) Following injury, the basement membrane is disrupted and repair processes are initiated. c) Epithelial cells are activated and secrete pro-fibrotic mediators such as growth factors and chemokines. The disrupted membrane allows proteins and inflammatory markers to leak into the airspaces. d) Fibroblasts are activated and recruited to the site of injury and wound healing response is initiated. e) Scar tissue is established but due to incomplete re-epithelisation the fibrotic response continues. Predisposing gene variants and viral inclusions are hypothesised predispositions to AT2 cell dysfunction in IPF (du Bois, 2010).

1.1.5.3.1 Role of cytokines and growth factors in the pathogenesis of IPF

Epithelial damage results in the release of a variety of cytokines, growth factors and pro-fibrotic mediators. Repetitive cycles of epithelial cell injury and epithelial apoptosis promote the migration, proliferation and activation of fibroblasts and their differentiation into myofibroblasts causing an accumulation of myofibroblasts and excessive synthesis of extracellular matrix. In turn, myofibroblasts secrete pro-fibrotic mediators which causes further alveolar epithelial cell injury and death thereby creating a vicious cycle of pro-fibrotic epithelial-fibroblast interactions (Sakai and Tager, 2013).

1.1.5.3.1.1 Interleukin-1β

Interleukin-1β (IL-1β) is a member of the interleukin family of cytokines. It is produced by macrophages, activated epithelial cells and fibroblasts and is a potent cytokine that induces many proinflammatory effects throughout the body, including the lung (Schmitz et al., 2005). Release of IL-1β from activated alveolar macrophages stimulates the surrounding parenchyma, which includes epithelial cells. Activation of epithelial cells then results in the release of chemokines such as monocyte chemotactic protein (MCP-1), which are capable of recruiting additional inflammatory cells (Dinarello, 1996). The IL-1β-induced activation of epithelial cells is implicated as a key pathogenic pathway in lung diseases including interstitial pulmonary fibrosis, cystic fibrosis and asthma (Levine, 1995). Furthermore, activation of epithelial cells by IL-1β can lead to the secretion of growth factors that cause fibroblast proliferation, collagen production and remodelling of the lower airway. Therefore, Il-1β is capable of eliciting a pro-fibrotic response in addition to a pro-inflammatory response (Dinarello, 1996). A number of animal and human studies have revealed the presence of IL-1β in chronic inflamed tissues and in tissues undergoing fibrogenesis (Phan and Kunkel, 1992). IL-1β has also been shown to be elevated in IPF patients, in serum and bronchoalveolar lavage fluid, compared with healthy control (Barlo et al., 2011; Pan et al., 1996; Zhang et al.,

1993). Studies on animal models have confirmed the role of IL-1β in pulmonary tissue injury and repair. Inhibition of IL-1β at the initiation of animal models of fibrosis caused attenuation of the disease (Piguet et al., 1993). Suggesting a causative link between cytokines involved in the acute phase of inflammation and the conversion to fibrosis. In addition, over expression of IL-1β in rodent epithelial cells caused increased expression of TGF-β1. Resulting in progressive interstitial fibrosis characterised by the presence of myofibroblasts and significant extracellular accumulations of collagen and fibronectin (Kolb et al., 2001a). IL-1β promotes a pro-fibrotic effect by inducing platelet-derived growth factor (PDGF) secretion from alveolar macrophages, epithelial cells and myofibroblasts. PDGF stimulates fibroblast proliferation, ECM synthesis and myofibroblast differentiation (Mia et al., 2014). However, the direct effect of IL-1β on fibroblasts remains unclear. It is known that fibroblasts exposed to IL-1β increase the expression of MMPs and subsequently the breakdown of collagen (Furuyama et al., 2008). This anti-fibrotic effect shows a dual role for IL-1β in fibrosis, as this should diminish the excessive accumulation of ECM. A recent study concluded that IL-1β alone did not contribute to the formation of myofibroblasts but is able to attenuate TGF-β1-induced fibroblast to myofibroblast differentiation (Mia et al., 2014). Furthermore, IL-1β has been shown to induce the expression of COX-2 resulting in the subsequent production of PGE_2 in lung fibroblasts (Coward et al., 2009). Therefore, the production of PGE_2 may limit the pro-fibrotic effects caused by IL-1β as well as other pro-fibrotic cytokines.

1.1.5.3.1.2 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a secreted by activated macrophages and epithelial cells. It is a pro-inflammatory cytokine with pleiotropic effects with a central role in cell-cell adhesion and stimulating the cytokine and chemokine production cascade (Zhang et al., 1997). TNF-α stimulates several factors such as TGF-β1, IL-1β, PDGF as well as increasing fibroblast proliferation. Several studies have demonstrated that TNF-α is present in areas of lung

fibrosis. In Bleomycin mouse models of lung fibrosis TNF-α levels have been shown to be markedly increased as well as increased cytokine production and collagen secretion (Zhang et al., 1997). Furthermore, TNF-α knock out mice fail to develop fibrosis after treatment with Bleomycin (Liu et al., 1998). In patients with IPF, TNF-α is abundantly expressed (Zhang et al., 1993). However, although evidence suggests this proinflammatory cytokine is involved in the pathogenesis of IPF, trials using anti-TNF-α therapies in patients with IPF have had little success (Pantelidis et al., 2001; Vassallo et al., 2002).

1.1.5.3.1.3 Platelet-derived growth factor

PDGF is produced by a wide variety of cells within the lungs including macrophages, fibroblasts, epithelial cells and endothelial cells. PDGF expression has shown to be increased in IPF patients and in animal models of fibrosis (Maeda et al., 1996; Vignaud et al., 1991). The role of PDGF in the pathogenesis of IPF is supported by several reports that place this molecule downstream of pathways activated by profibrotic cytokines; TGF-β1, TNF-α and IL-1β (Battegay et al., 1995; Kolb et al., 2001b; Raines et al., 1989).

1.1.5.4 Defective coagulation in the pathogenesis of IPF

In addition to the production of pro-fibrotic mediators, another aberrant pathological process in IPF is coagulation. Coagulation, the process of blood clotting, is an important component of wound healing and activation; however the coagulation cascade has several pro-fibrotic effects (Selman and Pardo, 2006). Activated epithelial cells cause activation of the clotting cascade resulting in the deposition of fibrin and the formation of a fibrin clot. Excessive fibrin deposition and impaired fibrinolysis, the breakdown of fibrin, is a feature of IPF and experimental models of fibrosis (Chambers, 2003). Epithelial injury also promotes the synthesis of activated factor X, which in turn activates TGF-β1, promoting fibroblast to myofibroblast differentiation (Scotton et al., 2009). Consequently, disordered coagulation

results in an anti-fibrinolytic, hypercoagulable microenvironment, promoting extravascular fibrin deposition and fibrotic tissue remodelling (Kotani et al., 1995). Thus, it is possible that an imbalance between pro-fibrinolytic and anti-fibrinolytic factors could promote the development of IPF.

As previously mentioned the historical concept that IPF is due to unchecked inflammation is now thought to be incorrect, however, data implies that the inflammatory response may exacerbate the pathogenesis of IPF (Coward et al., 2010a). Neutrophils, monocytes, and lymphocytes have all been shown to be elevated in IPF (Baran et al., 2007; Obayashi et al., 1997; Wynn, 2008). Evidence suggests that the alveolar epithelium contributes to a Th2-like pattern of cytokines in the lung microenvironment (Wallace and Howie, 1999). Exaggerated inflammatory responses can lead to excessive tissue injury which overwhelms repair processes promoting a Th2-like response and further promoting fibrosis. A pro-fibrotic Th2 response involves the secretion of IL-4 and IL-13, two putative fibrogenic cytokines which induce fibroblast to myofibroblast differentiation resulting in deposition of extracellular matrix proteins (Selman and Pardo, 2006).

All of the above mentioned pathways contribute to the pro-fibrotic microenvironment in the IPF lung (Figure 1-5). Overall, there is an imbalance of pro-fibrotic mediators, such as increased PDGF and TGF-β1, and anti-fibrotic mediators, such as collagenases and Prostaglandin E2 (PGE_2). It is likely that multiple abnormalities in a myriad of biological pathways affecting inflammation and wound repair including matrix regulation, re-epithelisation and the coagulation cascade all modulate the defective epithelial-mesenchymal cross talk to promote fibrosis.

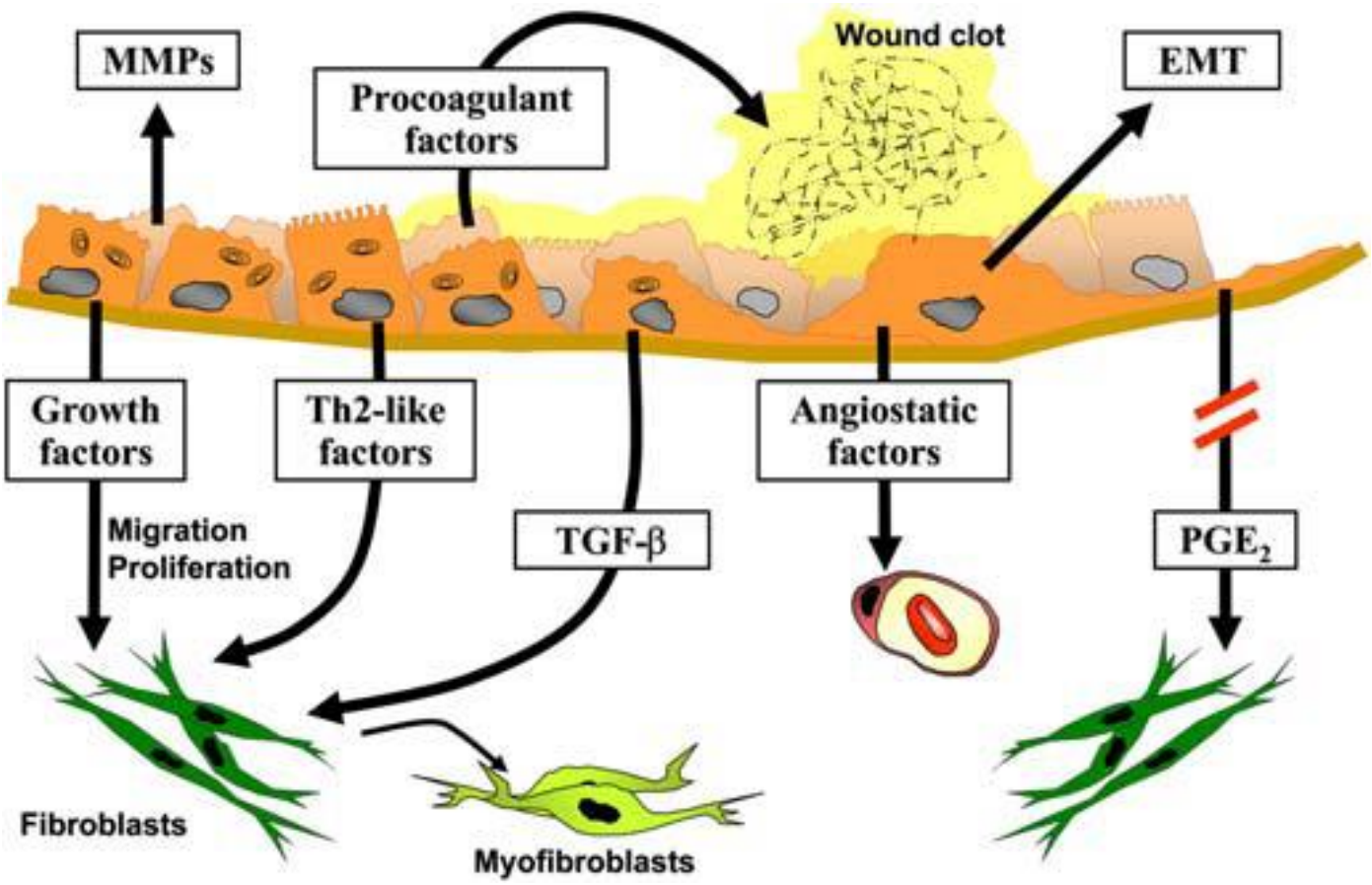


Figure 1-5 Pro-fibrotic Microenvironment in IPF Lung

After injury activated epithelial cells secrete a variety of growth factors and mediators that create a pro-fibrotic environment in IPF lungs, such as PDGF and TGF-β1. Increased procoagulant and angiostatic factors are also secreted from damaged epithelial cells. In addition, the secretion of anti-fibrotic mediators such as Prostaglandin E2 is inhibited (Selman and Pardo, 2006).

1.2 Myofibroblasts and IPF

The fibroblast is the most abundant cell type in normal connective tissue and plays a central role in the synthesis, degradation and remodelling of extracellular matrix in both health and disease (Evans et al., 2003). Fibroblasts undergo various phenotypic conversions between distinct but related cell types in order to perform various biological functions. This phenotypic plasticity is a prerequisite in order to repair damaged tissue. Fibroblast plasticity was first documented over 40 years ago when a subset of specialised fibroblasts were identified (Gabbiani et al., 1971). These cells were termed myofibroblasts as they possess features intermediate between fibroblasts and smooth muscle cells. Myofibroblasts have extracellular cellular matrix (ECM)-synthesising features of a fibroblast with cytoskeletal characteristics of a smooth muscle cell (Thannickal and Horowitz, 2006). Since their first description, great progress has been made in understanding myofibroblast biological characteristics and their participation in physiological and pathological situations.

The presence of myofibroblasts is a consistent finding in the pathology of several fibrotic conditions within the lung, liver and kidney (Hinz, 2010). It is well documented that myofibroblasts are the key effector cells in the pathogenesis of IPF. The presence of myofibroblasts in fibrotic lesions in animal models of fibrosis correlates with the development of active fibrosis and their persistence and localisation to the fibroblast foci in human disease is associated with disease progression (Zhang et al., 1994). Therefore, the persistence of myofibroblasts is a pathological repair process that when dysregulated can become detrimental to tissue repair resulting in aberrant tissue remodelling (Thannickal and Horowitz, 2006).

1.2.1 Features and Functions of Myofibroblasts

Myofibroblasts have been defined as an intermediate between fibroblasts and smooth muscle cells and are therefore characterised by their ability to express fibroblast markers such as Fibroblast Specific Protein-1 (FSP-1) (Lawson et al., 2005), contractile proteins, such as α-SMA and vimentin, (Eyden, 2008) and their secretion of ECM proteins, particularly Collagen I (Gabbiani, 2003).

The most widely used marker of myofibroblasts, in research and clinical diagnostics, is the expression of α-SMA stress fibres (Hinz et al., 2007a; Zhang et al., 1996). The incorporation of α-SMA into myofibroblasts enhances their contractile activity which is necessary for their contraction and normal wound healing (Hinz et al., 2007a). Another widely used marker of myofibroblast differentiation is the production and secretion of several extracellular matrix proteins, most prominently the Collagens of type I, III, IV and V (Hinz, 2010). Myofibroblasts are the key cellular source of collagen and secrete significantly greater amounts compared to fibroblasts (Ramos et al., 2001). Another ECM molecule secreted by myofibroblasts is fibronectin. Extra type III domain A (ED-A) fibronectin is an isoform of fibronectin arising from alternative splicing of fibronectin mRNA. ED-A fibronectin is specifically expressed during wound healing and fibrosis and its deposition precedes α-SMA expression after TGF-β1 stimulation (Leask and Abraham, 2004).

The primary function of the myofibroblast is to regulate tissue repair during wound healing however, this can severely impair organ function when extracellular matrix protein secretion becomes excessive (Hinz et al., 2007a). In normal conditions fibroblasts express little or no α-SMA and have low ECM production. After tissue injury, locally released cytokines from epithelial cells activate fibroblasts which then migrate to the damaged tissue and synthesise and deposit ECM. Following activation of myofibroblasts the apoptosis is essential to prevent

their accumulation and excessive ECM deposition for normal restoration of tissue architecture. In IPF, the accumulation of myofibroblasts form fibroblast foci and the pro-fibrotic microenvironment also facilitates their survival. Myofibroblasts isolated from lungs of IPF patients and cultured *ex vivo* have been shown to be more resistant to apoptosis compared with fibroblasts and this enhances their persistence at sites of injury (Ramos et al., 2001).

Myofibroblasts isolated from lungs of IPF patients are key sources of several pro-fibrotic cytokines including TGF-β1 (Goodwin and Jenkins, 2009) and monocyte chemotactic protein-1 (MCP-1) (Phan, 2002a) and thus myofibroblasts themselves contribute to the pro-fibrotic environment. Although myofibroblasts are resistant to apoptosis they secrete angiotensin peptides that induce apoptosis in adjacent epithelial cells (Uhal et al., 1998). This increases epithelial cell damage and perpetuates abnormal epithelial repair resulting in increased fibroblast proliferation, activation and differentiation into myofibroblasts creating a vicious cycle of epithelial damage and abnormal repair. Therefore, the pro-fibrotic role of the myofibroblast is more than their ability to synthesise and secrete ECM proteins.

The presence of myofibroblasts in fibroblast foci and their role in the pathogenesis of has been demonstrated in lung tissues from patients with IPF and animal models (Hinz et al., 2007b; Phan, 2002b; Scotton and Chambers, 2007). The failure of IPF to resolve correlates with the persistence of the myofibroblast (Kuhn and McDonald, 1991) and clearly more studies are needed to uncover the regulatory mechanisms involved in myofibroblast differentiation. Consequently, this study will focus on fibroblast to myofibroblast differentiation. Further understanding the pathways leading to myofibroblast differentiation may provide a number of molecular targets worthy of investigation for the treatment of IPF.

1.2.2 Origins of Myofibroblasts

Due to the importance of the myofibroblast in IPF studies have focussed on mechanisms underlying its *de novo* appearance and disappearance. The understanding of myofibroblast origin and differentiation remains uncertain and depending on the experimental model used, conclusions on myofibroblast origin appears contradictory (Hinz et al., 2007a; Phan, 2002a).

Evidence from both animal models and human studies demonstrates that resident lung fibroblasts, upon appropriate stimulation (e.g. TGF-β1), are a key source of myofibroblasts in IPF (Roy et al., 2001; Zhang et al., 1994) (Figure 1-6). As described previously, TGF-β1 secreted by epithelial cells after injury potently induces fibroblast to myofibroblast differentiation. Human lung fibroblasts stimulated with TGF-β1 have been used as an *in vitro* model of fibroblast to myofibroblast differentiation. This differentiation is associated with increased α-SMA expression and increased collagen production (Evans et al., 2003).

However, recent research has demonstrated alternative or additional precursors of myofibroblasts in the lung and other tissues (Figure 1-6). One alternative is that epithelial cells undergo differentiation into myofibroblasts by a process termed epithelial-mesenchymal transition (EMT). During EMT, epithelial cells lose their characteristic markers such as E-cadherin and Zona occludens-1 and acquire mesenchymal markers such as FSP-1, α-SMA and Collagen I (Grunert et al., 2003; Kim et al., 2006). Although the concept of EMT has been recognised for over 20 years only recent evidence has supported the role of EMT in IPF. Isolated rat alveolar type II cells have been shown to undergo EMT *in vitro* in response to prolonged treatment with TGF-β1 (Willis et al., 2005). In addition, cells co-expressing epithelial markers and α-SMA were abundant in lung tissue samples collected from IPF patients supporting the theory that epithelial cells can serve as a novel source of myofibroblasts in IPF (Willis et al., 2005). Elegant lineage tracing studies have also provided

strong support for EMT in IPF (Kim et al., 2006). Kim et al demonstrated co-expression of epithelial and mesenchymal markers in IPF lung biopsy suggesting that these cells can undergo transdifferentiation into myofibroblasts (Kim et al., 2006). Additionally, transcription factors that are necessary for EMT, such as Twist1, are increased in epithelial cells isolated from patients with IPF (Pozharskaya et al., 2009). EMT is transient and reversible, and mesenchymal-epithelial transition (MET), the reverse process of EMT can also occur under certain physiological conditions. However, the signals that induce MET remain largely unknown (Miyazono, 2009). It has been suggested that a similar process occurs with endothelial cells termed endothelial-mesenchymal transition (endo-MT) (Kalluri and Neilson, 2003). The signals which promote endo-MT and the contribution of endothelial cells to myofibroblasts requires further investigation.

A third hypothesis is that circulating fibrocytes, a subpopulation of bone marrow derived leukocytes with fibroblast characteristics, are also able to differentiate into myofibroblasts (Ebihara et al., 2006). Fibrocytes comprise of 0.1-1% of the circulating population in healthy individuals and express markers of hematopoietic cells, such as CD45, major histocompatibility complex II (MHC-II) and CD34, and have mesenchymal characteristics, including collagen production (Bucala et al., 1994). Fibrocytes participate in tissue remodelling by producing ECM proteins and secreting matrix metalloproteinases (Chesney et al., 1998). Subsequent studies have demonstrated that specific chemokine receptor/chemokine ligand biological axes are crucial for the recruitment of fibrocytes to sites of tissue injury or repair. The majority of fibrocytes express the chemokine receptor CXCR4. (Abe et al., 2001). In order for fibrocytes to enter the lungs from the peripheral circulation they must be actively recruited. One likely mechanism for achieving this is via the chemokine receptor, CXCR4, and its cognate ligand, CXCL12, which has been shown to regulate cellular migration in other systems (Abe et al., 2001; Phillips et al., 2003). Human fibrocytes cultured *in vitro* migrate in response to CXCL12 and thus the CXCR4-CXCL12 axis may be necessary to traffic peripheral blood

fibrocytes into the lungs (Phillips et al., 2004). In a murine model of bleomycin-induced fibrosis, isolated human fibrocytes injected into the tail were able to migrate to the lungs in response to bleomycin. Furthermore, CXCL12 was elevated in the lungs of bleomycin treated mice compared with control mice supporting the notion that a CXCL12 gradient could promote the recruitment of fibrocytes to the lung. Furthermore, levels of fibrocytes detected in lungs directly correlated with collagen deposition (Phillips et al., 2004). Collectively, these data demonstrate that circulating fibrocytes may contribute to ECM deposition and the promotion of fibrosis *in vivo*. This is supported further with data from patients with IPF. Patients with IPF have increased circulating fibrocytes compared with normal controls accounting for 6-10% of the circulating population (Mehrad et al., 2007). It has also been demonstrated that a blood fibrocyte count higher than 5% in IPF patients is associated with poor survival and thus may be useful as a clinical biomarker for disease progression (Moeller et al., 2009). Interestingly, epithelial cells from IPF patients strongly express CXCL12 which could provide the chemotactic gradient needed for trafficking fibrocytes into the lung (Andersson-Sjoland et al., 2008).

Whether fibrocytes are capable of differentiating into fully functioning myofibroblasts, especially in patients with IPF, remains the subject of an interesting debate. Fibrocytes isolated from human peripheral blood spontaneously differentiate into myofibroblasts *in vitro* and this is augmented following treatment with TGF-β1. TGF-β1 treated fibrocytes resulted in increased expression of α-SMA and there was no appreciable change in cell morphology when compared with myofibroblasts (Hong et al., 2007). Co-expression of fibrocyte and mesenchymal markers, such as pro-collagen I and α-SMA, further support the notion that circulating fibrocytes can differentiate into myofibroblasts in IPF patients (Andersson-Sjoland et al., 2008). Based on these observations it strongly suggests that fibrocytes have a role in the pathogenesis of IPF and likely contribute to the pro-fibrotic microenvironment.

Damaged organs recruit myofibroblast precursors from several different sources (Figure 1-6). It is unknown whether this is to satisfy the temporary high demand for cells with tissue remodelling activity or if myofibroblasts from different origins exhibit different characteristics and functions during tissue repair. The relative contribution from each of these origins to the population of myofibroblasts in IPF remains unknown. However, in a murine model of bleomycin-induced lung fibrosis EMT accounted for 33% of myofibroblasts and fibrocytes accounted for approximately 20% (Tanjore et al., 2009). Similar contributions has also been demonstrated in a murine model of renal fibrosis (Iwano et al., 2002). Although several cellular types are able to differentiate into myofibroblasts, evidence suggests that the resident lung fibroblasts are the main precursor cell for myofibroblasts. Therefore, this study will primarily focus on fibroblast to myofibroblast differentiation.

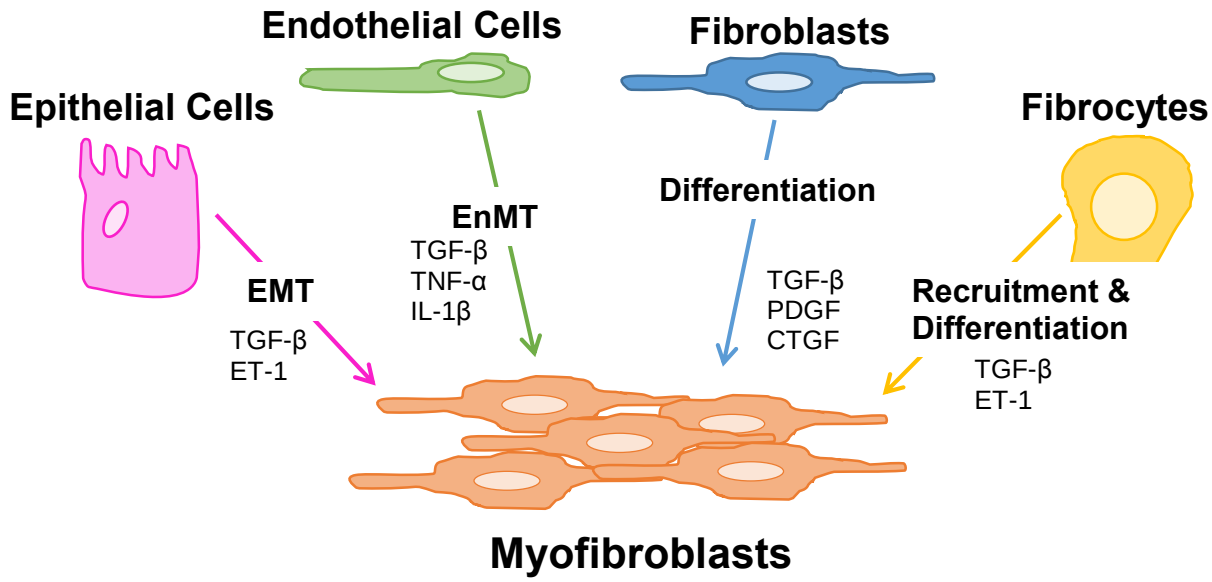


Figure 1-6 Origins of Myofibroblasts in IPF

The myofibroblast is a key effector cell in the pathogenesis of IPF. Myofibroblast are thought to originate from several sources. The most established explanation for their de novo appearance is differentiation from quiescent residing fibroblasts in response to growth factors such as TGF-β1, PDGF and CTGF. Under certain circumstances epithelial cells can also differentiate into myofibroblasts via a process termed epithelial-to-mesenchymal transition (EMT). Epithelial cells have been shown to undergo EMT in response to TGF-β1 and ET-1. Endothelial-to-mesenchymal transition might also represent another source of myofibroblasts. The third potential origin of myofibroblast in IPF is differentiation from circulating fibrocytes. Similar to resident cells, including fibroblasts and epithelial cells, they respond to TGF-β1 and ET-1 by transdifferentiating into myofibroblasts.

1.2.3 Differences between F-IPF and F-NL

Fibroblasts isolated from lungs of patients suffering from IPF (F-IPF) have different characteristics compared with fibroblasts isolated from patients with non-fibrotic lungs (F-NL). F-IPF are pro-proliferative and anti-apoptotic (Maher et al., 2010), they express significantly higher levels of α-SMA (Ramos et al., 2001) and have increased ECM protein production (Huang et al., 2007; Leask and Abraham, 2004). The most intensely studied difference between F-NL and F-IPF is that F-IPF are defective in their capacity to synthesise the anti-fibrotic mediator PGE_2. This defect is because the enzyme responsible for PGE_2 synthesis, cyclooxygenase 2 (COX-2), is significantly reduced in F-IPF (Coward et al., 2009). COX-2 expression is induced in response to several stimuli including TGF-β1 and interleukin-1β (IL-1β). COX-2 induction, by mediators present in the inflammatory milieu of the lung, may represent an important mechanism by which fibroblasts can increase their capacity for PGE_2 synthesis and limit fibroblast proliferation and differentiation into myofibroblasts. Since F-IPF share many features characteristic of myofibroblasts it is likely that F-IPF mainly consist of myofibroblasts, whereas, F-NL mainly consist of fibroblasts.

1.2.4 Inducers and Inhibitors of Fibroblast to Myofibroblast Differentiation

Given the various pathways leading to myofibroblast differentiation it is evident that a vast range of pro-fibrotic mediators regulate fibroblast to myofibroblast differentiation. Table 1-1 summarises mediators that induce and inhibit fibroblast to myofibroblast differentiation. Mediators that control myofibroblast differentiation are mainly produced by fibroblasts themselves and injured epithelial cells. As myofibroblasts have a key role in the pathogenesis of IPF mediators that regulate fibroblast to myofibroblast differentiation represent potential targets for novel anti-fibrotic drug therapy.

Myofibroblast Modulating Factors	
Myofibroblast Inducing Factors	**Myofibroblast Suppressing Factors**
TGF-β1	PGE_2
CTGF	IFN-γ
PDGF	HGF
TNF-α	FGF
ET-1	Plasmin
MCP-1	
PAMPS	

Table 1-1 Myofibroblast Modulating Factors in IPF

This table summarises the most researched mediators that either directly or indirectly regulate fibroblast to myofibroblast differentiation. Growth factors, cytokines, pathogen associated molecular pathogens (PAMPs) and mechanical stress can all regulate fibroblast to myofibroblast differentiation. Transforming Growth Factor β (TGF-β1),Connective Tissue Growth Factor (CTGF), Platelet Derived Growth Factor (PDGF), Tumour Necrosis Factor-α (TNF-α), Endothelin-1 (ET-1), Monocyte Chemoattractant Protein-1 (MCP-1), Prostaglandin E2 (PGE_2), Interferon-γ (IFN-γ), Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF).

1.2.4.1 Myofibroblast Inducing Factors

Transforming growth factor beta (TGF-β1) has been the most intensely studied mediator of fibroblast to myofibroblast differentiation. There is ample evidence that TGF-β1 can induce fibroblast to myofibroblast differentiation in lung fibroblasts (Evans et al., 2003), renal fibroblasts (Bottinger and Bitzer, 2002), cardiac fibroblasts (Liu et al., 2006b), hepatic fibroblasts (Bissell et al., 1995) and dermal fibroblasts (Stratton et al., 2001), as well as EMT (Willis et al., 2005). Hence, TGF-β1 is a potent inducer of fibrosis in several organs (Goodwin and Jenkins, 2009; Kim et al., 2006).

Several mediators act to enhance the effects of TGF-β1. One downstream mediator of TGF-β1 is connective tissue growth factor (CTGF) (Sanchez-Elsner et al., 2001). CTGF is a key angiogenic factor that is induced by a number of other pro-fibrotic mediators such as thrombin, a protein involved in the coagulation cascade. The biological role of this factor is still unclear but studies have demonstrated CTGF can stimulate fibroblast matrix production and myofibroblast differentiation (Leask and Abraham, 2003).

Platelet derived growth factor (PDGF) is a potent mitogen and chemoattractant for mesenchymal cells, including myofibroblasts, and stimulates the production of ECM proteins such as collagen and fibronectin (Bonner, 2004). The main source of PDGF production is from alveolar macrophages and epithelial cells, however, myofibroblasts can also secrete PDGF resulting in an autocrine feedback loop for fibroblast proliferation, ECM deposition and myofibroblast differentiation (Scotton and Chambers, 2007). In addition, PDGF can induce TGF-β1 expression suggesting that some of the effects of PDGF are mediated via TGF-β1 (Ask et al., 2006).

Tumour necrosis factor-α (TNF-α) has been shown to increase fibroblast proliferation, collagen production and myofibroblast differentiation and signals indirectly via TGF-β1 and PDGF signalling pathways (Kapanci et al., 1995).

Other studies suggest that Endothelin-1 (ET-1), a potent vasoconstrictor and mitogenic peptide, induces fibroblast activation, proliferation and differentiation into myofibroblasts (Fonseca et al., 2011). In addition, ET-1 can act synergistically with a number of pro-fibrotic mediators capable of inducing fibroblast to myofibroblast differentiation including TGF-β1 and PDGF (Swigris and Brown, 2010).

Monocyte chemotactic protein-1 (MCP-1) is a cytokine produced in response to inflammatory stimuli by a variety of cells including airway epithelial cells. MCP-1 is known to promote fibrosis through its ability to recruit fibroblasts, increase ECM, down regulate PGE_2 (Moore et al., 2003) and stimulate the production of TGF-β1 (Hartl et al., 2005).

Cytokines are not the only mediators capable of inducing fibroblast to myofibroblast differentiation. It was recently suggested that conserved pathogen-associated molecular patterns (PAMPs), found on infectious agents, maintain myofibroblasts in an activated state (Meneghin and Hogaboam, 2007). PAMPs are defined as pathogen by-products, such as lipoproteins, bacterial DNA and double stranded RNA, which are recognised by pattern recognition receptors (PRRs) expressed on fibroblasts. The interaction between PAMPs and PRRs activates the release of pro-inflammatory cytokines and chemokines. Fibroblasts express a variety of PRRs, including Toll-like receptors (TLRs). Toll ligands are able to bind to TLRs on fibroblasts and activate their differentiation into myofibroblasts (Otte et al., 2003).

More recently it has been suggested that the matrix itself promotes fibroblast to myofibroblast differentiation. Fibroblasts form loose attachments to the matrix, but as the matrix stiffens during wound repair the phenotype of the fibroblast changes to that of a myofibroblast (Hinz, 2010). Tissue stiffness increases as a consequence of ECM-remodelling activities of myofibroblasts following injury. Therefore, myofibroblasts generate the conditions to promote further fibroblast to myofibroblast differentiation resulting in an autocrine feedback loop. It has been proposed that this phenotypic change in response to increased matrix stiffness is through activation of TGF-β1. The mechanoregulation of TGF-β1 activation is thought to be via integrin-mediated contraction which mechanically changes the conformation of latent TGF-β1 (Wipff and Hinz, 2008). *In vivo* data has demonstrated that the activation of TGF-β1 by αvβ6 is of critical importance in IPF (Jenkins et al., 2006; Kim et al., 2006).

1.2.4.2 Myofibroblast Suppressing factors

Although several mediators are known to stimulate myofibroblast differentiation knowledge of mediators that inhibit fibroblast to myofibroblast differentiation is more limited. Prostaglandins are some of the earliest known agents that suppress the fibrotic response (Leask and Abraham, 2004). This fibroblast-suppressive function is mainly mediated by prostaglandin E2 (PGE_2). In normal tissue repair TGF-β1 induces PGE_2 production which is secreted by both epithelial cells and fibroblasts (Coker et al., 1997). PGE_2 acts in multiple ways to control wound healing and prevent fibrosis including regulation of wound closure in airway epithelium, inhibition of fibroblast migration (Narumiya et al., 1999), proliferation (Elias et al., 1985), collagen synthesis (Goldstein and Polgar, 1982) and differentiation (Leask and Abraham, 2004) and increases fibroblast apoptosis (Maher et al., 2010). Other growth factors, such as hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) and mediators involved in the coagulation cascade such as plasmin (Bauman et al., 2010) are also able to inhibit fibroblast to myofibroblast differentiation by their ability to up regulate PGE_2 production.

Interferon (IFN)-γ is a cytokine involved in the innate and acquired immune response produced by T cells and NK cells (Boehm et al., 1997). The anti-fibrotic effects of IFN-γ include; inhibition of fibroblast proliferation and differentiation into myofibroblasts. This is mediated by counter regulating TGF-β1 expression thus limiting fibroblast proliferation, differentiation and collagen synthesis (Scotton and Chambers, 2007). TGF-β1 and IFN-γ are secreted by inflammatory cells at the site of tissue injury, since they exert opposite effects their antagonistic interactions are of great importance in the regulation of connective tissue homeostasis (Leask and Abraham, 2004).

IP-10, an IFN-γ inducible protein, is a potent chemoattractant for leukocytes and a strong inhibitor of angiogenesis. Recent studies have shown that IP-10 is repressed in F-IPF compared with F-NL (Coward et al., 2009). IP-10 repression has also been demonstrated in a murine model of bleomycin induced fibrosis (Keane et al., 1999).

Based on the above biological observations identifying mediators that can inhibit fibroblast to myofibroblast differentiation has been the focus of several clinical trials including the use of IFN-γ and anti-TNF-α treatment (du Bois, 2010). However, no clinically effective compound has yet been identified.

1.3 TGF-β1 and Myofibroblast Differentiation

As previously mentioned TGF-β is a key mediator in the development of fibrosis in a number of organs due to its potent pro-fibrotic effects on both fibroblasts and epithelial cells. TGF-β promotes epithelial cell apoptosis and migration, EMT, collagen synthesis, fibroblast

proliferation and fibroblast to myofibroblast differentiation (Goodwin and Jenkins, 2009). Although there are several mediators capable of inducing fibroblast to myofibroblast differentiation it is evident that TGF-β1 is the most potent (Scotton and Chambers, 2007). As a result, this study will focus on TGF-β and its effect on fibroblast to myofibroblast differentiation.

TGF-β is a member of the TGF-β superfamily, a highly conserved group of 45 different proteins including TGF-β, inhibins, activins and bone-morphogenetic proteins (BMPs). Members of this superfamily regulate fundamental development and physiological processes such as cell proliferation, survival and differentiation and are frequently implicated in diverse pathological conditions (Leask and Abraham, 2004).

At present three mammalian isoforms of TGF-β1 have been identified; TGF-β1, TGF-β2 and TGF-β3 (Assoian et al., 1983; Cheifetz et al., 1987; Derynck et al., 1985). Each isoform is encoded by separate genes located on separate chromosomes and have distinct but related functions. All three isoforms are expressed in humans, however, TGF-β1 is the most abundantly expressed. Although a variety of cell types produce and respond to TGF-β, tissue fibrosis is primarily attributed to the TGF-β1 isoform (Wynn, 2008).

1.3.1 TGF-β1 Expression and Activation

TGF-β1 is ubiquitously expressed by all cells and tissues within the body. Although TGF-β1 has been documented to be a potent inducer of fibrosis large amounts of TGF-β1 protein are present in healthy adults without apparent effects. This is due to the fact that TGF-β1 must be activated in order to have a biological effect.

TGF-β1 is synthesised and secreted as pro-TGF-β1 in the form of a small latent complex (SLC), consisting of bioactive TGF-β1 that is covalently linked to the latency-associated peptide (LAP). Furthermore, SLCs can also associate with latent TGF-β1 binding proteins (LTBP-1, -3 and -4) forming large latent complexes (LLC) (Figure 1-7). LLCs are sequestered to the ECM via the LTBPs which bind to ECM proteins such as fibrillin and fibronectin. The LTBPs regulate location and the tissue specificity of TGF-β1 as the LTBPs bind preferentially to different isoforms of TGF-β1 (Saharinen and Keski-Oja, 2000). Failure to locate TGF-β1 in the appropriate location alters the effectiveness of activation of TGF-β1 (Annes et al., 2003). The latent complexes prevent TGF-β1 from binding to its receptors and thus inhibit subsequent signalling (Chen et al., 2005). Therefore, in order for TGF-β1 to exert its biological effects it must become activated by dissociating from the latent complexes (Annes et al., 2003).

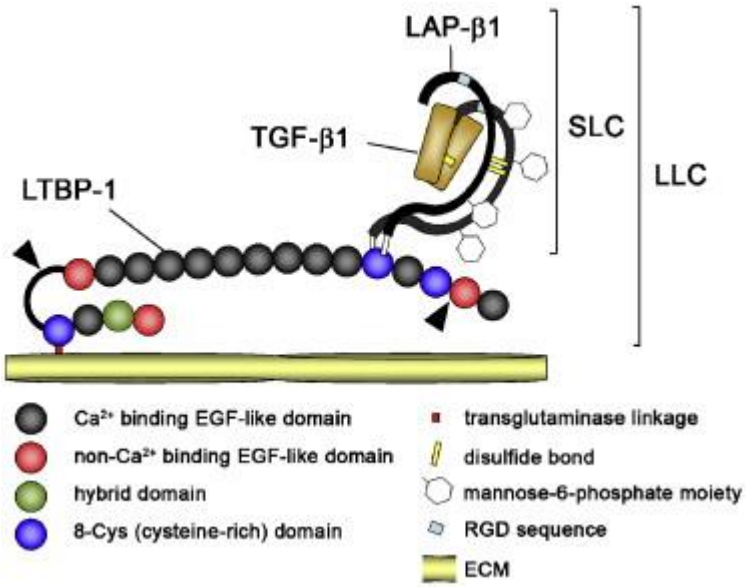


Figure 1-7 The Structure of Latent TGF-β1

Schematic diagram illustrating the structure of both the small latent complex (SLC) and the large latent complex (LLC). TGF-β1 is secreted as SLC consisting of TGF-β1 associated to the latency associated peptide (LAP). Once the SLC is associated with latent TGF-β1 binding proteins (LTBP) this is then termed the LLC. Following proteolytic release from the LTBPs by furin, TGF-β1 remains non-covalently associated with the LAP forming the SLC. The LAP and LTBP contain several different domains which act as recognition sites for various proteins, for instance, the RGD domain on the LAP acts as a recognition site for integrins (Wipff and Hinz, 2008).

In order to be activated the LLC must be proteolytically cleaved by furin, or extracellular proteases such as plasmin, from the LTBP. Once the LTBP has been cleaved TGF-β1 and the LAP form the SLC. The SLC can then be activated *in vitro* by extremes of temperature or pH, or by a variety of proteases such as thrombin (Taipale et al., 1992) and tryptase (Tatler et al., 2008) that cleave the LAP away from TGF-β1. In vivo, TGF-β1 can be activated by a number of proteases including plasmin, tryptase, thrombin, elastase, matrix metalloproteinase (MMP)-2 and MMP-9 and by interactions with thrombospondin or integrins (Jenkins, 2008). Most of the mechanisms of TGF-β1 described are ubiquitous; however the $\alpha_v\beta_6$ integrin is restricted to the epithelium and thus can only activate latent TGF-β1 in direct association with epithelial cells.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits. The mammalian genome encodes 18 α subunits and 8 β subunits resulting in 24 αβ integrin combinations. Although $\alpha_v\beta_6$ was the first integrin found to mediate TGF-β1 activation, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ all facilitate the activation of TGF-β1 *in vitro*. The role of integrin-mediated TGF-β1 activation *in vivo* has only been confirmed for the $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrin (Munger et al., 1999; Travis et al., 2007; Xu et al., 2009). Each αv integrin binds ligands through an RGD (Arg-Gly-Asp) sequence which is found in the LAP of TGF-β1 (Xu et al., 2009).

1.3.1.1 TGF-β1 activation in Idiopathic Pulmonary Fibrosis

TGF-β1 is a key mediator in IPF and thus its activation has been heavily implicated in IPF and airway remodelling. The mechanisms of TGF-β1 activation appear to differ depending on the cellular and anatomical compartments. Epithelial cell activation of TGF-β1 by the $\alpha_v\beta_6$ integrin appears to be central in IPF, mesenchymal activation of TGF-β1 by the $\alpha_v\beta_5$ and $\alpha_v\beta_8$ integrins appear to predominate in airway remodelling.

Several studies have demonstrated that the activation of $\alpha_v\beta_6$ is of critical importance in the pathogenesis of tissue fibrosis. Both $\alpha_v\beta_6$ null mice and mice with impaired TGF-β1 signalling are protected from bleomycin-induced lung fibrosis (Bonniaud et al., 2004; Li et al., 2011; Munger et al., 1999). In addition, administration of an anti- $\alpha_v\beta_6$ antibody prevents pulmonary fibrosis (Puthawala et al., 2008). Although over expression of $\alpha_v\beta_6$ in the normal lung is not sufficient to promote fibrosis (Huang et al., 1998; Häkkinen et al., 2004) , levels of the β_6 integrin subunit are increased after bleomycin-induced injury and in patients with lung fibrosis (Horan et al., 2008; Xu et al., 2009). TGF-β1 upregulates β_6 intergrin subunit, suggesting a self-amplifying paracrine loop (Araya et al., 2007). Thus, lung injury promotes $\alpha_v\beta_6$ integrin-mediated TGF-β1 activation, which in turn induces a self-amplifying loop through increased β_6 integrin expression. What may terminate or regulate this process is currently unknown. A key feature of $\alpha_v\beta_6$ integrin-mediated TGF-β1 activation is the requirement for cell-cell contact between cells expressing the integrin and TGF-β1 receptor. Therefore, the failure to restore the epithelial cell membrane integrity may be a key step in promoting fibrogenesis by this pathway, as the disruption of the epithelial membrane will allow epithelial-mesenchymal interactions.

Other studies have suggested that the matrix itself can facilitate integrin-mediated TGF-β1 activation. Increasing matrix stiffness can result in activation of TGF-β1 via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Furthermore, $\alpha_v\beta_5$ integrin-mediated TGF-β1 activation only occurs on culture substrates with stiffness comparable to fibrotic tissue (Wipff and Hinz, 2008). Therefore, initial epithelial injury may cause TGF-β1 activation via $\alpha_v\beta_6$ but as the matrix composition changes further activation of TGF-β1 may occur via mesenchymal integrins such as $\alpha_v\beta_5$.

1.3.2 TGF-β1 Signalling

Once activated TGF-β1 binds to a heterodimeric receptor complex consisting of TGF-β1 type I and TGF-β1 type II receptor which both possess serine/threonine kinase activity (Roberts, 1999). TGF-β1 signals through these receptors to activate a specific family of transcription factors, called Smads, which propagates TGF-β1 signalling from the activated receptor to the nucleus. Activation of Smads by TGF-β1 is invariant in most cell types and therefore this is known as the canonical TGF-β1 signalling pathway. The great diversity of TGF-β1 signalling is due to the combinatorial interactions of type I and type II receptors and Smads in various oligomeric complexes. In addition, these Smad complexes are complemented by sequence-specific transcription factors resulting in context dependent transcriptional regulation (Derynck and Zhang, 2003).

TGF-β1 also activates a variety of non-canonical signalling pathways. Other non-Smad TGF-β1 signalling pathways include c-Jun N Terminal Kinase (JNK), phosphoinositide 3-kinase-Akt-mTor (PI3K) pathway, the small GTPases Rho, Rac and Cdc42, and the mitogen activated protein kinase (MAPK) pathway. These pathways can induce Smad-independent responses as well as regulating Smad-mediated responses. This cross talk between the canonical and non-canonical signalling pathways enables cells to exhibit tight, complex controls over the TGF-β1/Smad signalling cascade (Stork and Schmitt, 2002).

1.3.2.1 Canonical TGF-β1 Signalling

Following activation of the TGF-β1 receptors, TGF-β1 signals within the cell through the Smad family of transcriptional activators (Roberts, 1999; Verrecchia and Mauviel, 2002). There are three subsets of Smads within the family: receptor regulated Smads (R-Smads), common partner Smads (Co-Smads) and inhibitory Smads (I-Smads). Following TGF-β1 receptor

activation, the R-Smads, Smad-2 and -3, are directly phosphorylated by the TGF-β1 receptor I kinase. There are several other R-Smads (Smad-1, -5 and -8) but these are involved in the signalling of other members of the TGF-β1 superfamily. Smad-2/3 can then form a heterotrimeric or heterodimeric complex with the co-Smad, Smad-4, and the resultant complex then translocates to the nucleus to regulate gene transcription (Verrecchia and Mauviel, 2002). An overview of the Smad signalling pathway is illustrated in Figure 1-8.

Smads themselves do not activate transcription instead Smads assist in the formation of a functional transcriptional complex on target promoters with other co-activators and repressors. Phosphorylated Smad-3 utilises specific transcription factors and co-activators such as CREB-binding protein (CBP) and p300, for nuclear DNA binding and initiation of gene transcription (Derynck and Zhang, 2003; Shen et al., 1998). Smad-4 itself acts as a co-activator and enhances ligand-induced transcription by stabilising the interaction of R-Smads with DNA and CBP/p300 (Derynck and Zhang, 2003). Smad-2, however, requires a nuclear DNA binding protein, Fast-1, in association with Smad-4, before it is able to induce transcription (Liu et al., 1999). The activity of these co-activators and co-repressor is adjusted by other signal transduction cascades which vary depending on the promoter or cell type of interest to tightly control TGF-β1-induced transcription (Leask and Abraham, 2004).

In order to further modulate or terminate TGF-β1-induced gene expression, Smad activity can also be regulated. Inhibitory Smads, such as Smad-6 and Smad-7, prevent R-Smad phosphorylation and subsequent gene induction (Nakao et al., 1997). Phosphorylated TGF-β1 receptor I recruits Smad-7 which then competes with Smad-2 and Smad-3 for binding to the activated receptor thus inhibiting the canonical TGF-β1 Smad pathway via a negative feedback loop (Itoh et al., 1998).

1.3.2.2 Non-canonical TGF-β1 Signalling

In addition to Smad-mediated transcription, TGF-β1 also activates several other signalling pathways including the MAPK pathways such as extracellular regulated kinase (ERK), p38 and c-Jun N terminal kinase (JNK) and phosphatidylinositol-3-kinase (PI3K) pathways. Some of these pathways can regulate Smad activity whereas others induce responses unrelated to Smad transcription (Chapnick et al., 2011). Unlike Smad signalling however, these non-canonical pathways of TGF-β1 are often cell type specific and context dependent (Derynck and Zhang, 2003).

In mammalian cells all three MAPKs, ERK, JNK and p38, are activated by TGF-β1. In some cases activation may be due to Smad-dependent transcription responses, however, the rapid activation of MAPKs (5 to 15 minutes after stimulation) suggests this is an independent effect of Smad-transcription and due to direct activation by TGF-β1 (Derynck and Zhang, 2003). Smad-independent activation is supported by studies using Mv1Lu (a mink lung epithelial cell line). TGF-β1 caused JNK activation, but not MAPK activation, demonstrating selectivity by TGF-β1 towards different MAPK signalling pathways. TGF-β1 treatment caused JNK activation in both R1B cells (mink lung epithelial cells deficient in TGF-β1R1) and MDA-MB-468 cells (breast carcinoma cells with a homozygous deletion of Smad-4 gene) suggesting that Smad4 is not involved in JNK activation (Engel et al., 1999; Yu et al., 2002). Therefore, these data demonstrate that the rapid activation of JNK occurs in a Smad-independent manner.

The mechanisms of ERK, JNK or p38 MAPK activation by TGF-β1 and its biological consequences are poorly characterised. TGF-β1 induced activation of MAPK, p38 and JNK pathways have been implicated in the regulation of apoptosis, cell migration and EMT (Heldin et al., 1997). Rapid activation of Ras by TGF-β1 in intestinal epithelial cells indicates the

involvement of Ras in TGF-β1-induced ERK activation (Janda et al., 2002; Yue and Mulder, 2000) and TGF-β1-induced Ras activation in mammary epithelial cells is associated with EMT (Janda et al., 2002). JNK and p38 MAPK signalling pathways are activated by various MAPK kinase kinases (MAPKKKs) in response to several stimuli (Derynck and Zhang, 2003). It has recently been shown that TGF-β1-activated kinase (TAK1), a MAPKKK family member, and ubiquitin ligase tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) are crucial for the activation of the p38 and JNK MAPK pathways (Sorrentino et al., 2008; Yamaguchi et al., 1999). However, further investigation is required to fully understand the activation of MAPKs by TGF-β1.

TGF-β1-induced EMT in mammary epithelial cells has been shown to be via Ras activation of the phosphatidylinositol-3-kinase (PI3K) pathway as indicated by phosphorylation of its downstream effector Akt (also known as Protein Kinase B) (Bakin et al., 2000). The underlying mechanism of how TGF-β1 activates PI3K is still unclear but other studies have demonstrated that PI3K activation can be direct, with possible involvement of RhoA (Bakin et al., 2000), but can also result from TGF-β1-induced TGF-α expression and consequent EGF receptor activation (Vinals and Pouyssegur, 2001).

TGF-β1 can also activate small GTPases; Rho, Rac and Cdc42 (Mu et al., 2012). The molecular mechanism of TGF-β1-induced small GTPase activation is poorly understood. It is believed that TGF-β1 activation of these small GTPases contributes to the regulation of cell adhesion and cell migration and thus may be critical events leading to EMT however further investigation to confirm this is necessary (Mu et al., 2012).

To date, the research on non-Smad signalling pathways has primarily focussed on epithelial cells and the role of EMT in cancer. There is very little understanding of the pathways involved in non-Smad signalling in fibroblasts and fibrosis. As previously mentioned, unlike the canonical pathway, the non-canonical signalling pathway is extremely cell type dependent and context specific. Therefore, it is necessary to further understand the non-Smad TGF-β1-induced signalling pathways in fibroblasts, their involvement in fibroblast to myofibroblast differentiation and their importance in the pathogenesis of IPF.

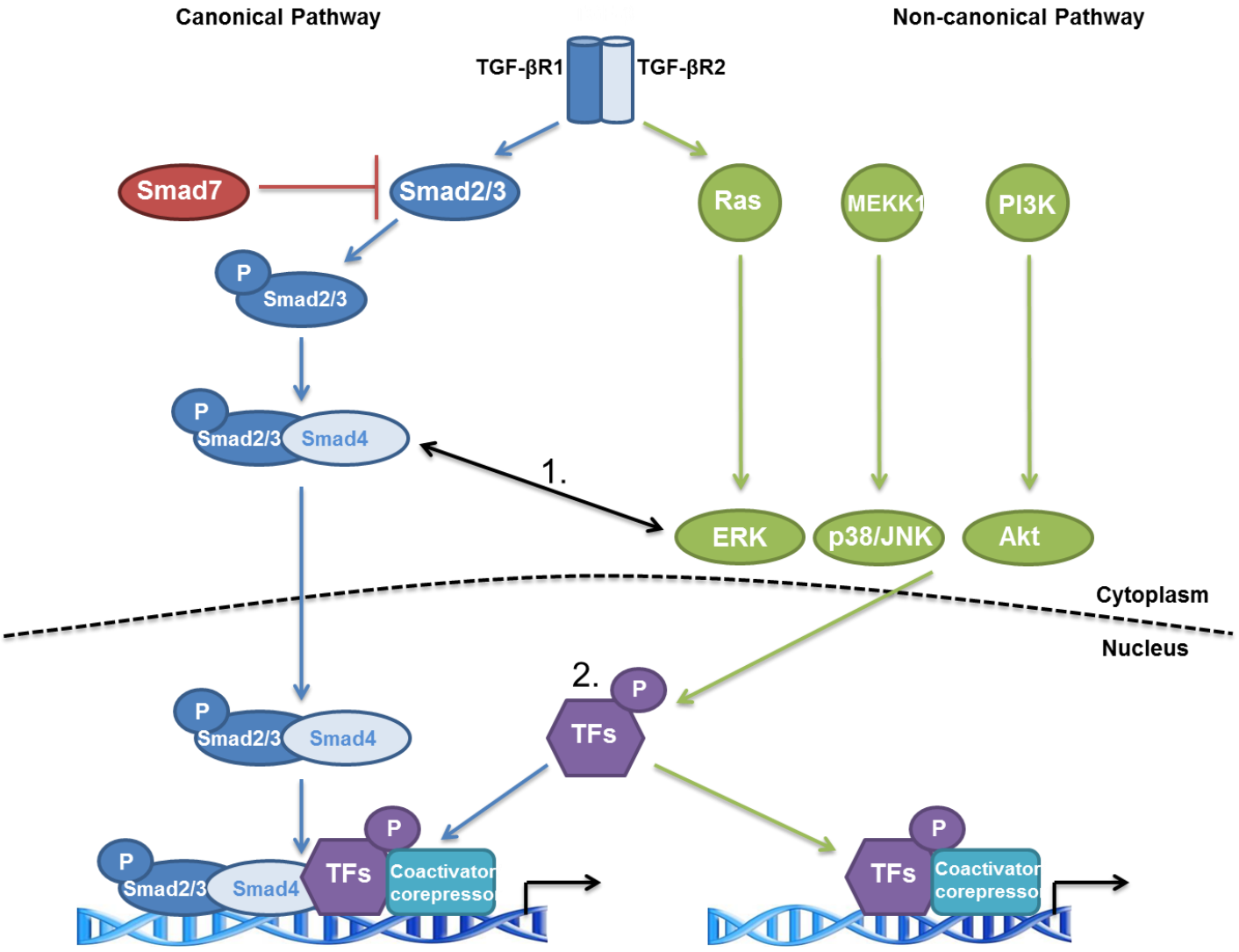


Figure 1-8 Schematic diagram of TGF-β1 signalling pathways

The left hand side of this diagram demonstrates the Smad-dependent TGF-β1 signalling pathway (blue) whilst the right hand side demonstrates non-canonical TGF-β1 signalling pathways (green). The details of both pathways are elaborated within the text. Briefly, both pathways are initiated with TGF-β1 binding to its cell surface receptor. Activation and phosphorylation of the TGF-β1 receptor results in activation of Smad2/3 which then associates with Smad 4. This Smad2/3-Smad4 complex translocates to the nucleus and associates with coactivators or corepressors to regulate gene transcription. In the non-canonical TGF-β1 signalling pathway, activation of the TGF-β1 receptor activates non-Smad signalling pathways such as Ras, p38 and PI3K. The two common points of integration between canonical and non-canonical TGF-β1 signalling are 1) direct phosphorylation of Smads by non-canonical pathway and 2) phosphorylation of co-activators or transcription factors of R-Smads. The cross talk between the canonical and non-canonical pathways can act to enhance or suppress the Smad signalling pathway.

1.3.2.3 Canonical and Non-canonical TGF-β1 Cross Talk

Although TGF-β1 signalling via the canonical Smad and non-Smad signalling pathway at first appears to diverge, these pathways can in fact cooperate and converge or counteract each other in order to define specific cellular responses to TGF-β1. There is extensive cross-talk between the canonical and non-canonical TGF-β1 signalling pathways enabling highly regulated cellular responses.

One example of this cross talk involves ERK activation, which has been proposed to both complement and antagonise Smad signalling depending on the cell type and context (Moustakas and Heldin, 2005). MAPKs phosphorylate a number of nuclear transcription factors, many of which can physically interact with Smads and regulate TGF-β1 signalling. For example, in mouse mammary epithelial cells activation of Ras, via epidermal growth factor (EGF), inhibits TGF-β1 signalling. Treatment with EGF activates ERK-1/2 which then phosphorylates Smad-1, Smad-2 and Smad-3 at different sites to the TGF-β1 I receptor site. This EGF-induced phosphorylation prevents Smad translocation to the nucleus and thereby inhibits TGF-β1 signalling (Kretzschmar et al., 1999). In contrast, in mink lung epithelial cells, EGF induces Smad-2 phosphorylation and promotes Smad-2 nuclear translocation and transcriptional activity (de Caestecker et al., 1998). Thus, Smad proteins may be phosphorylated by kinases of the MAPK pathway on different sites leading to either their activation or their repression depending on the cell type and context.

It would be interesting to determine the factors involved in TGF-β1 canonical and non-canonical cross talk, how these are activated and regulated in pulmonary fibroblasts and if the perturbation of this cross talk could be linked to the pathogenesis of IPF.

1.3.3 Effect of TGF-β1 on Myofibroblast Differentiation

It is well documented that TGF-β1 can induce the phenotypic modulation of fibroblasts to myofibroblasts in several fibrotic diseases both *in vitro* (Desmouliere et al., 1993) 1993) and *in vivo* (Sime et al., 1997). Human lung fibroblasts treated with TGF-β1 have increased α-SMA and collagen production (Evans et al., 2003) and TGF-β1 treatment induces relatively stable alterations in the fibroblast phenotype. Chronic treatment with TGF-β1 in foetal lung fibroblasts increases in α-SMA which was still present 8 days after the removal of TGF-β1 (Evans et al., 2003; Garrison et al., 2013).

The regulatory mechanisms of TGF-β1-induced fibroblast to myofibroblast differentiation remain unclear. TGF-β1 promotes its pro-fibrotic effects via both its canonical and non-canonical signalling pathways. Smad-3 has been shown to be a key mediator of the fibrotic response. Elevated levels of Smad-3 exist in several models of fibrosis and Smad-3 knock-out mice are protected from bleomycin-induced pulmonary fibrosis (Liu et al., 2003). Likewise, transient overexpression of active TGF-β1 in lungs, using adenoviral vector-mediated gene transfers, resulted in progressive pulmonary fibrosis in wild-type mice, whereas no fibrosis was seen in the lungs of Smad-3 knock mice (Bonniaud et al., 2004). These experiments demonstrate the direct implication of Smad-3 activation downstream of TGF-β1 in the development of pulmonary fibrosis.

TGF-β1 causes excessive ECM deposition by promoting the expression of ECM genes including fibronectin and fibrillar collagens. In human lung fibroblasts overexpression of Smad-2, Smad-3 and Smad-4 proteins is associated with increased production of all collagen types. However, transfection of Smad-3, but not Smad-2, resulted in increased TGF-β1-induced α-SMA expression (Evans et al., 2003). Non-Smad signalling is also thought to be necessary to induce fibroblast to myofibroblast differentiation. TGF-β1-induced α-SMA expression is

dependent on both ERK and p38 phosphorylation in human lung fibroblasts (Sakai and Tager, 2013). The involvement of non-Smad pathways in fibroblast to myofibroblast has received relatively little attention and thus it is necessary to determine if non-Smad signalling pathways can either positively or negatively regulate TGF-β1-induced fibroblast to myofibroblast differentiation.

1.3.4 Importance of TGF-β1 in IPF

There is extensive experimental evidence suggesting a key pathogenic role for TGF-β1 in IPF as well as other fibrotic diseases such as renal fibrosis and liver fibrosis (Leask and Abraham, 2004). TGF-β1 is upregulated in tissue samples from both animal models of IPF and patients (Khalil et al., 1991) and has been shown to promote epithelial cell apoptosis (Hagimoto et al., 2002) and migration (Yu et al., 2008), increase collagen synthesis and promote fibroblast to myofibroblast differentiation (Scotton and Chambers, 2007) and EMT (Kim et al., 2006).

Studies have demonstrated that over expression of active TGF-β1, but not latent TGF-β1 resulted in prolonged and severe pulmonary fibrosis characterised by excessive ECM protein production and the presence of cells with a myofibroblast-like phenotype (Sime et al., 1997). Other studies using inhibitors of TGF-β1 signalling have been shown to ameliorate bleomycin-induced fibrosis in hamsters (Wang et al., 1999) and TGF-β1 receptor inhibitor 1 (ALK-5) was effective at blocking fibrotic progression in a rat model of fibrosis (Bonniaud et al., 2005). Furthermore, TGF-β1 deficient mice display severely impaired late-stage wound repair, such as reduced re-epithelisation and excessive collagen deposition, compared with control mice (Bottinger et al., 1997).

Due to the potent role of TGF-β1 in IPF it is an attractive anti-fibrotic target, however, its therapeutic use may be limited due to the diverse roles of TGF-β1 in normal tissue homeostasis. Mice deficient in TGF-β1 suffered a severe wasting syndrome and an inflammatory response resulting in tissue necrosis, organ failure and death highlighting the potential challenges regarding the use of TGF-β1 as a target for anti-fibrotic therapy (Bottinger et al., 1997). Therefore, anti-fibrotic therapies that suppress excessive wound healing, such as PGE_2 or non-Smad signalling pathways that can negatively regulate pro-fibrotic TGF-β1 signalling, would have more therapeutic benefit for patients with IPF.

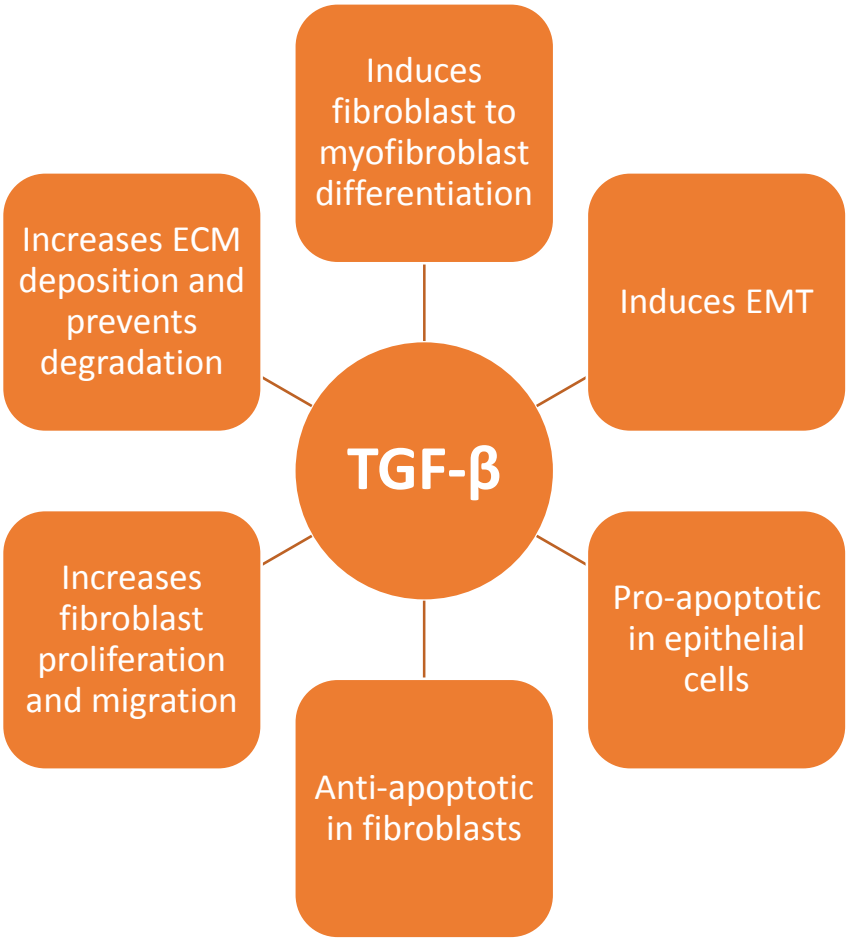


Figure 1-9 Pro-fibrotic effects of TGF-β1

Transforming growth factor-β (TGF-β1) is a potent pro-fibrotic cytokine and a key regulator of tissue fibrosis. TGF-β1 induces myofibroblast differentiation from both resident fibroblasts and epithelial cells via epithelial-mesenchymal transition (EMT). TGF-β1 is also a key regulator of ECM proteins such as collagen and stimulates the production of protease inhibitors that prevent the breakdown of ECM. TGF-β1 promotes fibroblast proliferation, migration and apoptosis resistance in fibroblasts. In contrast, TGF-β1 promotes epithelial apoptosis.

1.4 PGE_2 in IPF

PGE_2 is a major eicosanoid product secreted by fibroblasts, epithelial and stimulated inflammatory cells. It is commonly considered as a pro-inflammatory mediator and is actively involved in the pathogenesis of several diseases, such as rheumatoid arthritis and cancer (Kim and Kim, 2010). The lung represents a peculiar site for the action of PGE_2 as it has therapeutic benefits. PGE_2 can influence the behaviour of all cell types relevant to pulmonary fibrosis, including leukocytes, epithelial cells and mesenchymal cells (Huang and Peters-Golden, 2008). PGE_2 acts in multiple ways and has been shown to decrease fibroblast proliferation (Korn et al., 1980), reduce collagen deposition (Liu et al., 2004), increase collagen degradation (Baum et al., 1980), decrease fibroblast chemotaxis (Kohyama et al., 2001), inhibit TGF-β1-induced fibroblast to myofibroblast differentiation (Thomas et al., 2007) and inhibit EMT (Zhang et al., 2006).

Aside from inhibiting fibroblast functions, PGE_2 is an anti-apoptotic/pro-survival mediator in epithelial cells (Tessner et al., 2004) but a pro-apoptotic mediator in fibroblasts (Huang et al., 2009), and thus plays a key role in the 'apoptosis paradox' in IPF. According to this paradox, IPF is characterised by increased epithelial cell apoptosis and increased myofibroblast resistance to apoptosis (Moodley et al., 2004). Epithelial cells have a large capacity for synthesising PGE_2 but in damaged epithelial cells PGE_2 production is reduced resulting in a vicious cycle of continued epithelial apoptosis and increased persistence of myofibroblasts creating a pro-fibrotic microenvironment (Sakai and Tager, 2013).

Both fibroblasts and epithelial cells cultured from the lungs of IPF patients have an impaired ability to synthesise PGE_2 despite an increase in pro-inflammatory mediators known to increase its production such as TGF-β1, IL-1β and TNF-α (Coward et al., 2009; Keerthisingam et al., 2001; Olman, 2003). In fact, bronchoalveolar lavage collected from IPF patients have

50% less PGE_2 compared with normal controls (Borok et al., 1991). The relevance of this impairment is highlighted by the fact that inhibition or gene deletion of cycloxygenase-2 (COX-2), a key enzyme for PGE_2 synthesis, augmented bleomycin-induced fibrosis in mice (Hodges et al., 2004; Keerthisingam et al., 2001). In contrast, when endogenous PGE_2 is over produced, or when exogenous PGE_2 is administered, it protects against fibrosis in a mouse model of bleomycin-induced fibrosis (Dackor et al., 2011). A clinical trial involving short term administration of aerosolized PGE_2 in patients with IPF demonstrated increased levels of PGE_2 in bronchoalveolar lavage similar to normal controls, however no functional effects were studied and it is therefore difficult to assess its potential as a long term treatment (Borok et al., 1991).

In IPF, the reduced production of PGE_2 leads to an imbalance in eicosanoid synthesis and results in the overproduction of pro-fibrotic leukotrienes (LTs) derived from the 5-lipooxygenase pathway (Figure 1-11) (Borok et al., 1991). LTs stimulate fibroblast proliferation, collagen synthesis and myofibroblast differentiation (Charbeneau and Peters-Golden, 2005). The impact of these *in vitro* actions on fibroblast function is supported by both animal and human studies. Bronchoalveolar lavage and lung homogenates from IPF patients contained more LTs compared with non-fibrotic lung (Charbeneau and Peters-Golden, 2005). In contrast, 5-LO knock-out mice, which are deficient in leukotriene production, are protected from bleomycin-induced fibrosis (Peters-Golden et al., 2002). Therefore, through both their actions on fibroblasts, LTs are pro-fibrotic and PGE_2 is anti-fibrotic suggesting that an imbalance in eicosanoid synthesis significantly contributes to the pro-fibrotic microenvironment.

Given the diverse and potent anti-fibrotic effects of PGE_2, approaches to administer it directly or to increase PGE_2 synthesis seems promising as a therapeutic treatment. The anti-fibrotic

effects of PGE_2 are not limited to pulmonary fibroblasts and have been documented in other fibrosing conditions, such as renal and cardiac fibrosis. However, despite the anti-fibrotic effects of PGE_2 its use as a therapeutic agent is limited. Systemic delivery of PGE_2 would likely be intolerable due to potent pro-inflammatory effects. PGE_2 is actively involved in the pathogenesis of several diseases ranging from rheumatoid arthritis to cancer (Vancheri et al., 2004). Inhalation of PGE_2 has been shown to illicit cough in some patients (Gauvreau et al., 1999) and its short half-life results in an arduous dosing regimen. In addition, another potential hurdle was raised by a recent study demonstrating that fibroblasts isolated from some patients with IPF are resistant to the anti-fibrotic effects of PGE_2 owing to the down regulation of the PGE_2 receptor EP2 (Huang et al., 2008a). Understanding the mechanism through which PGE_2 exerts its inhibitory effects on fibroblasts, and in particular how PGE_2 inhibits TGF-β1-induced fibroblast to myofibroblast differentiation, will potentially disclose new therapeutic targets for the treatment of IPF. Therefore, further investigation is necessary to fully characterise the signalling pathway of PGE_2 and identify if other selective agents can mimic the anti-fibrotic effects of PGE_2.

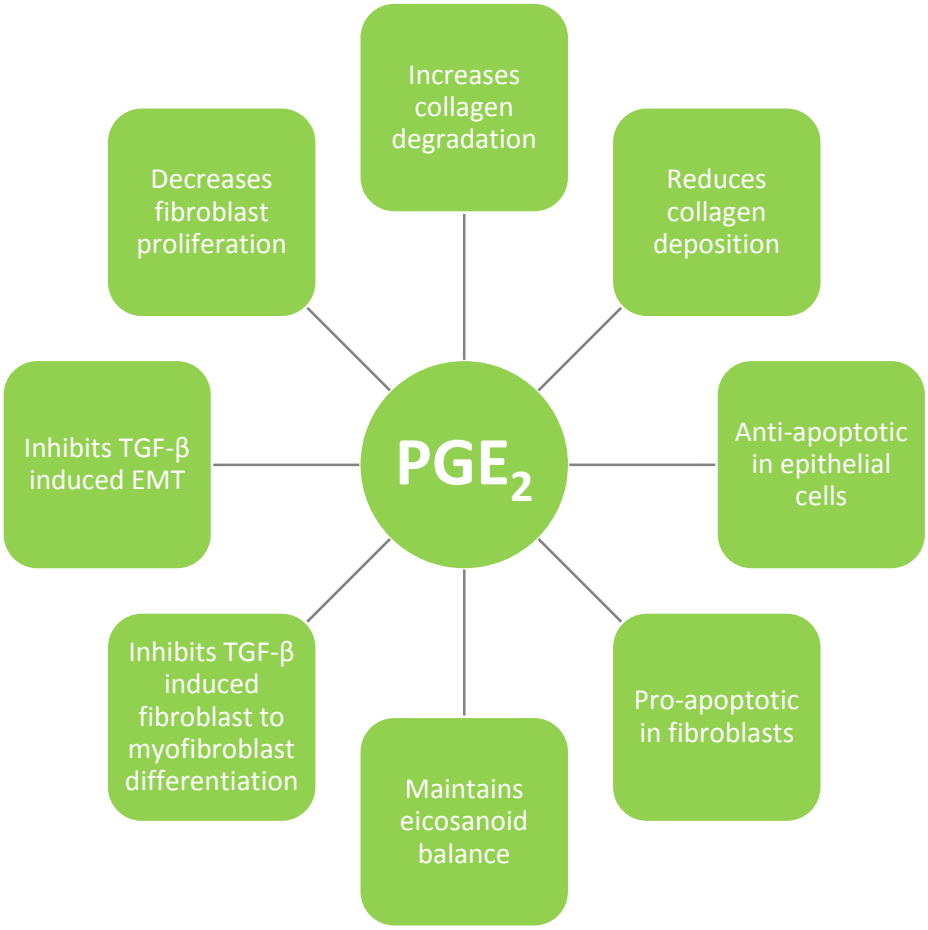


Figure 1-10 Anti-fibrotic effects of PGE_2

PGE_2 has been shown to have several inhibitory effects on fibroblasts and is a potent anti-fibrotic mediator. PGE_2 inhibits collagen production, fibroblast proliferation and prevents TGF-β1-induced fibroblast to myofibroblast differentiation and EMT. In addition, PGE_2 increases collagen degradation. Furthermore, PGE_2 is pro-apoptotic in fibroblasts and anti-apoptotic in epithelial cells.

1.4.1 PGE_2 Effect on Myofibroblast Differentiation

Although numerous mediators are known to promote fibroblast to myofibroblast differentiation PGE_2 is one of few factors identified to inhibit fibroblast to myofibroblast differentiation (Kolodsick et al., 2003). In normal conditions, the homeostatic control of cell proliferation and survival is finely tuned by multiple mechanisms of feedback. PGE_2 is part of an important feedback mechanism in which fibroblasts and epithelial cells increase their PGE_2 production in response to TGF-β1 to limit fibroblast to myofibroblast differentiation. However, fibrotic fibroblasts exhibit a marked reduction to up-regulate PGE_2 synthesis in response to TGF-β1 (Coward et al., 2009) and thus are unable to self-limit tissue repair and prevent fibrosis.

1.4.2 PGE_2 Production

PGE_2 is derived from the 20-carbon fatty acid arachidonic acid (AA). The initial step in PGE_2 synthesis involves the hydrolysis of cell membrane phospholipids via phospholipase A2 (PLA2) to free AA from the cell membrane. Free AA is then converted into a variety of oxygenated metabolites by several parallel metabolic pathways, the most studied of which is the 5-lipoxygenase (5-LO) which converts AA into leukotrienes and the cyclooxygenase (COX) pathway which converts AA into prostaglandins (PGs) (Figure 1-11). The COX pathway initially converts AA into an unstable intermediate, Prostaglandin H_2 (PGH_2), which is then metabolised via the corresponding prostanoid synthase into active prostanoids, such as PGE_2 and Prostacyclin (PGI_2). Individual cell types generate specific profiles of eicosanoids that reflect their complement of terminal synthase enzymes.

There are two isoforms of COX; COX-1 and COX-2. The former is constitutively expressed in many tissues, whereas COX-2 is tightly regulated and only expressed after induction by certain stimuli, such as IL-1β, lipopolysaccharide (LPS) and TGF-β1 (Bradbury et al., 2003). Due to the constitutive expression of COX-1 prostaglandin synthesis can proceed within

minutes and can be further amplified following COX-2 induction in response to certain stimuli allowing both immediate and delayed responses (Charbeneau and Peters-Golden, 2005).

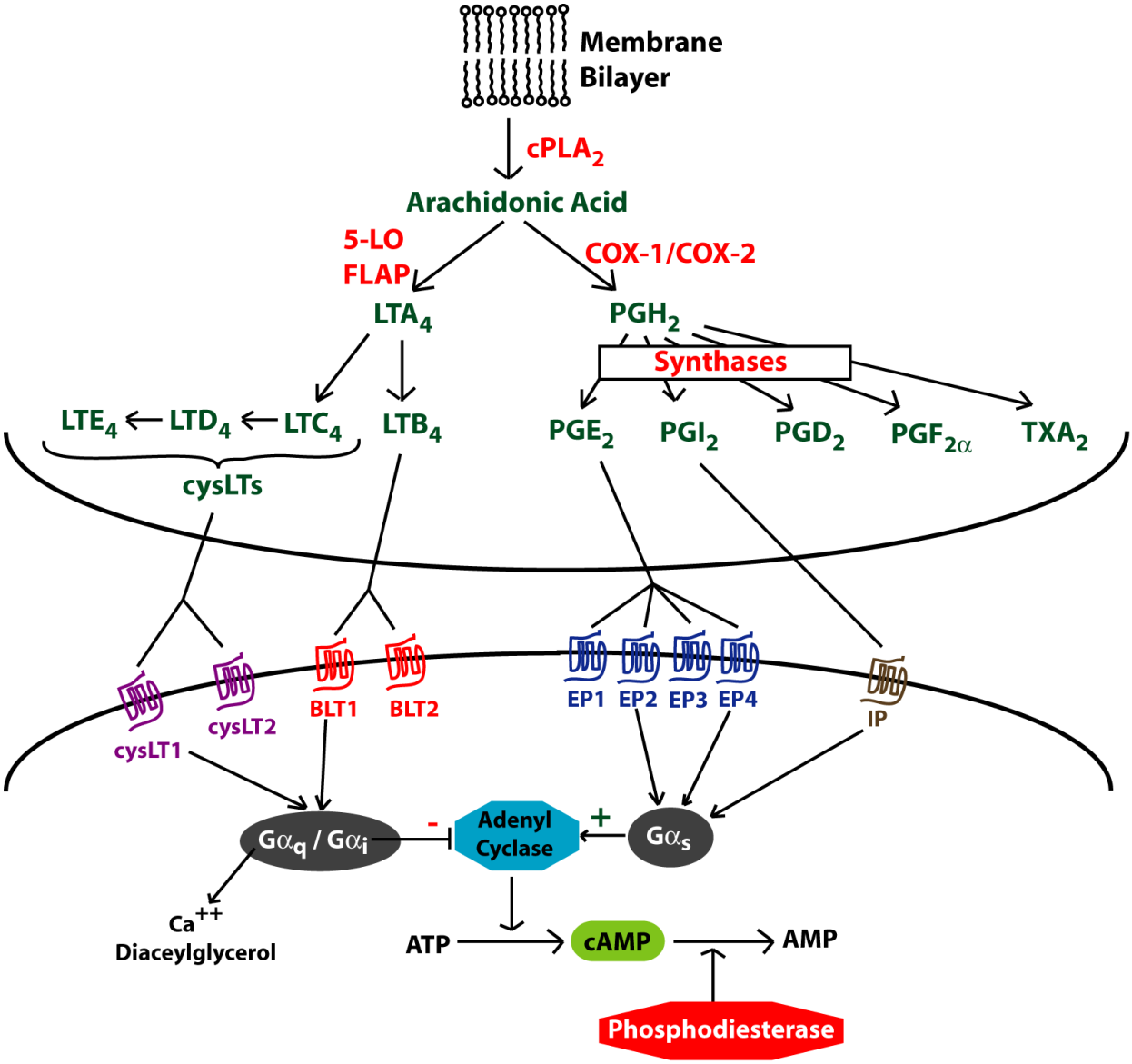


Figure 1-11 Biosynthetic Pathways and Receptors for Prostaglandins and Leukotrienes

Arachidonic acid is metabolised into Prostaglandins (PGs) and Leukotrienes (LTs) via the COX pathway and the 5-LO pathway, respectively. PGE_2 synthesis is initiated by the mobilisation of arachidonic acid from the membrane by phospholipase 2 (PLA2). Arachidonic acid is then converted into an unstable intermediate, Prostaglandin H2 (PGH2), by cyclooxygenase (COX) enzymes, either COX-1 or COX-2. PGH2 is further converted into five different prostaglandins, including Prostaglandin E2, via prostanoid synthases. Leukotrienes are metabolised from arachidonic acid by 5-Lipooxygenase (5-LO) and the 5-LO activating protein (FLAP) into Leukotriene A4 (LTA4). LTA4 is converted into LTB4 or cysteinyl-leukotrienes (cysLTs): LTC4, LTD4 and LTE4, via LTA4 hydrolyase and LTA4 synthase

respectively. Prostaglandins signal via G-protein-coupled receptors, EP1 – EP4, whereas LTs signal via cysLT receptors or BLT receptors. (Huang and Peters-Golden, 2008).

1.4.2.1 COX-2 Expression in F-IPF

Fibroblasts derived from patients with IPF have a limited capacity to synthesis PGE_2 compared with control fibroblasts. This is due to an inability to up-regulate COX-2 expression at both the mRNA and protein level (Coward et al., 2009; Keerthisingam et al., 2001). COX-2 deficient mice have limited induction of PGE_2 synthesis and are more susceptible to pulmonary fibrosis after bleomycin treatment (Keerthisingam et al., 2001). In contrast, overexpression of COX-2 in the lungs of mice using gene therapy, leads to an increase in PGE_2 synthesis and inhibits fibroblast proliferation (Jenkins et al., 2002). Furthermore, reduced activity of COX-2 has also been demonstrated in bronchial epithelial cells suggesting that diminished COX-2 expression may be a generalised abnormality in pulmonary cells of IPF patients and not just limited to fibroblasts (Petkova et al., 2003).

F-IPF are unable to induce COX-2 expression after treatment with several different stimulants suggesting this is a defect at the level of enzyme synthesis rather than a receptor or signalling abnormality in response to specific stimulants (Coward et al., 2009). The molecular mechanisms underlying the limitation in COX-2 induction is not yet fully understood. It has been demonstrated that COX-2 expression is reduced in F-IPF due to decreased histone acetylation causing COX-2 to be epigenetically repressed (Coward et al., 2009). The mediator or upstream effects that cause this epigenetic change remains unknown. Previous studies focus on the inhibitory effects of PGE_2 on fibroblasts but to date there is little understanding if PGE_2, or other agents, are capable of restoring COX-2 expression.

1.4.3 PGE_2 Receptors

The activity of PGE_2 is mediated by four E prostanoid receptors, EP1-EP4, which are coupled to distinct intracellular signalling machinery (Figure 1-11). These are specific transmembrane G-protein-coupled receptors that signal via alterations in intracellular Ca^{2+} and cAMP concentrations to activate a range of protein kinases and signalling pathways (Narumiya et al., 1999). Although the exact roles of each receptor type are not definitively established, it is known that stimulation of G_s-coupled EP2 and EP4 receptor increases intracellular cAMP concentrations, the G_i-coupled EP3 receptor decreases cAMP concentrations and the G_q-coupled EP1 receptor mediates increased intracellular Ca^{2+} levels (Narumiya et al., 1999). Two receptors for cysLTs (cysLT1 and cysLT2) and LTB4 (BLT1 and BLT2) have also been identified (Heise et al., 2000; Lynch et al., 1999; Yokomizo et al., 2000). The activation of LT receptors results in increased intracellular Ca^{2+} and decreased cAMP levels. The opposing effects of PGs and LTs in fibrosis reflect the activation of opposing signal transduction pathways. The eicosanoid receptor diversity, and the possibility that multiple receptors are expressed in a single cell, might explain the diverse biological responses and cellular specificity elicited by PGs and LTs in different cells and tissues. It is also possible that, in areas of actively on-going fibrosis or inflammation, receptor expression changes in response to pro-fibrotic or pro-inflammatory mediators which then alters the effect of eicosanoids on that particular cell. For example, one study demonstrated down regulation of the EP2 receptor in a subset of fibroblasts from a mouse model of fibrosis and some patients with IPF, resulting in these cells becoming less responsive to PGE_2 (Huang et al., 2008a; Moore et al., 2005).

1.4.4 PGE_2 Signalling

Due to the potent anti-fibrotic effects of PGE_2 the mechanism by which it exerts its inhibitory effects in lung fibroblasts has received great attention. Studies have demonstrated that the suppressive effects of PGE_2 on fibroblasts are mainly mediated by EP2 receptor activation

and subsequent increases in cAMP (Kolodsick et al., 2003; Liu et al., 2004). Northern blot analysis of mRNA distribution has shown that EP2 is abundantly expressed within the lungs (Breyer et al., 2001) and EP2 mRNA has been shown to be expressed at significantly higher levels compared with EP4 mRNA in pulmonary fibroblasts (Huang et al., 2007). Furthermore, studies have demonstrated EP2 receptor antagonists block the inhibitory effects of PGE_2 suggesting that EP2, and not EP4, is the major inhibitory receptor (Kolodsick et al., 2003). Although both the EP2 and EP4 receptor increase cAMP production there are significant differences between these receptors. For example, the EP2 receptor only signals via cAMP whereas, the EP4 utilises the phosphatidylinositol-3-kinase (PI3K) pathway in addition to cAMP signalling (Regan, 2003). In addition, EP4 receptors undergo rapid PGE_2 induced desensitisation whereas the EP2 receptor does not (Nishigaki et al., 1996).

Studies using cAMP elevating agents, such as Forskolin, a direct adenylyl cyclase activator, and Isoproterenol, a β_2-adrenergic receptor agonist, inhibit fibroblast proliferation and decrease collagen I mRNA (Liu et al., 2004). These studies highlight the importance of the PGE_2-EP2-cAMP signalling cascade in regulating multiple fibroblast functions. Although increased cAMP seems to be crucial to inhibit several fibroblast functions, the anti-fibrotic ability of various agonists does not always correlate with their ability to increase cAMP. Therefore, it is possible that unidentified PGE_2-induced signalling pathways, distinct from cAMP, may also contribute to the inhibitory actions of PGE_2 (Kolodsick et al., 2003).

1.4.4.1 cAMP

cAMP is a ubiquitous second messenger that regulates many physiological processes ranging from learning and memory to contractility and relaxation of the heart. At a cellular level, cAMP plays an important role in virtually every known function such as metabolism, gene expression, cell division and growth, differentiation and death (Cheng et al., 2008). Various extracellular

signals converge in the cAMP signalling cascade and therefore it is tightly regulated at several levels to maintain specificity.

cAMP generation and degradation is regulated by two families of enzymes; adenylyl cyclases (AC), which catalyse its synthesis from ATP, and cyclic nucleotide phosphodiesterases (PDEs) which catalyse its degradation (Dunkern et al., 2007; Liu et al., 2004; Liu et al., 2005a). These enzymes are differentially expressed and regulated in a cell type and stimulus-specific manner (Tasken and Aandahl, 2004). ACs and PDEs can be regulated both positively and negatively by several other signalling pathways such as the MAPK pathway and this cross talk between other signalling pathways provides further modulation of signal strength and cell-type specificity (Sassone-Corsi, 2012).

There are nine isoforms of adenylyl cyclases, AC1-9, and most tissues and cell types express more than one AC isoform (Cooper, 2003). Specific isoforms of ACs are tightly regulated by several other signalling pathways including G-protein subunits, Ca^{2+} and PKA (Cooper, 2003). Most ACs are activated by G-protein coupled receptors, such as EP2 receptor and β_2-adrenoceptor, by interactions with the α subunit of the G_s-receptor (α_s). α_s is released from the heterotrimeric αβγ G protein upon binding of ligand and binds to and activates AC. Alternatively, AC can be inhibited by ligands that stimulate G-protein coupled receptors coupled to G_i.

In addition to the complex regulation of cAMP production, degradation of cAMP is also tightly regulated by phosphodiesterases. There are eleven families of PDEs, PDE1-PDE11, each of which are differentiated by their substrate specificity. PDEs 1, 2, 3 and 4 are expressed in many tissues whereas other isoforms are more restricted (Francis et al., 2011). The majority

of PDE families preferentially degrade cAMP. Some PDE families can degrade cyclic guanine monophosphate (cGMP) as well as cAMP, known as dual-specificity PDEs, whereas other PDEs exclusively hydrolyse either cAMP or cGMP (Figure 1-12) (Lugnier, 2006). In many cells PDE3 and PDE4 account for most of the cAMP-hydrolysing activity of the cell (Conti et al., 2003).

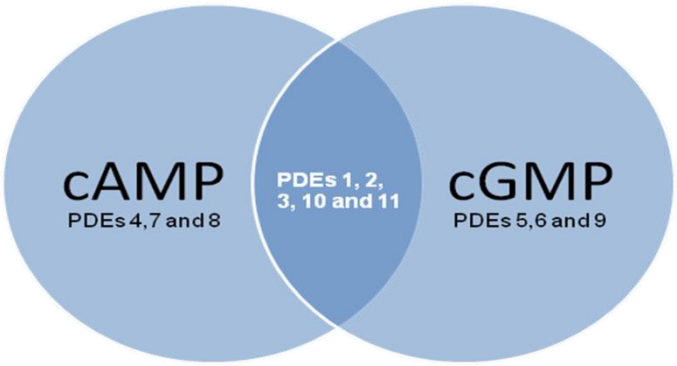


Figure 1-12 Overview of the Phosphodiesterase Families

Phosphodiesterase (PDE) are categorised into 11 families (PDE1-11) according to their preference for either cAMP or cGMP hydrolysis. Certain PDEs are highly specific for the hydrolysis of cAMP (PDE4, PDE7 and PDE8), cGMP (PDE5, PDE6 and PDE9) or both (PDE1, PDE2, PDE3, PDE10 and PDE11). Dual specificity PDEs can differ significantly in preference for cAMP or cGMP.

1.4.4.2 cAMP Signalling

Upon binding of a ligand the G-protein coupled receptor activates AC which catalyses the synthesis of cAMP from ATP (Cheng et al., 2008). cAMP has two main effectors: cAMP-dependent protein kinase (PKA) and exchange protein activated by cAMP (Epac) (Sassone-Corsi, 2012).

1.4.4.2.1 PKA

cAMP exerts its effects mainly by activating PKA. PKA is a serine-threonine kinase and is implicated in the regulation of a wide range of cellular processes (Taylor et al., 1992). Under basal conditions PKA is localised to the cytosol as an inactive enzyme. PKA is a heterotetramer composed of two regulatory (R) and two catalytic (C) subunits which are differentially expressed and are able to form different isoforms of PKA. There are several isoforms of each subunit, RIα, RIβ, RIα, RIIβ and Cα, Cβ, Cγ which possess distinct physical and biological properties (Taylor et al., 1992). There are two general classes of PKA, designated as PKA (I) and PKA (II), due to differences in their regulatory subunits. These isoforms are differentially expressed in a variety of cells and exert distinct roles in regulating cellular processes (Constantinescu et al., 2002). PKA is activated by cAMP binding to two sites on each R subunit, which causes their dissociation from the catalytic subunits (Taylor et al., 1992). The catalytic subunit can then affect a wide range of cellular events by phosphorylating an array of cytoplasmic and nuclear protein substrates including enzymes and transcriptional factors (Cheng et al., 2008). For example, PKA phosphorylates and causes the deactivation of phospholipase C (PLC), activates MAP kinases and decreases the activity of Raf-1 and Rho. In addition PKA can regulate various ACs and PDEs controlling both positive and negative feedback loops (Tasken and Aandahl, 2004).

Regulation of transcription by PKA is achieved by translocation of the catalytic subunit into the nucleus and direct phosphorylation of the cAMP response element binding protein (CREB) (Taylor et al., 1992). CREB phosphorylation is crucial for its transcriptional activity as it allows CREB to interact with transcriptional co-activators or co-repressors. Activation of CREB is critical for the regulation of a wide range of cellular functions, including survival and proliferation (Mayr and Montminy, 2001). In order to initiate gene transcription CREB co-localises with the CREB-binding protein (CBP). This complex then binds to a cAMP response element (CRE) on gene promoters allowing transcriptional regulation of downstream genes (Mayr and Montminy, 2001) (Figure 1-13).

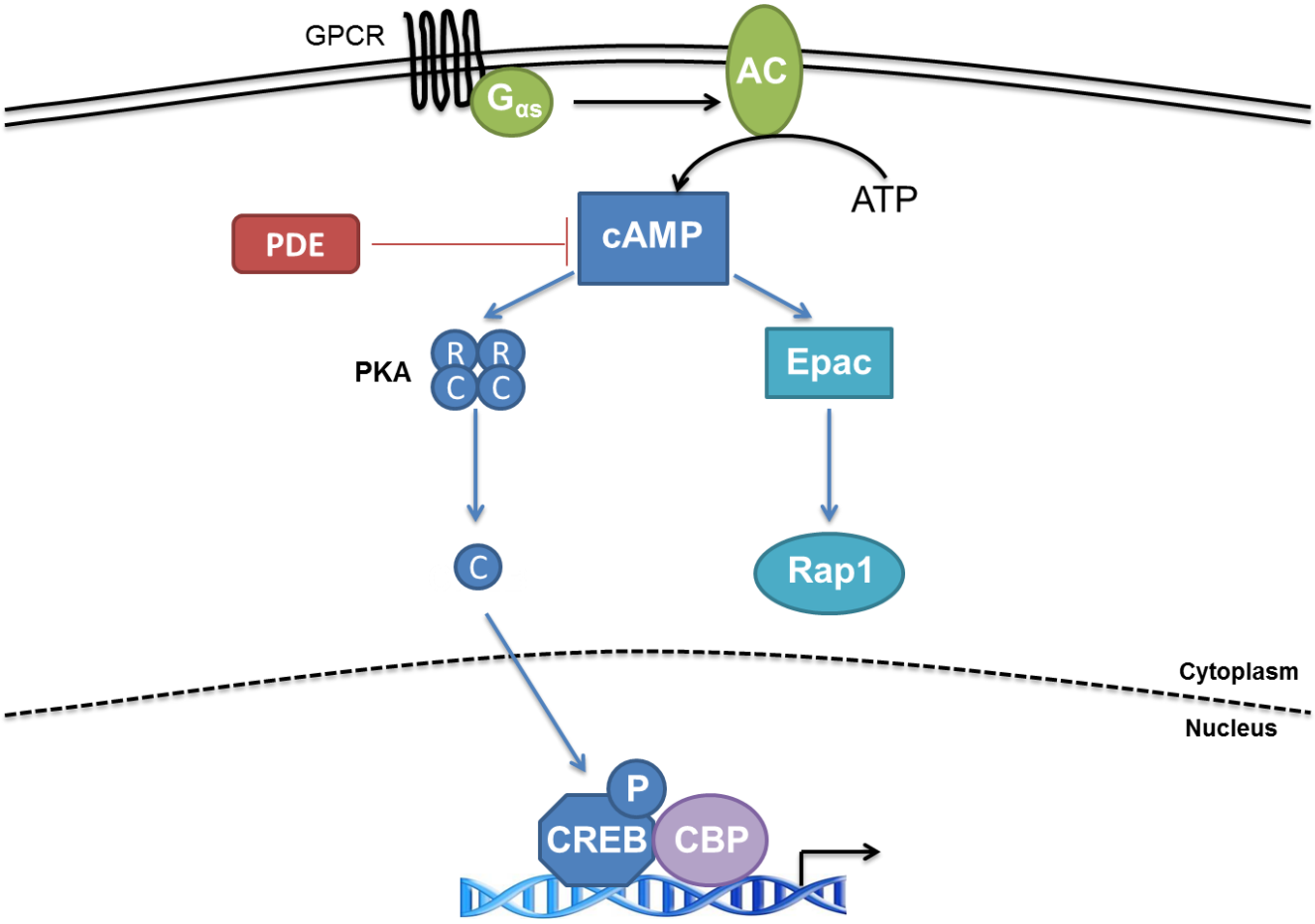


Figure 1-13 cAMP signalling via PKA and Epac

Binding of a ligand to a G-protein coupled receptor (GPCR) activates the $G_{\alpha s}$ protein which dissociates from the GPCR and activates adenylyl cyclase (AC). AC catalyses the production of intracellular cAMP from ATP. The degradation of cAMP is regulated by phosphodiesterases (PDE). cAMP binds to and activates Protein Kinase A (PKA) causing the catalytic subunit to translocate to the nucleus and phosphorylate the cAMP response element binding protein (CREB). Phosphorylated CREB binds to the CREB binding protein (CBP) and this complex binds to the cAMP response element (CRE) on the gene promoter. cAMP can also activate exchange protein activated by cAMP (Epac). Epac causes subsequent activation of the small G protein, Rap1, however downstream signalling of Epac is currently poorly understood.

1.4.4.2.2 Epac

Although many of the physiological effects of cAMP can be ascribed to the action of PKA experimental observations suggested the existence of “PKA-independent” mechanisms and exchange protein activated by cAMP (Epac) was recently identified as a novel cAMP effector (de Rooij et al., 1998). Signalling through PKA and Epac has been shown to have distinct, synergistic and even antagonistic effects on cellular function. In fact, some of the functions originally attributed to PKA are now recognised to be mediated by Epac (Cheng et al., 2008).

There are two isoforms of Epac, Epac1 and Epac2, which possess one and two cAMP binding sites respectively. Epac1 is ubiquitously expressed in all tissues, whereas Epac2 is more limited in its distribution (Cheng et al., 2008). Epac functions as a guanine nucleotide exchange factor and activates the small GTPase protein, Rap1, through its ability to promote the exchange of GDP for GTP (de Rooij et al., 1998). Rap1 was first identified as an antagonist of Ras, a small GTPase binding protein (Kitayama et al., 1989), and is implicated in a number of biological processes from cell proliferation and differentiation to cell adhesion (Stork, 2003).

The existence of two highly coordinated cAMP effectors provides a mechanism for more precise and integrated control of cAMP signalling. The binding affinity of cAMP for PKA and Epac has been found to be very similar (K_d ~ 2.9 µM) and as such it has been proposed that PKA and Epac are activated in response to moderate increases of cellular cAMP and PKA and/or Epac activation depends on compartmentalisation of cAMP and the availability of these effector proteins (Dao et al., 2006)

Extensive studies have established that Epac is involved in a host of cAMP related cellular functions such as cell adhesion, differentiation, proliferation, gene expression and apoptosis

(Cheng et al., 2008). Knowledge of Epac and downstream signalling in fibroblasts, at present, is still limited. However, one study has demonstrated that PKA and Epac exert distinct and independent effects on different cell functions in lung fibroblasts. The activation of Epac1 and Rap1 is responsible for the inhibition of fibroblast proliferation, independently of PKA, whereas activation of PKA inhibits collagen production (Huang et al., 2008a). Studies using canine kidney epithelial cells (Insel et al., 2012) and rat cardiac fibroblasts (Yokoyama et al., 2008) show that treatment with pro-fibrotic agents, such as TGF-β1, decreases the expression of Epac. Conversely, overexpression of Epac inhibits pro-fibrotic responses implying the importance of Epac in regulating the fibrotic response (Yokoyama et al., 2008). The mechanism that regulates Epac expression and inhibition of its expression via TGF-β1 remains to be determined. Interestingly, Rap1 targeted siRNA did not affect Epac induced inhibition of collagen synthesis (Yokoyama et al., 2008) suggesting a role for other downstream mediators of Epac.

The role of PKA and Epac in fibroblast to myofibroblast differentiation, or whether PGE_2 signals via PKA and/or Epac has not yet been investigated. Understanding the importance of PKA and Epac in PGE_2 signalling is necessary to identify alternative therapeutic targets which act in a similar manner to PGE_2.

1.4.4.3 cAMP and TGF-β1 signalling

In addition to directly regulating many important cellular processes, cAMP integrates and interacts with an array of intracellular signalling pathways in order to achieve an integrated response.

The cAMP pathway is known to integrate with the TGF-β1 signalling pathway. One example of cross talk between these two pathways is evidence by the fact that cAMP causes inhibition

of TGF-β1-induced pro-fibrotic gene transcription. This is hypothesised to be due to direct competition between CREB and Smads for transcriptional co-activators (Figure 1-8) (Liu et al., 2006a). Interestingly, one study demonstrated that fibroblasts isolated from fibrotic lung are less responsive to the anti-fibrotic effects of cAMP, with regard to proliferation and collagen synthesis, compared with normal fibroblasts (Liu et al., 2005b). The levels of cAMP and PKA expression did not vary between fibrotic and non-fibrotic fibroblasts but there was reduced cAMP-stimulated CREB phosphorylation in fibroblasts isolated from patients with IPF. The authors hypothesise that the reduced amount of phosphorylated CREB resulted in increased availability of transcriptional co-activators for Smad proteins allowing Smad transcription of pro-fibrotic genes to preferentially take place (Liu et al., 2005a). Although TGF-β1 primarily signals via Smad proteins, non-canonical TGF-β1 signalling pathways such as ERK/MAPK, Rho/JNK and PI3K/Akt can also be inhibited by cAMP, however, the exact mechanism remains unknown (Liu et al., 2006a). The mechanism by which cAMP inhibits TGF-β1 signalling in fibroblasts, either via canonical or non-canonical signalling pathway, is an area where further research is required.

1.5 Effect of cAMP Stimulants on Myofibroblast Differentiation

It is evident that cAMP is a key negative regulator of fibroblast function (Kolodsick et al., 2003; Liu et al., 2004). Therefore, increasing cAMP by stimulating G-protein receptors, increasing AC activity, decreasing PDE activity, or using cAMP analogues could all potentially have inhibitory and anti-fibrotic effects on fibroblasts.

G-protein-coupled receptor agonists, such as β_2-agonists salmeterol and isoprotenol, and iloprost, a stable prostacyclin derivative, have been shown to inhibit fibroblast proliferation, decrease TGF-β1-induced α-SMA expression and down regulate collagen deposition in

human lung fibroblasts (Baouz et al., 2005; Goulet et al., 2007; Liu et al., 2004; Stratton et al., 2002).

Several studies using pulmonary fibroblasts have also demonstrated that direct activation of adenylyl cyclase by forskolin, or over expression of adenylyl cyclase, inhibited proliferation, decreased α-SMA expression, decreased collagen synthesis and inhibited TGF-β1-induced myofibroblast differentiation (Failla et al., 2009; Huang et al., 2007; Kolodsick et al., 2003; Liu et al., 2004). One would expect that increasing all adenylyl cyclase isoforms, due to their ability to increase cAMP, would have anti-fibrotic effects. However, a recent study demonstrated that over expression of adenylyl cyclase 6 increased signalling by β_2-agonists and iloprost, but not PGE_2 signalling (Liu et al., 2010). This study was also supported using an *in vivo* model of lung fibrosis. Transgenic mice that over expressed adenylyl cyclase 6 treated with prostacyclin had decreased fibrosis and collagen deposition compared with wild-type mice in a model of bleomycin-induced fibrosis (Liu et al., 2010). Therefore, further investigation is necessary to determine if over expression of a different adenylyl cyclase isoform could enhance the anti-fibrotic effects of PGE_2.

In addition, inhibiting the degradation of cAMP, using PDE4 inhibitors, such as roflumilast and rolipram, has been shown to attenuate fibroblast chemotaxis, reduce α-SMA and collagen expression and increase COX-2 mRNA and protein in various lung fibroblast cell lines (Dunkern et al., 2007; Togo et al., 2009). Furthermore, TGF-β1-induced α-SMA expression in primary human lung fibroblasts is inhibited with PDE4 inhibitors alone and synergistically with the addition of PGE_2. PGE_2 plus the PDE4 inhibitor caused intracellular cAMP levels to increase synergistically (Dunkern et al., 2007). Interestingly, PDE4 inhibitors were more effective at inhibiting pro-fibrotic activity in the presence of TGF-β1. This is likely due to augmented PGE_2 production as TGF-β1 increases PGE_2 expression in normal pulmonary

fibroblasts (Togo et al., 2009). However, whether PDE4 inhibitors would elicit anti-fibrotic effects in myofibroblasts, which are deficient in their ability to produce PGE_2, remains unknown.

Many of the above studies have focussed on the anti-fibrotic effects of cAMP stimulants in commercially available cell lines or normal pulmonary fibroblasts and their ability to prevent TGF-β1-induced fibroblast to myofibroblast differentiation. However, many patients with IPF only present clinically after significant fibrosis has already occurred (Raghu et al., 2011). Therefore, determining whether or not cAMP stimulants have the same anti-fibrotic potential in already differentiated myofibroblasts, which have an established pro-fibrotic phenotype, would be more useful to assess the therapeutic benefit and requires further investigation.

1.5.1 Potential Therapeutic effects of cAMP Stimulants in IPF

Based on the above *in vitro* data the use of cAMP stimulants appears to be an attractive anti-fibrotic therapeutic approach, however, no such therapies have yet been clinically evaluated.

cAMP elevating agents, such as β_2-agonists, iloprost and roflumilast, are already used clinically for the treatment of various lung diseases including asthma, pulmonary hypertension and Chronic Obstructive Pulmonary Disease (COPD) due to their bronchodilatory effects (Baouz et al., 2005; Goulet et al., 2007; Racke et al., 2008). Therefore, there is great potential to extend the use of these cAMP elevating agents to IPF patients. Some cAMP elevating agents may also have additional benefits for IPF patients. For example, pulmonary hypertension is a secondary effect of IPF, and as such treatment with iloprost may have anti-fibrotic potential in addition to its ability to reduce arterial blood pressure (Galie et al., 2002). Iloprost has been shown to reduce TGF-β1-induced CTGF production and collagen synthesis

in normal dermal fibroblasts and as such may have potential to treat scleroderma, an autoimmune disorder characterised by fibrosis of the skin (Stratton et al., 2001). Despite this, studies have not evaluated the effect of iloprost, or other cAMP elevating agents, on the prevention or reversal of myofibroblast differentiation in primary human fibroblasts or evaluated their use clinically.

Several PDE inhibitors are also used clinically for a variety of lung diseases and are well tolerated (Rabe, 2011). *In vitro* data supports the concept that cAMP elevating agents could be used in conjunction with PDE inhibitors as a novel therapeutic treatment to further increase cAMP production. One study evaluated the effect of prostacyclin analogues, alone and in conjunction with PDE inhibitors, in an animal model of pulmonary hypertension. The study demonstrated that prostacyclin treatment in combination with PDE inhibitors resulted in significant synergistic effects on vascular remodelling and attenuated pulmonary vasoconstriction in rat lungs. In contrast, the use of a prostacyclin analogue alone did not significantly attenuate the development of pulmonary hypertension (Phillips et al., 2005). This study provides further rationale for the use of PDE inhibitors, in conjunction with cAMP elevating agents, as a novel treatment for IPF patients.

Although cAMP analogues are not currently available for clinical use, several cAMP analogues have been developed which can selectively activate PKA or Epac (Holz et al., 2008) which, if developed further, could be a potential therapeutic option for IPF patients. The role of PKA versus Epac in modulating fibroblast functions, such as fibroblast migration, contraction and differentiation into myofibroblasts, remains relatively unknown and requires further investigation. Understanding the mechanisms by which myofibroblast differentiation is controlled via PKA, Epac and their downstream signalling components will assist in the

development of these as therapeutic agents and enhance our understanding of the pathogenesis of IPF.

Increasing intracellular cAMP concentrations by means of selective inhibitors of phosphodiesterases or activators of adenylyl cyclase is an attractive therapeutic approach for the treatment of IPF and as such further investigation into their anti-fibrotic effects on primary pulmonary fibroblasts and myofibroblasts is of great interest. These alternative therapeutic targets may confer more specificity than PGE_2 treatment alone, reduce the arduous dosing regimen necessary for PGE_2 and would still be effective in patients with EP2 receptor defects as suggested by previous studies (Huang et al., 2008a). A more targeted therapy may also avoid side effects caused by PGE_2 such as cough which was caused in some patients who inhaled PGE_2 (Gauvreau et al., 1999) and limit the systemic side effects of PGE_2 binding to EP1 and EP3 receptors in other organs (Borok et al., 1991).

1.6 Ras

As previously mentioned in this thesis, in addition to Smad signalling TGF-β1 is able to activate other signalling pathways, the nature of which depends on the cell type and the target of interest (Mulder, 2000). One example is that TGF-β1 can signal via Ras and activate certain Mitogen-Activated Protein Kinases (MAPKs), including the Extracellular Signal-Related Kinases, ERK-1 and ERK-2, and Jun-N-Terminal Kinase (JNK) (Engel et al., 1999; Hartsough et al., 1996; Mulder and Morris, 1992). However, the role of TGF-β1 signalling via Ras has received very little attention in the context of fibrosis.

Ras is a member of the superfamily of small GTP-binding proteins (G proteins). G proteins play a central role in cell biology by coupling signals generated within biological membranes

to intracellular effectors. G proteins are monomeric proteins with molecular masses of 20 to 40 kDa. More than 100 G proteins have been identified and the members of this superfamily are structurally classified into five distinct families: Ras, Rho, Rab, Sar1/Arf and Ran (Takai et al., 2001). The families are divided according to the degree of sequence conservation and different families are responsible for different cellular processes, for example, the Ras family is a key regulator of cell growth whereas the Rho family controls actin-cytoskeleton protein expression (Downward, 2003).

Ras proteins have been the subject of intense scrutiny since their pro-oncogenic effects were first identified more than 40 years ago (Malumbres and Barbacid, 2003). There are three major mammalian isoforms of Ras: Ha-Ras, K_i-Ras, and N-Ras. Although Ras proteins share a high degree of sequence homology data suggests they have distinct roles in cell physiology (Olson and Marais, 2000). Gene targeting experiments that selectively knocked out Ras from the mouse genome showed that K_i-Ras, but not Ha-Ras or N-Ras, is essential for development (Johnson et al., 1997). However, further investigation is necessary to determine the individual role of each Ras isoform.

In order for Ras to function properly it requires post-translational modifications. The purpose of this is to localise Ras proteins to the correct subcellular compartment and to allow Ras to bind to other regulators and downstream effectors (Takai et al., 2001). As Ras is a cytosolic protein the addition of a farnesyl group, via Farnesyltransferase, is necessary to allow Ras localisation to the inner membrane (Hancock et al., 1989).

Ras proteins operate as a molecular switch for several intracellular signalling cascades modulating cellular responses such as cell proliferation, differentiation and survival amongst

many other cellular functions by cycling between an active “on” and inactive “off” state (Omerovic et al., 2007).

1.6.1 Ras protein cycle: activation/inactivation

Ras proteins are membrane localised guanine-nucleotide binding proteins which are regulated by a guanosine diphosphate/guanosine triphosphate (GDP/GTP) cycle, being inactive when bound to GDP (Ras.GDP) and active when bound to GTP (Ras.GTP) (Figure 1-14) (Olson and Marais, 2000). Ras proteins are activated by receptor tyrosine kinases (RTKs). Following stimulation by ligands, RTKs recruit adaptor proteins and Guanine Nucleotide Exchange Factors (GEFs) to the plasma membrane to promote GDP/GTP exchange (Olson and Marais, 2000). The binding of GTP to Ras results in allosteric changes which increases the affinity of effector interactions and allows the initiation of downstream signalling (Takai et al., 2001).

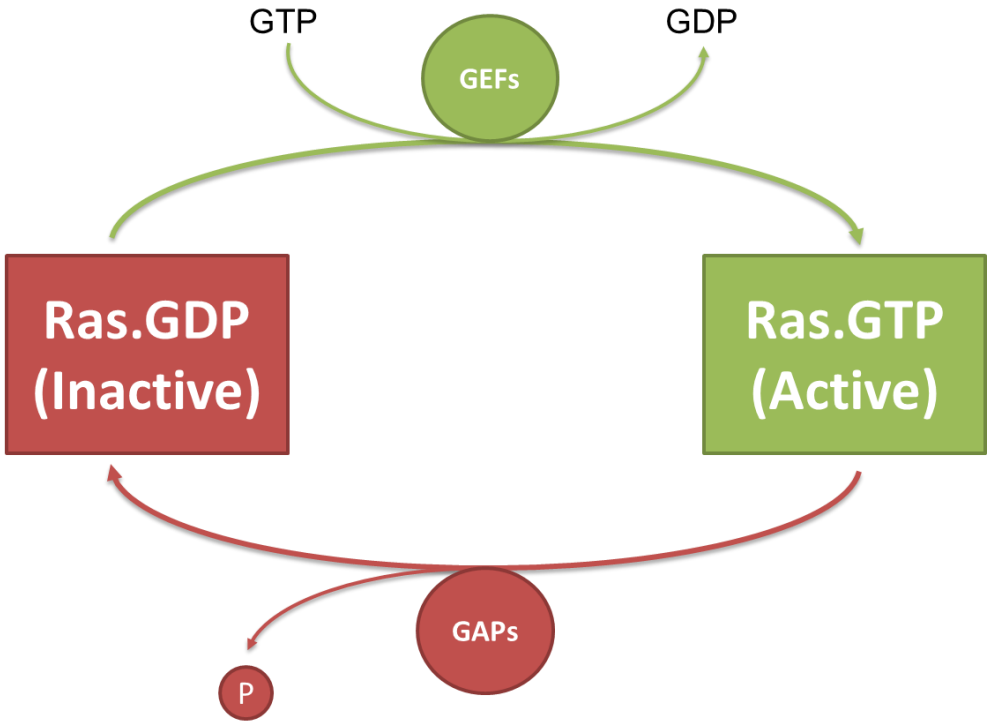


Figure 1-14 The GDP/GTP cycle of Ras

Ras acts as an on/off switch to regulate various intracellular signalling pathways. Ras cycles between an inactive (Ras.GDP) complex and an active (Ras.GTP) complex. The activation/inactivation of Ras depends on opposing actions of Guanine Nucleotide Exchange Factors (GEFs), which promote the exchange of GDP to GTP, and Guanine Activating Proteins (GAPs), which hydrolyse GTP to GDP.

Regulation of Ras activation is critical as both the extent and duration of activation is important. For example, in PC12 cells, a cell line derived from an adrenal gland tumour in rats, transient Ras signalling results in proliferation whereas sustained signalling causes the cells to differentiate (Marshall, 1995). Therefore, the Ras GDP/GTP cycle must be carefully balanced by opposing effects of Guanine Nucleotide Exchange Factors (GEFs), which activate Ras by catalysing the release of GDP and replacing it with GTP, and Guanine Activating Factors (GAPs), which promote the hydrolysis of GTP and inhibit Ras. It is the balance of these proteins that determines the activation state of Ras (Downward, 1996; Takai et al., 2001).

Nine Ras GEFs have been characterised, however, the model Ras GEF is Son of Sevenless (Sos) (Figure 1-15). Following ligand binding, and activation of RTK, Sos is recruited to the plasma membrane. Most RTKs do not bind to GEFs directly but use adapter proteins such as growth factor receptor bound protein-2 (Grb2) and Shc adaptor protein (Shc) (Figure 1-15) (Takai et al., 2001). RTKs serve as docking sites for Grb2 and the Shc/Grb2 complex then recruits Sos from the cytosol, forming a receptor-adaptor-GEF complex. Sos then stimulates Ras by converting Ras.GDP to Ras.GTP (Takai et al., 2001).

Ras activation is opposed by the effects of Ras GAPs which promote the hydrolysis of bound GTP by Ras to GDP ensuring that Ras is rapidly inactivated after stimulation (Olson and Marais, 2000). Although Ras has intrinsic GTPase activity GAPs increase Ras's GTPase activity by 10,000-fold. In the same way that multiple GEFs have been identified, eight Ras GAPs have been characterised, including p120 Ras GAP and NF1, providing a range of possibilities for initiating down-stream Ras signalling (Campbell et al., 1998; Omerovic et al., 2007).

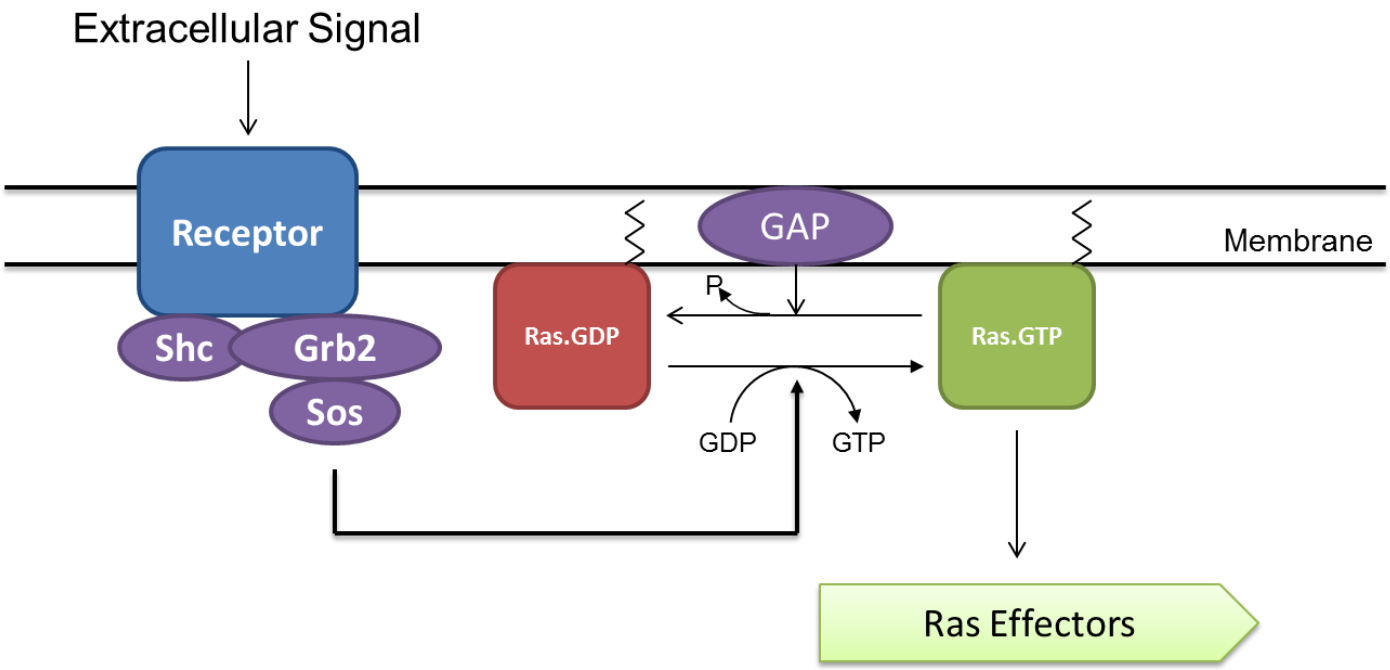


Figure 1-15 The Activation of Ras

Tyrosine Kinase Receptor activation results in the recruitment of adaptor proteins such as, Shc and Grb2. The Shc/Grb2-receptor complex then recruits Guanine Nucleotide Exchange Factor (GEF) Sos. Recruitment of Sos to the plasma membrane results in activation of Ras by converting Ras.GDP to Ras.GTP. Ras.GTP activates a cascade of kinases, for example, the MAPK signalling cascade which controls gene transcription.

1.6.2 Ras Signalling Pathways

Ras proteins have been shown to regulate cell proliferation (Pruitt and Der, 2001), differentiation (Noda et al., 1985), morphology (Whitman and Melton, 1992) and apoptosis (KauffmanZeh et al., 1997). In order to control this wide range of cellular functions a number of Ras effector proteins are involved (Figure 1-16). At least twenty different effectors have been identified. Many of these proteins are GEFs for other GTPases enabling cross talk between a number of signalling pathways (Omerovic et al., 2007). The majority of Ras activators and effectors consist of groups of closely related protein families facilitating signalling convergence and divergence. Given the potential complexity of these interactions finely tuneable mechanisms must be in place to ensure that the correct pathways are engaged depending on the strength and type of initial input (Omerovic et al., 2007). Ras, Ras activators and downstream effectors are mainly studied in the context of cancer as Ras mutations resulting in constituently activated Ras is apparent in 30% of all cancers (Omerovic et al., 2007). The Ras-MAPK pathway is an important component of many cancerous cells and appears to be required for tumour metastasis, likely through its ability to induce EMT (Chapnick et al., 2011). However, the importance of Ras in fibroblast to myofibroblast differentiation or its role in pulmonary fibrosis has not been determined.

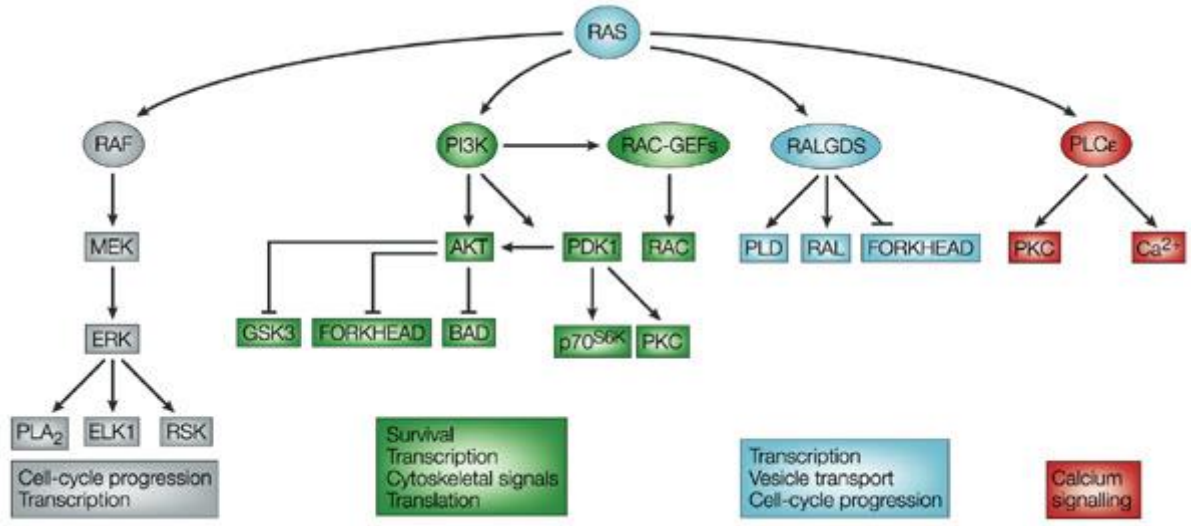


Figure 1-16 Signalling cascades downstream of Ras

Once in its active, GTP bound state, Ras interacts with numerous effector proteins. Raf protein kinases initiate the mitogen activated protein (MAPK) cascade, which results in ERK activation. This kinase has numerous substrates both in the nucleus and cytoplasm and mainly regulates cell cycle progression. Phosphatidylinositol-3-kinase (PI3K) activate target proteins such as Akt, which regulates cell survival. RALGDS proteins are nucleotide exchange factors for RAL, a Ras-related protein. Phospholipase Cε (PLCε) activates protein kinase C (PKC) and calcium mobilisation from intracellular stores (Downward, 2003).

The first mammalian effector of Ras characterised, and still the most intensively studied, is the protein serine/threonine kinase Raf (Figure 1-16). There are three closely related Raf proteins: C-Raf (also known as Raf-1), B-Raf and A-Raf (Marais et al., 1995). Ras.GTP binds to Raf proteins that enable recruitment of Raf to the plasma membrane. This is thought to be crucial for Raf activation (Marais et al., 1995). Once activated Raf phosphorylates and activates mitogen activated protein kinase kinases 1 and 2 (MEK-1 and MEK-2). These are dual specificity kinases that are capable of phosphorylating and activating the mitogen activated protein kinases (MAPKs). Three distinct groups of MAPKs have been identified; extracellular signal regulated kinases 1 and 2 (ERK-1 and ERK-2), c-Jun-N-terminal kinases/stress activated protein kinases (JNKs/Sapks) and p38 MAPKs (Mulder, 2000). MAPKs mediate signal transduction from the cytosol to the nucleus. Upon activation, MAPKs translocate to the nucleus to stimulate the activity of various transcription factors, such as ELK-1, to regulate gene transcription (Pruitt and Der, 2001).

In addition to the Ras/MAPK pathway, Ras activates several other effector pathways. Ras can interact directly with the catalytic subunit of type I phosphatidylinositol-3-kinases (PI3Ks) resulting in its activation (Rodriguezviciana et al., 1994). PI3K controls a large number of downstream enzymes. Much attention has been paid to Akt, a key anti-apoptotic protein, which phosphorylates various targets involved in the survival signalling pathway (Khwaja et al., 1997). In addition, PI3K activation stimulates Rac, a Rho family protein that is involved in the regulation of the actin cytoskeleton and transcription factor pathways including activation of nuclear factor κB (NF-κB) (Khwaja et al., 1997).

A third effector family for Ras includes three exchange factors for the Ras related Ral proteins; Ral guanine nucleotide dissociation stimulator (RALGDS), RALGDS-like gene (RGL/RSB2)

and RGL2/RLF. Through these proteins Ras stimulates Ral which is implicated in promoting cell cycle arrest and apoptosis (De Ruiter et al., 2001).

Phospholipase Cε (PLC) is another Ras effector which links Ras to activation of PKC and calcium mobilisation. PKC phosphorylates many downstream targets and multiple cellular functions have been ascribed to the activation of PKC including, regulating cell growth and gene transcription (Kelley et al., 2001).

Interestingly, there is differential activation of Ras effector proteins by different Ras isoforms, for example, K_i-Ras recruits and activates Raf more efficiently than Ha-Ras. Conversely, Ha-Ras activates PI3K more efficiently than K_i-Ras (Yan et al., 1998). Therefore, it is likely that Ras proteins are functionally distinct and the localisation of Ras to specific regions of the plasma membrane may allow specific and differential activation of the Ras effector proteins by different Ras isoforms.

1.6.3 Ras/MAPK, TGF-β1 and cAMP Crosstalk

As previously mentioned, in addition to signalling via Smads TGF-β1 activates Ras which can then activate several downstream pathways including the MAPK pathway (section 1.3.2.2). The ability of TGF-β1 to specifically activate numerous target genes is due, in part, to the interaction between both the canonical and non-canonical pathways.

There is ample evidence that the TGF-β1/Smad and Ras/MAPK pathway interact with each other and that the MAPK signalling cascade can regulate TGF-β1/Smad signalling. There are two common points of integration between the Ras/MAPK and TGF-β1 pathways: 1)

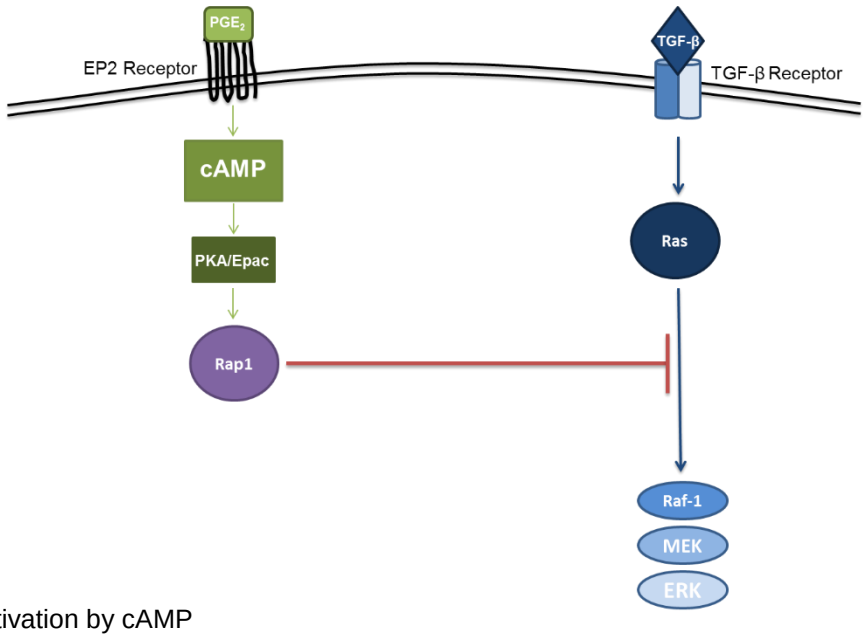
phosphorylation of co-activators of Smads and 2) phosphorylation of Smad2/3 directly by ERKs (Chapnick et al., 2011). Depending on the cell type, the MAP/ERK cascade might enhance or suppress Smad-dependent responses enabling TGF-β1 to have multiple effects in various cell types (Mulder, 2000). For example, the induction of collagen by TGF-β1 requires synergy between the TGF-β1-activated ERK and Smad signalling in human glomerular mesangial cells (Hayashida et al., 2003). This study demonstrated that the Ras/MAPK pathway is required for maximal induction of Smad activity following TGF-β1 treatment. R-Smad phosphorylation is enhanced via activation of the Ras/MAPK pathway allowing Smad and MAPK pathways to synergistically induce collagen synthesis (Hayashida et al., 2003). Furthermore, TGF-β1-induced activation of the Ras/MAPK pathway results in increased TGF-β1 expression and thus amplifies the TGF-β1 response which induces secondary TGF-β1 effects (Yue and Mulder, 2000). Other examples of crosstalk between the TGF-β1 and Ras/MAPK pathways include EMT in mammary epithelial tumour cells (Oft et al., 1996). TGF-β1-induced EMT is a fundamental mechanism that drives metastasis *in vivo* or invasion *in vitro* and constant TGF-β1 signalling is required to induce a stable phenotypic change. Acting alone neither pathway is successful in permanently converting epithelial cells to a mesenchymal phenotype, however, long term-expression and cooperation of TGF-β1 and Ras/MAPK causes complete EMT due to the induction of TGF-β1 autocrine signalling (Oft et al., 1996). Therefore, as crosstalk between TGF-β1 and Ras/MAPK signalling pathways is necessary for permanent EMT it may be that similar crosstalk is required for fibroblast to myofibroblast differentiation and as such Ras inhibition could be a novel therapeutic target for pulmonary fibrosis.

In addition to TGF-β1 signalling, the cAMP pathway also interacts with the Ras/MAPK cascade enabling essential crosstalk between these two fundamental pathways. The identification of cross talk between the Ras/MAPK and cAMP signalling pathways originated from studies investigating Raf. The cell type specific expression of Raf-1 or B-Raf allows cAMP to have cell

specific and varied biological outcomes (Stork and Schmitt, 2002). For example, in NIH3T3 fibroblasts (mouse embryonic fibroblast cell line) cAMP inhibited growth factor stimulated ERK whereas in PC12 cells (rat cell line derived from tumour in adrenal medulla) cAMP induced ERK activation. This is due to the differential expression of Raf-1, which is inhibited by cAMP, and B-Raf, which is activated by cAMP (Stork and Schmitt, 2002) (Figure 1-17).

The precise mechanisms for such actions remain to be elucidated. Studies have suggested that PKA can directly inhibit Ras/MAPK by phosphorylating Raf-1 (Mischak et al., 1996). An additional mechanism of Raf-1 inhibition is via the activation of the small G protein, Rap1, which is activated via both PKA (Schmitt and Stork, 2002) and Epac (de Rooij et al., 1998). Activation of Rap1 by cAMP has been demonstrated in a variety of cells including NIH3T3 fibroblasts (Schmitt and Stork, 2000). Rap1 has a dual function for controlling Ras/MAPK signalling depending whether Raf-1 or B-Raf is expressed. Rap1 antagonises Raf-1 (Carey et al., 2003; Stork and Schmitt, 2002) but activates B-Raf (Young et al., 1994). Raf isoforms vary in their cell-specific expression and subcellular localisation. Raf-1 is expressed in many tissues and highly expressed in muscle whereas B-Raf is expressed in endocrine cells (Kievit et al., 2001), endothelial cells (Wojnowski et al., 1997) and prostate cells (Chen et al., 1999). Interestingly, Yoshida and colleagues analysed transcripts for MAPK signalling in lung homogenates from IPF patients and reported increased expression of B-Raf compared with normal lung control subjects (Yoshida et al., 2002). Currently, it is unknown whether B-Raf, or Raf-1, is expressed in pulmonary fibroblasts. Studies are needed to determine whether cAMP can activate or inhibit ERK activity, via B-Raf or Raf-1 respectively, and to define the importance of this crosstalk in fibroblast to myofibroblast differentiation.

ERK inhibition by cAMP



ERK activation by cAMP

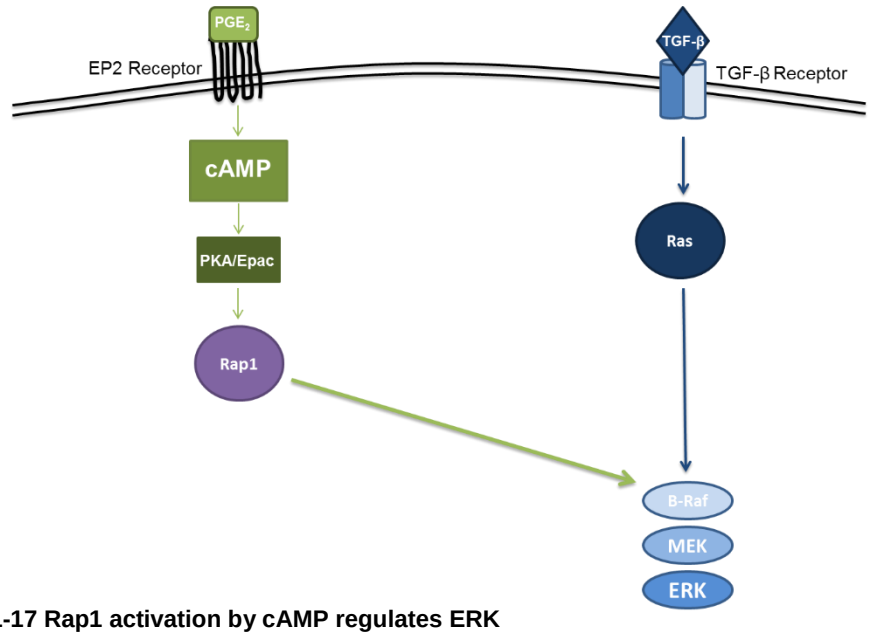


Figure 1-17 Rap1 activation by cAMP regulates ERK

A schematic diagram demonstrating how ERK can be inhibited or activated by the activation of Rap1. a) ERK inhibition by cAMP. Stimulation of a G-protein coupled receptor, for example the EP2 receptor via PGE_2, results in increased cAMP production and activation of Rap1. In Raf-1 expressing cells, Rap1 binds to Raf-1 and physically blocks its activation by Ras thereby inhibiting growth factor activation of ERKs. In addition, PKA and Epac can directly phosphorylate Raf-1 and prevent its activation via Ras. b) ERK activation by cAMP. In cells that express B-Raf, Rap1 activates B-Raf resulting in activation of ERK.

1.6.4 The importance of Ras in Fibrosis

Both the TGF-β1 and cAMP signalling pathway play a critical role during wound repair. These pathways operate in opposition to one another. TGF-β1 signalling promotes a pro-fibrotic phenotype and, in contrast, cAMP signalling promotes an anti-fibrotic phenotype. Despite the fact that the Ras/MAPK pathway represents an important signalling branch point between the TGF-β1 and cAMP pathway (Mulder and Morris, 1992) the role of Ras/MAPK signalling in the context of fibrosis has received little attention. However, a few studies have investigated the role of Ras in fibrosis and suggest that the inhibition of TGF-β1-induced Ras signalling has anti-fibrotic effects. Therefore, targeting the Ras signalling pathway could be a novel therapeutic target for IPF patients.

The Ras/MAPK signalling pathway has been shown to promote TGF-β1-induced collagen production and CTGF induction in human dermal and NIH3T3 fibroblasts (Stratton et al., 2002). Forced expression of Ha-Ras in dermal fibroblasts resulted in immediate up regulation of collagen. Furthermore, Ha-Ras stimulation resulted in increased Smad-3 phosphorylation which was independent of TGF-β1 production and activation (Smaldone et al., 2011). Conversely, deletion of Ha-Ras in a mouse model of renal fibrosis, resulted in reduced collagen accumulation, fibronectin production and myofibroblast differentiation compared with the wild-type control (Grande et al., 2010).

In contrast, signalling via the cAMP pathway is able to inhibit TGF-β1-induced Ras signalling. The inhibitory effect of cAMP is dependent on the activation of PKA and subsequent antagonism of the Ras/MAPK signalling cascade (Stratton et al., 2002; Stratton et al., 2001). Although many studies have focussed on the roles of MAPKs in inflammatory lung diseases, the significance of MAPK signalling in IPF has not been reported. One study has demonstrated that forskolin and isoproterenol treatment inhibited TGF-β1-stimulated collagen production

and α-SMA expression in cardiac fibroblasts via MAPK signalling. Increased cAMP production resulted in reduced ERK activity and reduced Smad-mediated recruitment of transcriptional coactivators (Liu et al., 2006a). In support of this study, a mouse model of bleomycin-induced fibrosis resulted in increased ERK activation and collagen deposition which were both significantly reduced following treatment with a MEK inhibitor (Galuppo et al., 2011). Western blot analysis of human lung biopsy samples also demonstrated increased ERK signalling in IPF samples compared with normal controls (Antoniou et al., 2010).

Another study has demonstrated that inhibition of downstream effectors of Ras inhibited specific fibroblast functions (Schmitt and Stork, 2002). This study investigated the roles of cAMP effectors in IMR-90 fibroblasts (foetal lung fibroblasts) and primary fibroblasts isolated from normal and non-fibrotic lungs and measured fibroblast proliferation and collagen production. Following PGE_2 treatment, activation of Epac resulted in the activation of Rap1 and inhibited fibroblast proliferation. Rap1 inhibition of cell growth has previously been attributed to the inhibition Raf-1 and ERK-1/2 in NIH3T3 fibroblasts (Schmitt and Stork, 2002). As such, it seemed logical to consider ERK-1/2 inhibition as a candidate target for the inhibitory effects of Epac. However, neither PGE_2 nor the Epac agonist was able to inhibit phosphorylated-ERK-1/2 suggesting that PGE_2, Epac and Rap1 inhibits cell proliferation independently of ERK-1/2 (Huang et al., 2008b). At present, how Epac and Rap1 inhibit cell proliferation remains unknown. The study also demonstrated that activation of PKA, but not Epac, was responsible for reducing collagen deposition. Whether or not inhibition of Epac/Rap1 or PKA is responsible for other anti-fibrotic effects such as α-SMA expression or COX-2 induction requires further investigation.

In addition to the MAPK signalling pathway, the PI3K/Akt pathway has also been shown to have a role in TGF-β1 signalling in the context of fibrosis (Lu et al., 2010; Martin et al., 2007).

Inhibition of PI3K in human lung fibroblasts abrogated TGF-β1-induced proliferation, α-SMA expression and collagen production demonstrating that the PI3K/Akt pathway may have an important role in fibroblast to myofibroblast differentiation and the pathogenesis of IPF (Conte et al., 2011). To further support the pro-fibrotic role of the PI3K/Akt pathway in fibrosis, fibroblasts isolated from IPF patients have increased activation of Akt compared with normal controls (Xia et al., 2010). Whether or not other downstream signalling pathways of Ras, such as PLCε or RALGDS, have a role in fibrogenesis needs further clarification.

Finally, studies have evaluated the effect of farnesylthiosalicylic acid (FTS), a specific Ras inhibitor, in animal models of fibrosis. FTS has been shown to act as a functional Ras antagonist *in vitro* by affecting Ras-membrane interactions preventing its activation and facilitating its degradation in the cytosol (Aharonson et al., 1998; Haklai et al., 1998). In a mouse model of muscular dystrophy, in which fibrosis is a prominent pathological feature in skeletal muscle, Ras expression and activity were increased compared with wild-type controls. FTS treatment significantly decreased both Ras expression and activity and also decreased ERK phosphorylation in skeletal muscle homogenates. FTS treated mice had a reduced fibrosis score, reduced collagen deposition and improved muscle strength (Nevo et al., 2011). Likewise, studies have demonstrated that Ras activity and expression is increased in liver cirrhosis. In an animal model of liver fibrosis, rats treated with FTS had lower histopathological fibrosis scores compared with cirrhotic rats indicating that FTS treatment promotes the regression of liver fibrosis (Reif et al., 2004). Increased Ras expression has also been associated with renal fibrosis. The study demonstrated that increased Ras activity was due to reduced levels of Rasal1, a Ras GAP which inactivates Ras, in fibroblasts isolated from both animal models and patients with renal fibrosis. Increased Ras activity was associated with increased proliferation of fibroblasts and increased collagen I and α-SMA expression. Furthermore, mice with experimentally induced fibrosis and treated with FTS had a lower fibrosis score compared with untreated mice (Bechtel et al., 2011). Therefore, FTS has been

shown to have a protective effect on renal fibrosis and muscular dystrophy and thus may also have therapeutic potential in IPF. Taken together these studies suggest that the activation of the Ras/MAPK signalling pathway may contribute to the pathogenesis of IPF.

In summary, although limited, there is strong data supporting the activation of MAPKs and other Ras signalling pathways in human fibrotic diseases. In addition to TGF-β1 other pro-fibrotic cytokines, such as PDGF, IL-13 and TNF-α, also signal via Ras (Hardie et al., 2010; Kim et al., 2002) Thus, inhibition of Ras or its downstream effectors is a logical target for a novel IPF treatment.

1.7 Epigenetic regulation of gene transcription

IPF is characterised by changes in expression of multiple pro-inflammatory and pro-fibrotic genes which code for the production of a diverse range of mediators resulting in the fibrotic microenvironment. Gene expression profiling studies have demonstrated that various transcriptional changes are present in the lung parenchyma of patients with IPF (Kaminski, 2003). Furthermore, myofibroblast differentiation is underpinned by changes in expression of hundreds of different genes that combine to generate the myofibroblast epigenome. Epigenetic regulation of gene expression has been extensively studied in the past in the context of malignancies but has only recently emerged as an important mechanism in the development of non-malignant diseases such as IPF. The regulatory mechanism at the level of transcription in fibroblast to myofibroblast differentiation requires further clarification and, even more importantly, understanding the mechanisms for switching off pro-fibrotic gene expression could be exploited in therapy.

Epigenetics is the stable and heritable change in gene expression resulting from modifications of the chromatin structure without altering the DNA sequence. Epigenetic modifications

include DNA methylation and post-translational histone modifications (Fischle et al., 2005). Epigenetic modifications are dynamic and can be altered in response to the environment, diet and ageing and as such epigenetics is now considered an important mechanism in many diseases (Yang and Schwartz, 2011).

To address the phenomenon of epigenetics we need to consider DNA in the context of chromatin. Chromatin is the complex of DNA and proteins that make up the contents of the cell nucleus. Genetic material requires extreme compaction into the nucleus as well as methods for regulating transcription, replication and repair. In eukaryotes, these roles are carried out by histone proteins, which assemble across the entire length of the DNA into nucleosomes.

Nucleosome proteins are structured as follows; two copies of histone proteins H2A, H2B, H3 and H4 come together to form the nucleosome octamer, which binds and wraps about 1.7 turns of DNA or 146 base pairs of DNA (Luger et al., 1997). Due to its appearance, this nucleosome structure is termed “beads on a string” fibre. The addition of one H1 protein further condenses the “beads on a string” fibre into a coiled fibre (Figure 1-18). The H1 protein also binds a further 20 base pairs of DNA and referred to as linker DNA. The coiled fibre can condense even further into chromosomes, which is DNA at its most condensed form. In a resting cell, when DNA is wound around the histones and condensed into chromatin this excludes the binding of RNA Polymerase II, which catalyses the transcription of DNA into messenger RNA. This conformation of chromatin is described as “closed” or heterochromatin and is associated with the suppression of gene expression. Gene transcription only occurs when the chromatin is opened up, also known as euchromatin, allowing DNA to unwind so that RNA Polymerases II and basal transcription factors can now bind to the DNA to initiate gene transcription.

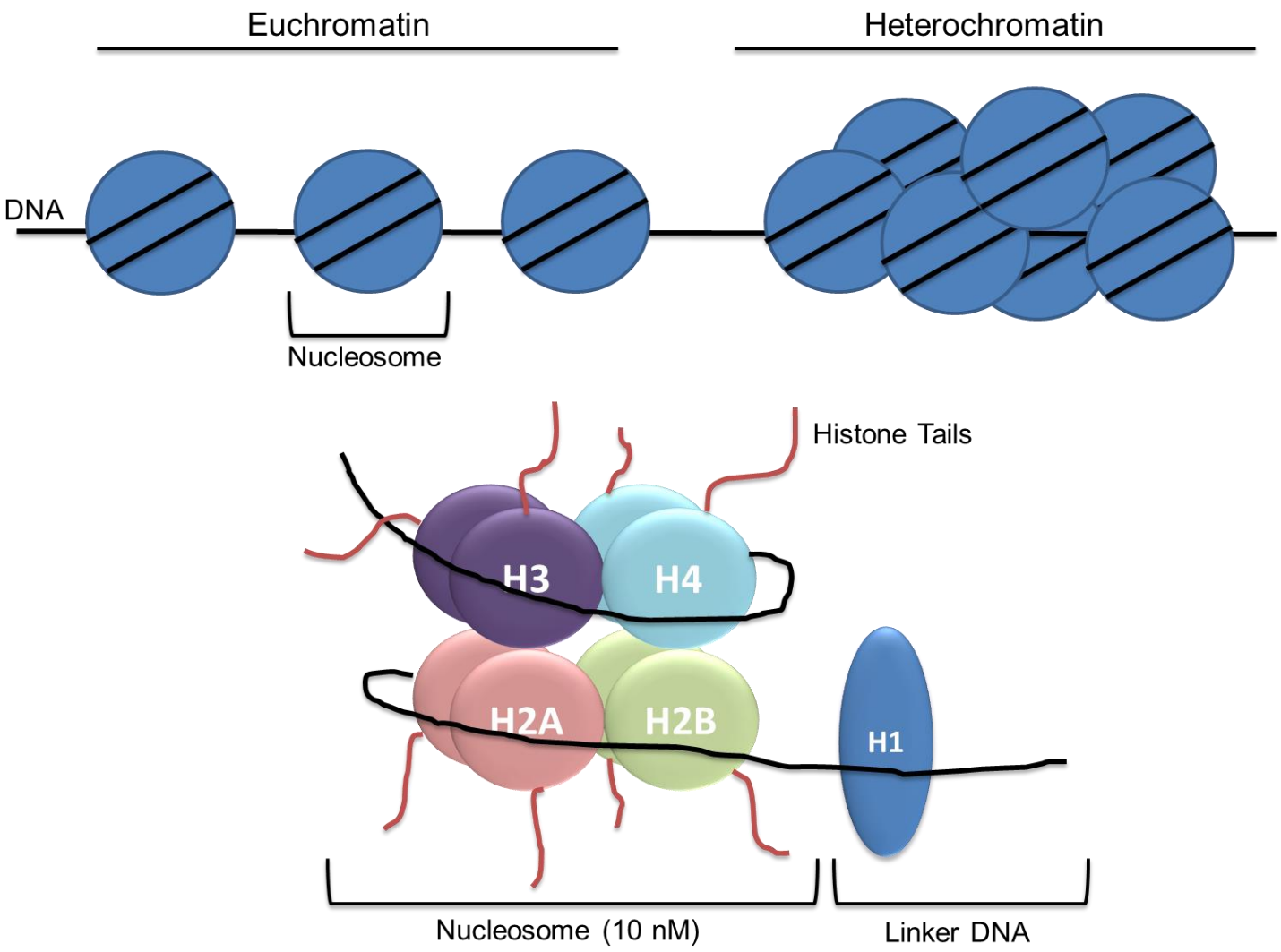


Figure 1-18 The Structure of a nucleosome, euchromatin and heterochromatin.

DNA is wrapped around nucleosomes which are composed of eight histone proteins with two copies of histone H2A, H2B, H3 and H4 forming the "beads on a string" fibre. The addition of H1 allows nucleosomes to condense further forming a highly condensed coiled fibre. Each histone molecule has a long tail rich in lysine residues which are the sites for posttranslational modifications including acetylation, methylation and phosphorylation. Modifications to the histone tails change the structure of chromatin in order to initiate or repress gene transcription. In its highly condensed form DNA is referred to as heterochromatin and gene transcription is

prevented due to the closed structure. However, when DNA is less condensed, known as euchromatin, gene transcription is active.

1.7.1 Histone Modifications

Due to the highly condensed structure of heterochromatin alteration of chromatin structure is critical for the regulation of gene expression. Such structures need to be dynamic and capable of regulating compaction and un-folding (Peterson and Laniel, 2004). In order to make chromatin more accessible histone proteins can be enzymatically modified by a variety of posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitinylation and sumoylation (Shahbazian and Grunstein, 2007). Adding to the complexity, modifications can occur on a variety of sites on each histone and it is thought that these different combinations result in distinct outcomes of chromatin regulated functions (Cheung et al., 2000). These modifications are reversible and thus modified chromatin can be returned to its compact state after transcription and/or DNA replication (Fischle et al., 2005). When cells are stimulated with extracellular mediators histones in the chromatin undergo posttranslational modifications which are linked to gene transcription and to the passage of epigenetic information from one cell generation to the next (Sterner and Berger, 2000).

1.7.1.1 Histone Acetylation/Deacetylation

Although histones can undergo several modifications the best characterised is histone acetylation, which is catalysed by histone acetyltransferase (HAT) enzymes. Each histone has a terminal 20 to 35 residue segment, known as the histone tail, that is rich in amino acids and extends from the surface of nucleosome which provides a platform to mediate interactions between proteins that function to remodel chromatin (Cheung et al., 2000). Histone tails are rich in lysine residues and thus are positivity charged which enables interaction with negatively charged DNA. Histone acetylation neutralises some of the histone tails positive charge and reduces histone to DNA contact and nucleosome to nucleosome interactions (Roth et al., 2001). This then destabilises the nucleosome structure and opens up the DNA to allow co-transcriptional proteins and polymerase II access to the DNA and the initiation of gene

transcription (Fletcher and Hansen, 1995). Just as acetylation of histones is associated with gene induction, the removal of acetyl groups by histone deacetyltransferases (HDACs) is associated with the repacking of chromatin and gene silencing (De Ruijter et al., 2003).

1.7.1.2 Histone Methylation

Methylation is another common modification of histones. Methylation occurs on multiple, but specific sites, on the histone tails and can be associated with gene activation or repression. In particular, histones H3 and H4 are methylated at a number of lysine (K) and arginine (R) residues. It is possible that methylation can alter chromatin structure however, a methyl group is relatively small and the addition of a methyl group does not affect the charge of histone tails. Therefore, it is unlikely that methylation alone will alter chromatin structure. It is more likely that the addition of a methyl group creates a binding site allowing other regulatory proteins to be recruited (Bannister and Kouzarides, 2005). Lysine side chains can be mono-, di- or tri-methylated, whereas the arginine side chains can be mono- or di-methylated (Margueron et al., 2005). At present there are 24 known sites of methylation on histones and if all three methylation states of lysine and arginine are taken into consideration there are potentially 3 x 10^{11} different methylation states. This highlights the complexity and diverse range of methylation marks controlling gene expression.

Histone methylation is regulated by histone methyltransferases (HMTases) and histone demethylases (HDMase). Methylation of specific residues results in either gene activation or repression, for example H3K9me3 (histone 3 lysine 9 trimethylation) is associated with gene repression. In contrast H3K4me3 (histone 3 lysine 4 trimethylation) is associated with transcriptional activation (Berger, 2007). Histone methylation leads to the recruitment of other proteins such as Heterochromatin Protein 1 (HP1) which are able to recruit additional HMTs, DNA methyltransferases and HDACs to direct changes in chromatin structure (Lachner et al.,

2001). Histone lysine methylation is an important epigenetic mark that regulates gene transcription and chromatin organisation. BIX01294, a specific inhibitor of G9a histone-lysine-N-methyltransferase which methylates lysine 9 of histone H3, has been used in several studies to investigate lysine methylation and epigenetic regulation(Chang et al., 2009).

1.7.2 DNA Methylation

DNA methylation is so far the most studied epigenetic mechanism. DNA is methylated by the addition of a methyl group to the 5'position of the cytosine residue in a cytosine-phosphoguanine (CpG) dinucleotide. This process is common throughout the genome but methylation of CpG islands, genomic regions that contain a high frequency of CpG sites, generally results in gene repression. Whereas, hypomethylation tends to result in gene transcription (Dwivedi et al., 2011).

DNA methylation is catalysed by DNA methyltransferases (Dnmts) (Robertson and Wolffe, 2000). In mammalian cells three Dnmt isoforms have been identified; Dnmt1, Dnmt3a and Dnmt3b. In general, Dnmt3a and Dnmt3b are thought to be responsible for *de novo* methylation and development whereas Dnmt1 maintains DNA methylation during replication (Okano et al., 1999; Prokhortchouk and Defossez, 2008).

Although it is well known that hypermethylation of CpG islands results in gene repression the mechanism is not fully understood. Studies suggest that DNA methylation is mediated by methyl-CpG-binding domain proteins, such as MeCP2, which can recruit repressive complexes containing HDACs and HMTases that interact to cause local histone deacetylation and methylation and consequent gene repression (Prokhortchouk and Defossez, 2008). It is not clear whether DNA methylation is the initial event triggering a cascade of HDAC and HMT

recruitment resulting in gene repression or alternatively, whether histone deacetylation is the initial event and DNA methylation follows in order to stabilise gene repression.

In general, transcriptionally active regions of DNA are hypomethylated, rich in acetylated histones and accessible to transcription factors. Transcriptionally inactive regions of DNA are comprised of hypermethylated DNA and deacetylated histones forming compact chromatin with an unfavourable configuration for transcription.

1.8 Evidence of Epigenetic Regulation in Myofibroblast Differentiation

Histone modifications and DNA methylation are key mechanisms for repressing gene expression and are particularly relevant in controlling cell differentiation. Epigenetic mechanisms are likely to be involved in IPF, especially given the association of IPF with cigarette smoking (Baumgartner et al., 1997) and the relationship between cigarette smoke and changes in DNA methylation and histone modifications (Belinsky et al., 2002). Furthermore, 625 CpG islands were reported to be differentially methylated between IPF and control lungs, supporting a role for epigenomic changes in IPF (Rabinovich et al., 2012). To date, very few studies have been published that investigate the role of epigenetic regulation in fibroblast to myofibroblast differentiation however studies suggest that myofibroblast differentiation is epigenetically controlled. Unravelling the epigenetic mechanisms of fibroblast to myofibroblast differentiation will improve our understanding of IPF pathogenesis and could potentially lead to new therapeutic strategies.

1.8.1 Epigenetic regulation of TGF-β1-induced Fibroblast to Myofibroblast Differentiation

Accumulating evidence demonstrates that histone acetylation/deacetylation is involved in fibrogenesis in various tissues. Previous studies have demonstrated the importance of histone

acetylation in dermal myofibroblast differentiation whereby inhibition of HDACs, particularly HDAC4, prevents TGF-β1-induced α-SMA mRNA and protein expression (Glenisson et al., 2007; Rombouts et al., 2002) and collagen expression (Ghosh et al., 2007). In addition, HDAC inhibition has been shown to inhibit EMT transition in human renal epithelial cells (Sam et al., 2006).

DNA methylation has also been suggested to be important in regulating TGF-β1-induced myofibroblast differentiation. TGF-β1-induced EMT in kidney epithelial cells becomes irreversible after treatment with TGF-β1 for 8 days suggesting a stable epigenetic change such as DNA methylation (Sam et al., 2006). A study has demonstrated that three CpG islands in the α-SMA gene promoter are differentially methylated in lung fibroblasts expressing α-SMA, compared with lung alveolar epithelial type II cells which showed uniformly high methylation and do not express α-SMA (Hu et al., 2010). Inhibition of DNA methylation resulted in a significant induction of α-SMA whereas ectopic expression of Dnmts suppressed α-SMA expression, even in cells treated with TGF-β1 (Hu et al., 2010).

1.8.2 Epigenetic regulation of COX-2 Expression

The COX-2 gene is an immediate-early gene that can be activated transiently and rapidly, and its expression is subject to multilevel regulation via both transcriptional and posttranslational mechanisms. Since COX-2 is an inducible gene it is controlled by transcription factor activation and binding to recognition sequences on the gene promoter as well as chromatin structure (Coward et al., 2010a). Previous studies of the COX-2 promoter have demonstrated that COX-2 is critically regulated by different transcription factors including CREB (Nie et al., 2005), C/EBP (CCAAT-enhancer-binding protein) and NF-κB (nuclear factor kappa light chain enhancer of activated B cells) (Gorgoni et al., 2001). Induced COX-2 gene transcription in human airway smooth muscle cells is closely associated with increased H4 acetylation (Nie et

al., 2005). In contrast, defective H3 and H4 acetylation is responsible for diminished COX-2 gene expression in IPF lung fibroblasts. This epigenetic abnormality is due to decreased recruitment of transcriptional co-activators with intrinsic HAT activity and increased recruitment of transcriptional co-repressor complexes containing HDAC activity to the COX-2 promoter (Coward et al., 2009). Whether or not histone methylation or DNA methylation is involved in COX-2 repression during fibroblast to myofibroblast differentiation still remains to be determined.

1.8.3 Epigenetic Regulation by cAMP

The cAMP signalling pathway exerts diverse effects on epigenetic regulation and gene transcription. Signalling molecules such as cAMP ultimately influence the activity or recruitment of transcription factors at gene promoters. Transcription factors then recruit various co-activators which modify chromatin structure and regulate gene expression For instance, forskolin, a cAMP agonist, prevents glucose-mediated L-type pyruvate kinase gene expression by inducing H3 and H4 deacetylation and H3K9 methylation (Burke et al., 2009). However, cAMP agonists can exert contrasting effects on gene transcription, for example, cAMP dependent PKA induces histone H3 phospho-acetylation in striatal neurons (Li et al., 2004).

The precise epigenetic mechanisms regulating fibroblast to myofibroblast differentiation are unclear and epigenetic effects mediated by cAMP, such as decreased α-SMA and increased COX-2 expression, in fibroblast to myofibroblast differentiation has not yet been explored. Unravelling the epigenetic effects of cAMP-mediated fibroblast to myofibroblast differentiation will improve our understanding of IPF and potentially lead to the development of novel therapeutic targets. In addition, the emergence of epigenetic therapeutics such as the Dnmt inhibitor 5-azadC and HDAC inhibitors such as romidepsin, both anticancer drugs undergoing

clinical trials, demonstrates there is great promise for this class of drug to be applied in the treatment of fibrosis.

1.9 Summary

Myofibroblasts have been identified as the key effector cells in the pathogenesis of IPF and their differentiation from lung fibroblasts and epithelial cells is an important source of myofibroblasts in IPF. Although F-IPF is a heterogeneous population it is likely to consist mainly of myofibroblasts originating from resident fibroblasts. F-IPF resembles the myofibroblast phenotype including but not limited to, repressed COX-2 expression and increased α-SMA and collagen expression. Myofibroblasts have repressed COX-2 expression resulting in the loss of PGE_2 production. PGE_2 is a potent anti-fibrotic mediator that inhibits fibroblast to myofibroblast differentiation via cAMP activation. Currently, how COX-2 expression is lost during myofibroblast differentiation is unknown. However, exogenous PGE_2 or cAMP agonists can prevent TGF-β1-induced fibroblast to myofibroblast differentiation (Kolodsick et al., 2003). The molecular mechanism in which PGE_2 exerts its anti-fibrotic effects has yet to be elucidated thus understanding this pathway in more detail will potentially disclose new perspectives in the treatment of IPF.

1.10 Hypothesis and Aims

We hypothesise that COX-2 gene expression and subsequent PGE_2 production is gradually lost during myofibroblast differentiation and that exogenous PGE_2 and other cAMP stimulating agents can compensate for the lack of endogenous PGE_2 and thus prevent and reverse myofibroblast differentiation (Figure 1-19). This study focusses on two key functional markers of myofibroblast differentiation; α-SMA and collagen expression. The overall aim of this study was to unravel the molecular mechanisms of myofibroblast differentiation and cAMP mediated inhibition of this process in IPF. This was achieved by addressing four specific aims:

1. Investigate if the loss of COX-2/PGE_2 is associated with fibroblast to myofibroblast differentiation

2. Examine the effect of exogenous PGE_2 and cAMP elevating agents on the prevention and reversal of fibroblast to myofibroblast differentiation

3. Investigate the molecular mechanisms of cAMP mediated regulation of fibroblast to myofibroblast differentiation

4. Examine the effect of epigenetic inhibitors on the prevention and reversal of fibroblast to myofibroblast differentiation

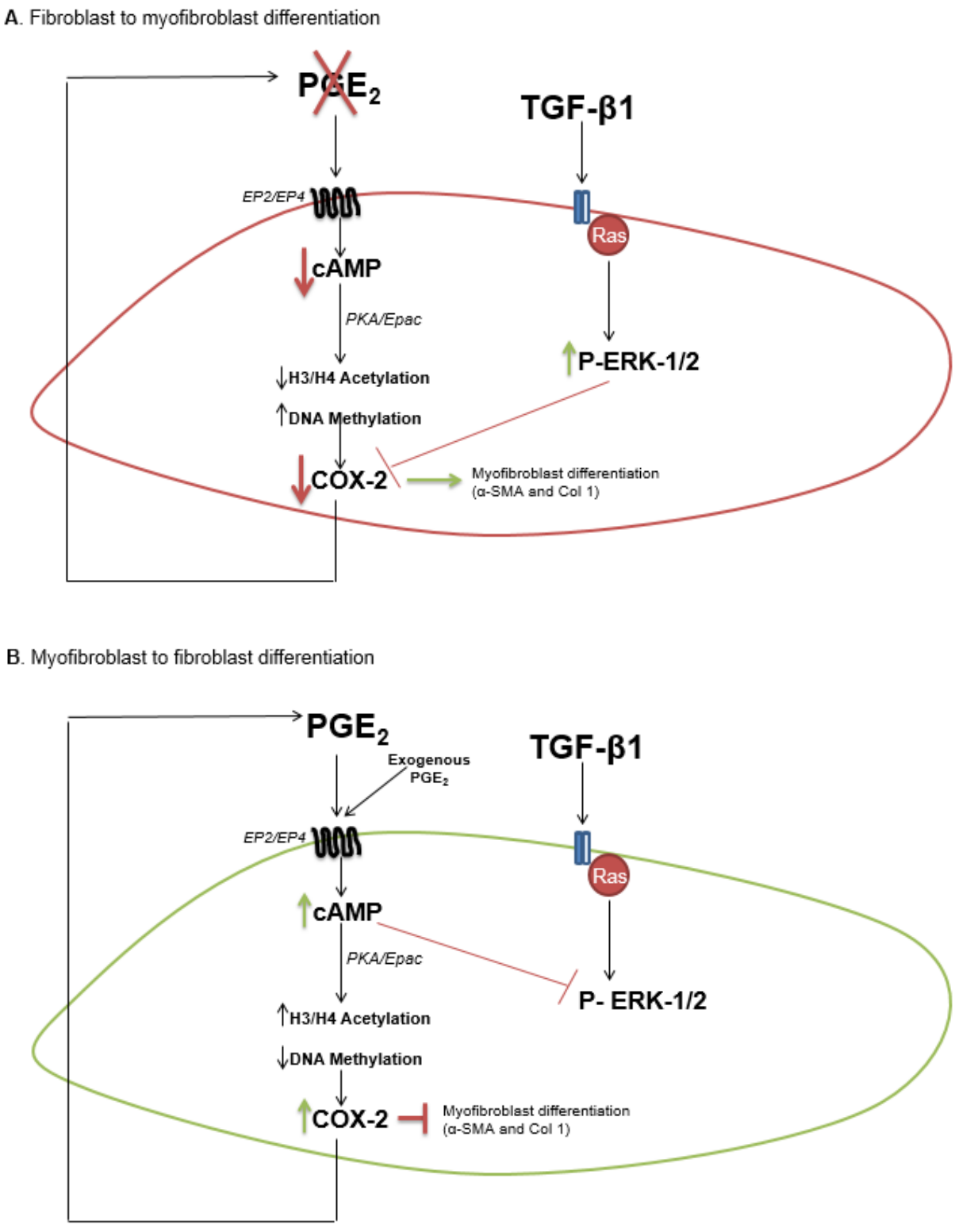


Figure 1-19 Schematic Diagram of Hypothesis

This diagram illustrates our proposed hypothesis. During myofibroblast differentiation (A), TGF-β1 treatment causes repression of COX-2 and subsequent PGE_2 production possibly via

Ras activation and the MAPK signalling cascade. The lack of PGE_2 results in decreased cAMP production and downstream signalling, decreased H3 and H4 acetylation and increased DNA methylation at the COX-2 promoter, causing epigenetic repression of COX-2, further decreasing the amount of PGE_2 synthesised. In addition, reduced cAMP will result in reduced ERK-1/2 phosphorylation allowing uncontrolled TGF-β signalling via ERK-1/2 to further reduce COX-2 expression. (B) Treatment with exogenous PGE_2, or other cAMP elevating agents, can compensate for the loss of endogenous PGE_2, causing increased cAMP production, increased H3 and H4 acetylation, decreased DNA methylation, resulting in COX-2 gene transcription to prevent and reverse fibroblast to myofibroblast differentiation. Inhibition of ERK-1/2 phosphorylation via cAMP will reduce TGF-β signalling via the ERK-1/2 signalling pathway.

METHODS

2 METHODS

2.1 Introduction

This chapter outlines the general methods used in this thesis. The source of human tissue and cells are described. All reagents, kits, primer sequences, buffer and media recipes are listed in the Appendix.

2.2 Cell Culture

2.2.1 Primary Human Lung Fibroblasts

Human fibroblasts were a kind gift from Dr Feghali-Bostwick at the University of Pittsburgh. Fibroblasts were cultured from the explanted lungs of patients with IPF (F-IPF) who underwent lung transplantation and from non-fibrotic lung (F-NL) (Table 2-1) tissue obtained from lung cancer patients at the University of Pittsburgh Medical Centre, under a protocol approved by the University of Pittsburgh Institutional Review Board. Approximately 2 cm^3 pieces of peripheral lung, collected from areas with fibrosis, were minced and fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal calf serum (FCS) (10% v/v final concentration) (see Appendix) at 37 ^oC, 5% CO_2 in a humidified incubator (Pilewski et al., 2005). At passage three the cell lines were transported to the University of Nottingham for further culturing.

Alterations is eicosanoid profiles have been reported to accompany the serial passage of fibroblasts (Polgar and Taylor, 1980) as such, F-NL and F-IPF were cultured to passage six and passage five respectively, in DMEM containing FCS (see Appendix). The main reason for using F-IPF at passage five was to ensure purity and differences present *in vivo*. F-IPF were tested at passage four and passage five and compared to F-NL passage five and passage 6.

Experiments demonstrated that there were no differences in IL-1β-induced COX-2 expression between passages. This is confirmed by several other studies that report COX-2 reduction in F-IPF compared to F-NL (Coward et al., 2009; Keerthisingam et al., 2001; Wilborn et al., 1995) and there is further evidence that the differences in maximal COX-2 activity between F-NL and F-IPF can persist through to passage 12 (Wilborn et al., 1995). The medium was changed every two days. Cells were grown until 100% confluent and growth arrested for 24 hours for all experiments in serum free DMEM (see Appendix). All cells were classified as being free from mycoplasma infection prior to experiments.

Cell line	Age (years)	Male/Female	Smoking History (Pack Years)
NL15	22	Male	U
NL16	49	Female	13
NL32	27	U	N/S
NL34	63	Male	U
NL36	U	U	U
NL37	50	Male	U
IPF14	46	Male	12
IPF48	65	Male	18
IPF52	67	Male	70
IPF55	58	Male	20
IPF57	40	Male	4
IPF103	71	Male	5
IPF108	63	Male	20
IPF110	70	Male	20
IPF111	74	Male	6
IPF112	58	Female	N/S
IPF114	67	Male	25

Table 2-1 Demographic data of F-NL and F-IPF

This table summarises the demographic data of the patients from whom fibroblast cell lines were generated. Pack years is a standard way to measure the number of cigarettes smoked over a period of time. It is calculated by multiplying the number of cigarettes smoked per day by the number of years the person has smoked. This is then divided by 20 (average number

of cigarettes in a packet) to calculate average pack years. Abbreviations: N/S Non-smoking, U Unknown.

2.2.2 Freezing Cells

Cells were cultured in 225 cm^2 flasks until fully confluent, washed with serum free DMEM and trypsinised using 0.25% Trypsin-Ethylenediaminetetraacetic Acid (EDTA). After centrifugation (1200 rpm, 5 minutes) the pellet was resuspended in 10% Dimethyl Sulphoxide (DMSO) plus 90% FCS to give 10^6 cells per ml. The suspension was aliquoted into 1 ml cryovials and placed in a Nunc Cryo 1 ^oC freezing container, this contains 100% isopropan-2-ol to freeze the cells a rate of 1 ^oC per minute. The cells were placed at -80 ^oC overnight then transferred to liquid nitrogen and stored until required.

2.2.3 Cell Counting

The number of cells in a cell suspension was determined using an Improved Neubauer haemocytometer and a light microscope. Cells were diluted 1:1 with Trypan blue in order to distinguish between live and dead cells. To calculate the total number of cells per ml the number of cells counted was divided by the number of fields counted, multiplied by the dilution factor (2) and then multiplied by 10^4. To determine the total number of cells this was then multiplied by the total volume of suspension.

2.2.4 Materials

A list of all materials, reagents and antibodies can be found in the appendix. PGE_2 was dissolved in ethanol (final concentration 1.0% v/v) and diluted in serum-free medium before use, IL-1β was dissolved in ddH20 with 0.01% BSA and diluted in serum free medium before use, TGF-β1 was reconstituted in 4mM HCl with 0.01% BSA, all other agents used were dissolved in DMSO (final concentration 1.0% v/v) and diluted in serum free medium before use. In all experiments, a group of control cells were incubated with the drug vehicles for the same period of time as the experimental cells were incubated with the drugs.

2.3 Bicinchoninic Acid (BCA) Protein Assay

The BCA assay is a colorimetric assay for determining the protein concentration in experimental samples. Total cell lysate was assayed for protein concentration using the BCA Protein Assay Kit (Thermo Scientific, Fisher). A standard curve of known Bovine Serum Albumin (BSA) concentrations was added in duplicate to a 96 well flat bottomed plate. 10 µl of sample was added in duplicate to the plate. Cell lysates were mixed with 200 µl of working reagent (BCA reagent A:B at 50:1, supplied with kit) then incubated for 30 minutes at 37 ^{o}C. After cooling to room temperature the absorbance at 570 nM was determined using a BMG plate reader. A standard curve was constructed and protein concentrations were calculated. Protein concentrations were determined immediately prior to separation by Western blot to avoid freeze thawing which can promote protein degradation.

2.4 Western Blot

2.4.1 Principle of Assay

Western blotting is an analytic technique used to detect specific proteins within cell lysates or homogenised tissue. The technique uses sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins according to their molecular weight. The separated proteins are then transferred, via an electric current, to a polyvinylidene fluoride (PVDF) membrane. The membrane is then probed with specific primary antibodies and a chemiluminescent secondary antibody in order to detect and semi-quantify proteins.

2.4.2 Cell Lysis

Cell media was removed and the cells were washed with ice-cold PBS. Cells were lysed on ice in 100 µl of RIPA buffer (see Appendix). The cell lysates were centrifuged (16,000 rpm, 30

minutes at 4 °C) to remove any insoluble protein. The cell lysates were aliquoted into clean 1.5 ml tubes and stored at -20 °C until ready for analysis.

2.4.3 Protein Sample Preparation

After protein concentration had been determined samples were diluted 4:1 in Lamellae buffer (see Appendix) and boiled at 100 °C for 10 minutes. The Western blot was carried out under reducing and denaturing conditions.

2.4.4 Gel Electrophoresis

The Protean Tetra gel casting system (BioRad) was set up according to the manufacturer's instructions. A 10% resolving gel (see Appendix) was prepared and poured into the gel casting system. The gel was left to set for approximately 30 minutes at room temperature. Stacking gel (see Appendix) was then prepared and poured on top of the resolving gel. A 10 or 15 well comb was inserted into the gel and left to set for a further 30 minutes at room temperature. Once set the comb was removed and apparatus was placed in the running tank filled with 1 x running buffer (see Appendix). The first lane was loaded with 10 µl of rainbow molecular maker (Rainbow™, Biorad) and the subsequent lanes were loaded with 20 µg of protein. The samples were subjected to electrophoresis at 150 v constant voltage for approximately one hour.

2.4.5 Protein Transfer

Proteins were transferred from the gel onto PVDF Immun-blot™ membrane (BioRad) in a transblot apparatus (BioRad). Sponge and filter paper were cut to size and soaked in 1 x transfer buffer (see Appendix). PVDF membrane was cut to size and soaked in methanol for 30 seconds before being soaked in 1 x transfer buffer. The gel was carefully removed from

the casting system and placed in 1 x transfer buffer. The gel and the PVDF membrane were sandwiched (sponge-filter paper-gel-PVDF membrane-filter paper-sponge) and any bubbles were expelled by rolling across the top. The transfer cassette was then placed into the transfer tank filled with 1 x transfer buffer. The system was run at 100 v for 45 minutes on ice. Once the transfer was complete the membranes were removed and washed in Tris buffered saline plus Tween (1 x TBST buffer, see Appendix) (3 x 5 minutes).

2.4.6 Protein Detection

To reduce any non-specific binding of the antibodies the membrane was blocked in 5% non-fat milk in TBST at room temperature for one hour or at 4 ^{o}C overnight on a rocker. The membrane was washed 3 x 5 minutes in TBST and incubated with the primary antibody, diluted in 5% non-fat in TBST, at room temperature for one hour or overnight at 4 ^{o}C on a rocker. After washing, the secondary antibody (horseradish peroxidise conjugated, goat anti-mouse or goat anti-rabbit (1:2000) was diluted in 5% non-fat milk in TBST and incubated with the secondary antibody for one hour at room temperature. The membrane was washed in TBST (3 x 10 minutes), blotted dry and placed face up on saran wrap. ECL™ Western blotting detection kit reagents (Amersham BioSciences) were mixed 1:1, enough to cover the membrane, and incubated for one minute at room temperature. The membrane was blotted dry and placed between transparent acetate sheets and developed in a dark room using hyperfilm ECL™ (Amersham BioSciences). The molecular weights of the bands were compared to the Rainbow™ protein molecular makers. To ensure that the protein samples were equally loaded the membrane was probed for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000) as a loading control. GAPDH is a housekeeping gene that is constitutively expressed in all cells and whose expression should not vary between cell types or with stimulation (Ferguson et al., 2005). Relative density was calculated by normalising the density of target bands against that of the GAPDH bands using Li-Cor Image Studio Lite analysis software.

2.5 cAMP Assay

2.5.1 Principle of Assay

The concentration of cAMP in samples was determined by a competitive binding radioimmunoassay (Gilman, 1970). The basic principle of the assay is the direct competition between cAMP present within the samples or standards and [^{3}H]-cAMP for association with a cAMP binding protein (usually protein kinase A (PKA)). The unbound cAMP is removed and precipitated by binding to charcoal. Unlabelled cAMP from the standards and samples competes with the radioactive labelled cAMP and thus the amount of radioactive cAMP bound by the protein kinase is inversely proportional to the concentration of cAMP in either the standards or samples.

2.5.2 cAMP Extraction

Cells were cultured in 24-well plates and, if necessary, pre-treated for three days with either PGE_2 or TGF-β1 before the removal of the cell media. The cells were washed in PBS and incubated in 500 µl fresh serum-free medium containing a general phosphodiesterase (PDE) inhibitor, 1 mM 3-isobutyl-1-methylxanthine (IBMX), for 30 minutes to prevent cAMP degradation. When measuring cAMP after the addition of PDE inhibitors, such as Roflumilast, cells were incubated in serum-free medium alone i.e. without IBMX. To stimulate cAMP production cells were treated with PGE_2 or other cAMP stimulating agents. cAMP production was terminated after 30 minutes with 100 µl ice cold 30% trichloroacetic acid (TCA). The solution was transferred to 1.5 ml tubes and mixed 1:1 with cAMP extraction buffer (see Appendix). The mixture was vortexed and centrifuged at 4 ^oC, 1500 rpm for 15 minutes. After centrifugation, the aqueous phase containing cAMP was collected and stored at -20 ^oC until ready for analysis and the organic phase containing TCA was discarded (Khym, 1975; Pang et al., 1998b).

2.5.3 cAMP Radiation Assay

100 µl of cAMP standards or samples were mixed with [^{3}H]-cAMP (0.825KBq/50 µl) and then incubated with binding buffer containing a limited amount of 3'5'cyclic AMP dependent protein kinase and BSA (see Appendix) at 4 ^{o}C overnight. The protein bound cAMP was separated from the unbound cAMP by using 2.6% charcoal solution containing 2% BSA (see Appendix) to absorb and precipitate the free cAMP. The samples were centrifuged at 4 ^{o}C, 4000 rpm for 15 minutes. After centrifugation, the supernatant containing protein bound cAMP was collected and mixed with 5 ml scintillation cocktail (Emulsifier Safe, Perkin Elmer). The radioactivity was counted using a Tri-carb T100 TR liquid scintillation analyser. The concentration of cAMP in the sample was calculated against the standard curve. The cAMP standards ranged between 1 pmol/100 µl to 16 pmol/100 µl. cAMP production in response to formoterol (Form), forskolin (FSK) and iloprost (Ilo) was conducted in the same way as PGE_2.

2.6 Immunocytochemistry

2.6.1 Principle of Assay

Immunocytochemistry is an immunological technique used to detect proteins or antigens in whole cells. The target protein, either cell surface or intracellular, is labelled with a specific antibody conjugated to a fluorochrome which is then visualised using a fluorescent microscope. Immunocytochemistry is a useful technique to not only quantify an antigen but to determine its cellular location.

2.6.2 Cell Staining

Cell surface expression of EP2, EP4 and β_2-agonist receptors was assessed by immunocytochemistry. Cells were grown on glass chamber slides until approximately 60% confluent so that the cells are not touching or overlapping in order for individual cell

quantification. Cells were washed in ice cold PBS and fixed in methanol for 10 minutes at 4 ^{o}C. The cells were blocked with blocking solution (see Appendix) containing goat serum and BSA for 30 minutes at 4 ^{o}C to inhibit non-specific binding of the antibodies. The blocking solution was removed and the cells were washed twice in ice cold PBS. Cells were incubated with the primary antibody overnight at 4 ^{o}C at the appropriate dilution (1 µg/ml EP2, 1 µg/ml EP4 and 1 µg/ml β2-AR). The cells were washed three times with PBS and then incubated with the secondary Fluorescein isothiocyanate (FITC) labelled antibody (1:1000 dilution) for 20 minutes at room temperature in the dark to prevent bleaching of the fluorochrome. In addition, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) at 5 µg/ml, to stain the cell nucleus. The cells were washed three times in PBS, allowed to dry then fixed and covered with FluoroSave^{TM} Reagent. The slides were analysed using a fluorescent microscope where ten individual cells were picked at random from five separate fields and the fluorescence intensity was measured and normalised to the cell surface area. As a control, cells were treated with dilution buffer containing no primary antibody, secondary antibody and DAPI to indicate any non-specific binding or false positives due to non-specific binding of the secondary antibody.

2.7 Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

2.7.1 Principle of Assay

RT-PCR is a common technique that measures the amount of specific messenger RNA (mRNA) within a cell or tissue and is a sensitive method for analysing gene expression. The first step of RT-PCR is to isolate RNA from the sample. RNA is naturally unstable and quickly digested by RNase enzymes and experimental work with RNA is therefore always performed on ice and stored at -80 ^{o}C. RNA is extracted using the NucleoSpin® RNA II kit (see Appendix). In brief, cells are lysed and RNases inactivated, contaminating DNA is removed by DNase and RNA then binds to the silica membrane within the columns. Washing steps allow the

removal of salts, metabolites and macromolecular cellular components and pure RNA is eluted via centrifugation.

Isolated RNA is then converted into complementary DNA (cDNA) via reverse transcription. Most mRNAs have a polyA tail at the 3' end composed of multiple adenosine residues. A universal primer composed of multiple tyrosine residues, known as Oligo dT Primer, binds to the poly A and allows reverse transcriptase to synthesise a complementary strand of DNA. The final step of RT-PCR is to amplify the cDNA using the enzyme DNA polymerase. The essential requirements for PCR includes specific primers for the gene of interest, a thermostable DNA polymerase, commonly from *Thermus aquarticus (Taq)*, deoxynucleoside triphosphates (dNTPs), which are utilised by the polymerase to amplify the DNA template and a fluorescent DNA binding dye, SYBR Green (Figure 2-1).

Figure 2-1. Conversion of mRNA to cDNA by Reverse Transcriptase

cDNA is a DNA copy synthesised from mRNA. a) The oligo dT Primer binds to the poly A tail found at the 3' end of the mRNA (orange). b) Together with the deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP) the reverse transcriptase enzyme synthesises a complementary DNA strand on the mRNA template. c) The cDNA requires to be converted into a double stranded DNA copy. The single stranded cDNA strand is dissociated from the single stranded mRNA strand and DNA polymerase synthesises double stranded cDNA. d) The cDNA can now be used for amplification by PCR.

The PCR reaction consists of three separate steps at different temperatures: denaturation, annealing and extension. Denaturation of cDNA separates the two strands of cDNA allowing the primers and DNA polymerase to bind to a single DNA strand. Denaturation occurs at approximately 90 - 95 ^{o}C. After denaturation the sample is cooled to enable binding of the primers. The annealing temperature of a sample depends on several factors including the length of primers and the percentage of guanine cytosine residues present and is usually 3 - 5 ^{o}C lower than the melting temperature of the primers. Therefore, primer pairs with similar melting temperatures are used for optimal results. Finally elongation utilises the DNA polymerase to extend the DNA from the primers and templates. Elongation is usually carried out at 72 ^{o}C which is the optimal temperature in which DNA polymerase can synthesis DNA. These three steps are repeated over a number of cycles resulting in exponential amplification of DNA until one or more of the reagents becomes limiting (Figure 2-2). The SYBR Green intercalates into the double stranded DNA product and the fluorescent signal is measured using a fluorescence-detecting thermocycler. The intensity of the fluorescence is directly proportional to the DNA in the sample and is determined after each cycle, therefore the accumulation of PCR product can be determined in real time throughout the course of the reaction. In order to standardised samples and minimise variability between samples the house keeping gene Beta-2-microglobulin (B2M), whose expression level is expected to remain constant between samples, is used as an endogenous standard. Quantification of target DNA is calculated by comparing the amount of housekeeping gene DNA to the target DNA using the $\Delta\Delta$Ct method for relative quantification. Normalised values to B2M ΔCts were initially calculated using the following equation:

$$\Delta Ct_{sample} = Ct_{gene} - Ct_{B2M}$$

The $\Delta\Delta$Ct was then determined using the formula:

$$\Delta\Delta Ct = Ct_{sample} - Ct_{control}$$

The expression of normalised genes (to B2M) compared to the mean of control samples was calculated using the formula:

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

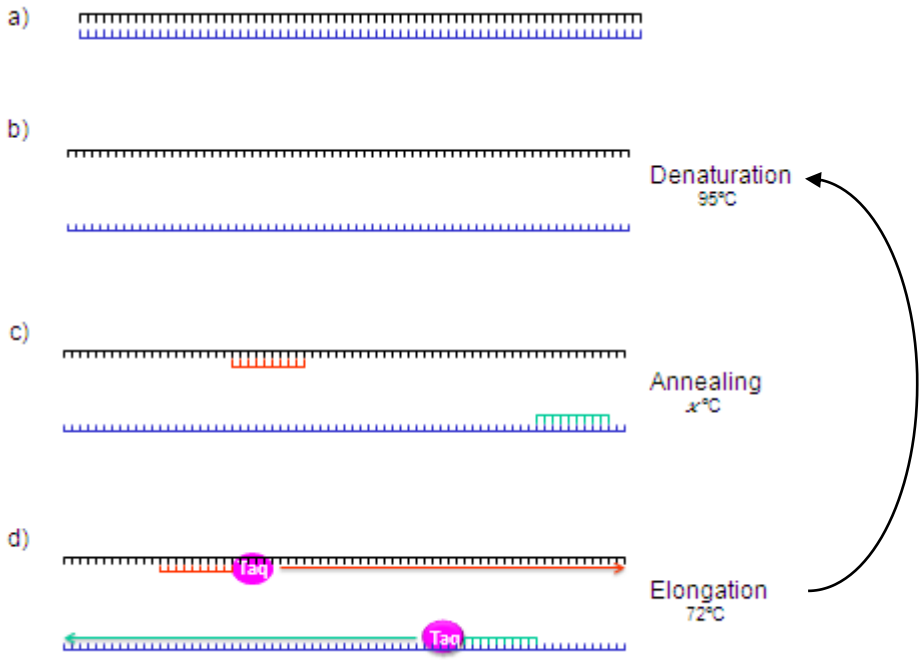


Figure 2-2. Schematic diagram of PCR Thermal Profile

Double stranded cDNA is the starting point for PCR amplification. b) The cDNA is heated in order to denature the double stranded cDNA resulting in single stranded cDNA. c) Gene specific primers bind to the single stranded cDNA. d) Taq polymerase adds complimentary nucleotides resulting in double stranded cDNA. The three step process of denaturation, annealing and elongation is repeated to yield a detectable PCR product.

At the end of the reaction, the PCR products are subjected to dissociation curve analysis to confirm production of a single amplification product. The thermocycler generated a thermal denaturation curve (dissociation curve) of the PCR product. A single melting temperature will result in a single peak and thus indicates the presence of a single product. The presence of multiple peaks suggests multiple products may be present due to non-specific binding of primers to the DNA or primer dimer formation (primers binding to each other). The presence of primer dimers is confirmed by peaks in the dissociation curve at relatively low melting temperatures.

2.7.2 RNA Isolation

Cells were grown to confluence in six well plates, treated as required and growth arrested for 24 hours. Total RNA was isolated using the NucleoSpin RNA II Kit (Macherey Nagel, Germany) following the manufacturer's protocol. All columns and buffers used were supplied with the kit. Briefly, cells were lysed in 350 µl of RA1 lysis buffer supplemented with 10 µl/ml β-mercaptoethanol and stored at -80 ^{o}C until required. Lysed samples were thawed at room temperature and 350 µl 70% ethanol was added and mixed thoroughly. The sample was added to the NucleoSpin filter column and centrifuged at 11,000 g for 30 seconds to allow RNA binding to the column, flow through was discarded. The membrane was washed with 350 µl Membrane Desalting Buffer (MDB) to remove contaminating salts and centrifuged at 11,000 g for one minute. DNA bound to the membrane was digested by adding 95 µl DNAse I reaction mixture (10 µl reconstituted DNase to 90 µl Reaction buffer) per sample for 15 minutes at room temperature. The membrane was washed in 200 µl RA2 buffer and centrifuged at 11,000 g for 30 seconds. The flow through was discarded. The membrane was washed in 600 µl of RA3 buffer and centrifuged at 11,000 g for 30 seconds. After discarding the flow through a further 250 µl of RA3 buffer was added and centrifuged at 11,000 g for 2 minutes to completely dry the membrane. The RNA was then eluted from the membrane by adding 20 µl nuclease free

water and centrifuged at 11,000 g for 1 minute. Eluted RNA was stored at -80 ^{o}C until required. RNA quantity and quality was determined using NanoDrop® UV-Vis spectrophometer.

2.7.3 Reverse Transcription

10.7 µl of RNA was added to 3 µl 200 µg oligo(dT) primers (final concentration 0.5 µg) and 5 µl of dNTPs (2 µM of each dNTP) and heated to 72 ^{o}C for five minutes. Following heating, RNA was reverse transcribed with 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase, 25 units of RNase Inhibitor and 5 µl M-MLV RT buffer. The volume of the reaction mixture was made up to 25 µl with nuclease free water. The reaction mixture was incubated at 42 ^{o}C for 90 minutes. Amplification was carried out with a PCT-100 programmable thermal controller (Bio-Rad Laboratories Ltd, Hertfordshire, UK). RT products were stored at -20 ^{o}C until required.

2.7.4 Quantitative PCR

RT products were subsequently used for PCR amplification. Quantitative PCR (qPCR) analysis was performed using primers and thermal cycling conditions as described in the Appendix. 2 µl reversed transcribed cDNA was used for qPCR using Kappa Taq mastermix containing SYBR green and amplified on an Mx3000P® qPCR system (Stratagene, California, US). Each reaction consisted of 2 µl cDNA, 10 µl Kappa Taq master mix, 200 nM of both sense and antisense primers and nuclease free water to a final volume of 20 µl. Thermocycler conditions included incubation at 95 ^{o}C for three minutes for enzyme reactivation followed by 40 cycles of two-step cycling at 95 ^{o}C for three seconds for denaturation and the appropriate annealing temperature (see Appendix) for 1 minute. Integration of the fluorescent SYBR gene into PCR product was monitored after each annealing step. Control (nuclease free water only) was included in every experiment and all samples and controls were assayed in duplicate. The cycle threshold (Ct), the point at which the PCR product is detectable above a fixed

threshold, was determined for both the target gene and the housekeeping gene. Changes in gene expression were normalised to changes in house keeping gene (B2M) and expressed as relative expression compared with the control sample. Amplification of one specific product was confirmed by melting curve analysis where a single melting peak confirmed the presence of a single PCR product and eliminated the possibility of primer-dimer association. For melting curve analysis to be performed the products were heated from 55 to 95 ^{o}C after the 40 cycles.

2.8 Active Ras Pull Down Assay

2.8.1 Principle of Assay

A pull down assay is an *in vitro* method used to determine an interaction between two proteins. Ras only binds to its downstream kinase, Raf-1 when in its active-GTP bound state via a Ras Binding Domain (RBD) interaction. Glutathione agarose resin binds a recombinant Raf-1-RBD GST-fusion protein via a GST/Glutathione interaction thus capturing active Ras and allowing the inactive, GDP-bound, Ras to be washed away. The captured active Ras is detected and measured via Western Blotting (see method 2.4) using a monoclonal anti-Ras antibody that detects all Ras isoforms (K-, N- and H-Ras).

2.8.2 Active Ras Pull Down Method

Following appropriate treatment cells were rinsed with ice-cold TBS (see Appendix) and scraped in 500 µl of Lysis/Binding/Wash Buffer (supplied with kit). Lysed samples were vortexed briefly, incubated on ice for five minutes and then centrifuged at 16,000 g for 15 minutes at 4 ^{o}C. A small sample of the cell lysate was used to determine the protein concentration using the BCA method (see method 2.3). Unstimulated cell lysates were treated with GTPγS and GDP as a positive and negative control for Ras activity. For 500 µl of cell lysate 10 µl 0.5M EDTA (pH 8.0) was added and vortex briefly. 5 µl 10mM GTPγS (positive)

and 5 µl 100mM GDP (negative control) was added. The sample was incubated for 15 minutes at 30 ^{o}C with gentle agitation. The reaction was terminated by placing the samples on ice and adding 32 µl of 1M $MgCl_2$.

For each affinity precipitation 500 µg of protein was used. 100 µl 50% resin slurry (supplied with kit) was added to the spin cup and centrifuged at 6,000 g for 10 – 30 seconds. Flow through was discarded. 400 µl Lysis/Binding/Wash Buffer was added to each tube with the resin, the tubes were inverted several times and centrifuged at 6,000g for 10 – 30 seconds. Flow through was discarded. 80 µg GST-Raf-1-RBD was added to the spin cup followed by 500 µg of total cell lysate. The spin cup was vortexed briefly and incubated at 4 ^{o}C with gentle rocking for one hour. The spin cup was centrifuged at 6,000 g for 10 – 30 seconds. The resin was washed three times by adding 400 µl Lysis/Binding/Wash buffer, inverting the tube three times and centrifuging at 6,000 g for 10 – 30 seconds. The spin cup was placed in a new collection tube. 50 µl 2X reducing sample buffer (1 part β-mercaptoethanol to 20 parts 2X SDS sample buffer (supplied with kit)) was added to the resin and incubated for two minutes at room temperature. The tube was centrifuged at 6,000 g for two minutes, the spin cup was discarded and the eluted samples were heated for five minutes at 100 ^{o}C. Samples were stored at -20 ^{o}C until ready for analysis.

2.8.3 Active Ras Detection

Gel electrophoresis was used to determine Active Ras in total cell lysates following the Western blot protocol as already described in methods 2.4. For each gel, 25 µl of sample was added per lane. An unfractionated cell lysate was used as a control to verify that the Western blot analysis was functioning properly. Following electrophoresis, the membrane was blocked in 3% BSA at room temperature for 1 – 2 hours and washed in TBST for 5 minutes. The anti-Ras antibody (supplied with kit) was diluted (1:200 dilution) in 3% BSA and 0.1% NaN3 in

TBST and incubated overnight at 4 ^{o}C. The membrane was washed in TBST (5 x 5 minutes). The secondary antibody was added and incubated for one hour at room temperature. The membrane was washed in TBST (5 x 5 minutes). The membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to X-ray Film.

2.9 PGE_2 EIA

2.9.1 Principle of Assay

The PGE_2 EIA kit is a competitive binding assay used to quantify PGE_2 levels in sample supernatants (Caymen Chemicals). The assay is based on competitive binding between PGE_2 present in the sample and a PGE_2-acetylcholinesterase (AChE) conjugate known as the PGE_2 tracer. The amount of PGE_2 within the sample competes with the PGE_2 tracer to bind to a monoclonal antibody. Therefore, the amount of PGE_2 tracer bound to the antibody will be inversely proportional to the amount of PGE_2 in the sample. The antibody-PGE_2 complex binds to a polyclonal antibody that has been previously attached to the well. Ellmans reagent, which contains the substrate for AChE is added to the well and the enzymatic reaction results in a distinct yellow colour. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of PGE_2 tracer bound to the well, which is inversely proportional to the amount of free PGE_2 within the sample.

2.9.2 PGE_2 EIA Method

Levels of PGE_2 in cell culture supernatants were determined by an EIA assay according to the manufacturer's instructions. All reagents and buffered were supplied with the kit. Cells were grown in six well plates until confluent. Supernatants were collected following appropriate stimulation and stored at -80 ^{o}C until required. 50 µl of standard, blank and sample was added to the plate and incubated for 18 hours at 4 ^{o}C. The wells are washed five times with wash

buffer. 200 µl Ellmans reagent is added to each well. The plate is incubated for 60 to 90 minutes on an orbital shaker and protected from light. The optical density is measured at 420 nm (reference Filter 570 nm) in a TECAN GENios (TEcan UK Ltd, Theale, Reading, UK). The absorbance is measured periodically until the blank is in the range of 0.3 - 1 absorbance units (blank corrected). Standards were diluted using two fold serial dilutions in reagent diluent to provide an eight point standard curve ranging from 1000 pg/ml to 7.8 pg/ml. Each plate contained a blank, a non-specific binding and maximum binding well and standards and samples were performed in duplicate. The concentration of PGE_2 in unknown samples was calculated using the standard curve. PGE_2 concentrations were than normalised to total protein concentration for each sample and the data were expressed as pg of PGE_2 per µg of total protein.

2.10 Cell Viability

The toxicity of all the chemicals used within this study was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay (van Meerloo et al., 2011). At the end of each experiment culture media was removed and replaced with 250 µl serum free media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (Sigma), then incubated for 20 minutes at 37 ^{o}C. This medium was removed and the plates dried overnight. 250 µl dimethyl sulfoxide (DMSO) was then added to dissolve the blue-coloured tetrazolium. The optical density was read at 550 nm in a TECAN GENios (Tecan UK Ltd, Theale, Reading, UK) microplate reader. Viability was set at 100% in control cells.

2.11 Statistical Analysis

Data is expressed as mean ±SEM from n cell lines for all experiments unless otherwise stated. Statistical analysis was performed using GraphPad Prism 5. When comparing between two data sets an unpaired Student's t-test was performed to determine the significance of differences between two means. One way ANOVA was performed with a Dunnet post test to compare a group of conditions to a single control. P values less than 0.05 were accepted as significant. For Western blot experiments all blots are shown and density analysis has been completed.

RESULTS

3 THE EFFECT OF PGE_2 ON FIBROBLAST TO MYOFIBROBLAST DIFFERENITATION

3.1 Introduction

Myofibroblasts are a consistent finding in the pathology of several fibrotic diseases and their differentiation from resident fibroblasts represents a critical step in the pathogenesis of IPF (Zhang et al., 1994). Due to their contractile phenotype and increased production of extracellular matrix proteins, such as collagen, myofibroblasts are key effector cells in fibrosis (Gabbiani, 2003). In addition, myofibroblasts secrete several pro-fibrotic mediators such as TGF-β1, TNF-α and PDGF (Gabbiani, 2003). Therefore, inhibition of fibroblast to myofibroblast differentiation may be an effective means to prevent the progression of fibrosis. Phenotypic features of the myofibroblast include repressed COX-2 expression, reduced PGE_2 production (Coward et al., 2009), increased α-SMA expression and increased collagen production (Gabbiani, 2003).

A variety of pro-fibrotic mediators, such as TGF-β1 and TNF-α, are potent inducers of fibroblast to myofibroblast differentiation in several fibrotic diseases both *in vitro* and *in vivo* (Desmouliere et al., 1993; Goodwin and Jenkins, 2009; Sime et al., 1997). TGF-β1 has been shown to increase α-SMA and collagen expression in fibroblasts and the myofibroblast phenotype persists several days after the removal of TGF-β1 suggesting that the effect of TGF-β1-induced myofibroblast differentiation is long lasting (Evans et al., 2003; Garrison et al., 2013).

In addition to the myofibroblasts secreting pro-fibrotic mediators there is a reduction of anti-fibrotic mediators (Keerthisingam et al., 2001; Wilborn et al., 1995). One of the best-studied anti-fibrotic mediators is PGE_2. PGE_2 has been shown to inhibit fibroblast proliferation (Korn

et al., 1980), collagen expression (Baum et al., 1980) and fibroblast to myofibroblast differentiation (Garrison et al., 2013). PGE_2 is a lipid mediator derived from the metabolism of arachidonic acid by COX-1 and COX-2 enzymes (Vancheri et al., 2004) and is the major prostanoid synthesised by fibroblasts (Wilborn et al., 1995). PGE_2 is recognised to have an important role in IPF as a local deficiency of PGE_2 has been implicated in the pathogenesis of IPF in humans (Wilborn et al., 1995) and animal models (Hodges et al., 2004). Furthermore, over expression of exogenous PGE_2 has been shown to protect against experimental induced fibrosis (Arras et al., 2005). PGE_2 signals via four G-protein coupled E prostanoid receptors: EP1, EP2, EP3 and EP4 (Narumiya et al., 1999). Previous studies have demonstrated that the anti-fibrotic effects of PGE_2, such as inhibition of proliferation and collagen production, are mainly mediated via cAMP signalling (Kolodsick et al., 2003). Two EP receptors, EP2 and EP4, are known to couple to adenylyl cyclase resulting in its activation and increasing cAMP (Huang et al., 2007). Studies have demonstrated that the inhibitory effects of PGE_2 in normal lung fibroblasts are mainly via EP2 and EP4 and subsequent cAMP accumulation (Huang et al., 2007; Kolodsick et al., 2003).

Il-1β is a pro-inflammatory cytokine that has been shown to have pro-fibrotic effects. Over expression of IL-1β in rodent epithelial cells caused increased expression of TGF-β1 resulting in progressive interstitial fibrosis characterised by the presence of myofibroblasts and secretion of collagen and fibronectin (Kolb et al., 2001a), despite its ability to induce COX-2 expression. The direct effect of IL-1β on fibroblasts and subsequent PGE_2 production remains unclear.

Studies from our laboratory (Coward et al., 2009) and others (Keerthisingam et al., 2001; Vancheri et al., 2000) have shown that fibroblasts isolated from patients with IPF have deficient PGE_2 production attributable to the impaired induction of COX-2. These studies

suggest that deficient PGE_2 production may contribute to the pathogenesis of IPF and therefore, reconstitution of this deficient anti-fibrotic mediator may have potential therapeutic benefit.

3.2 Aims

The aim of this chapter was to investigate if the loss of PGE_2 contributes to the pro-fibrotic phenotype of myofibroblasts and to determine if exogenous PGE_2 treatment can compensate for the loss of endogenous PGE_2 to prevent and reverse fibroblast to myofibroblast differentiation. We initially investigated the phenotypic differences between F-NL and F-IPF by assessing PGE_2, COX-2, α-SMA and Col 1 expression. As COX-2 is an inducible protein, IL-1β was used as a stimulus. We assessed the effect of IL-1β on F-NL and F-IPF and determined the effect of IL-1β on fibroblast to myofibroblast differentiation. We next assessed if TGF-β1-treated F-NL mimicked the phenotype of F-IPF and if this was a suitable *in vitro* model of fibroblast to myofibroblast differentiation. We also assessed if TGF-β1 treatment correlated with the loss of COX-2 during fibroblast to myofibroblast differentiation. We finally examined whether exogenous PGE_2 treatment could reverse fibroblast to myofibroblast differentiation and inhibit TGF-β1 -induced myofibroblast differentiation. This chapter also sought to determine which receptor PGE_2 signals via in order to further investigate the downstream signalling pathway and identify novel and specific therapeutic targets.

3.3 Experimental Protocol

F-NL and F-IPF were cultured to confluence in 6 well culture plates, medium was changed every 48 hours and confluent cells were growth arrested in serum free medium for 24 hours prior to all experiments. To measure COX-2 protein and mRNA expression cells were treated with 2 ng/ml IL-1β for 24 hours and 4 hours, respectively. After treatment cells were subject to protein extraction or total RNA isolation for Western blot and qPCR analysis, respectively, as described in Chapter 2. mRNA levels of COX-2, α-SMA, Col 1 and the internal control, β2-microglobulin (β2M), were determined by quantitative RT-PCR. The results are calculated as the ratio of the gene of interest mRNA and β2M mRNA and then fold change over untreated control.

In the time course experiments cells were incubated with PGE2 (1 μM), TGF-β1 (2 ng/ml), ONO-AE1-259 (EP2 agonist) (5 μM) and ONO-AE1-329 (EP4 agonist) (5 μM) for 0, 1, 2, 3 and 5 days, serum starved for 24 hours prior to IL-1β stimulation for 24 hours (2 ng/ml) (Figure 3-1).

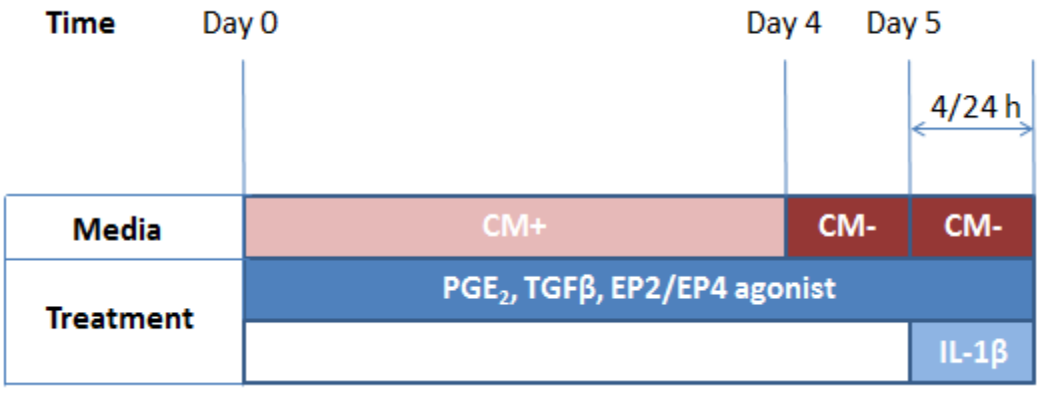


Figure 3-1 Experimental Protocol for time course experiments

Schematic diagram of the treatment and timelines for the time course experimental protocol. CM+ = media contain serum, CM- = serum free media.

In the concentration response experiments cells were treated for 3 days with 0.1 µM – 10 µM PGE_2, 1 µM – 10 µM ONO-AE1-259 and 1 µM – 10 µM ONO-AE1-329, serum starved for 24 hours prior to stimulation with IL-1β for 24 hours.

To test if TGF-β1-induced myofibroblast differentiation is a permanent phenotypic change F-NL were treated with TGF-β1 (2 ng/ml) for 3 days and then removed from the media for a further 3 days prior to 24 hours of IL-1β stimulation (2 ng/ml) (Figure 3-2).

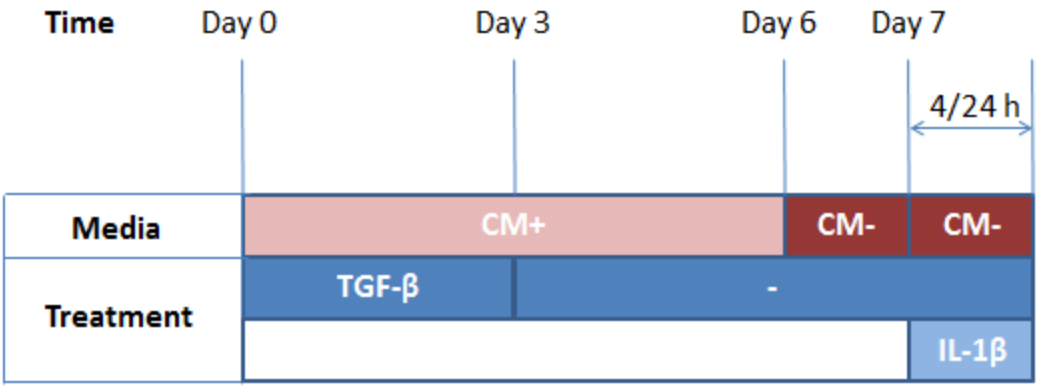


Figure 3-2 Experimental Protocol for TGF-β Removal Experiments

Schematic diagram of the treatment and timelines for the TGF-β experimental protocol. CM+ = media contain serum, CM- = serum free media.

To test the inhibition of PGE_2 on TGF-β1-induced fibroblast to myofibroblast differentiation, F-NL were pre-treated with 1 µM PGE_2 for one hour prior to the addition of 2 ng/ml TGF-β1 for 3 days (Figure 3-3).

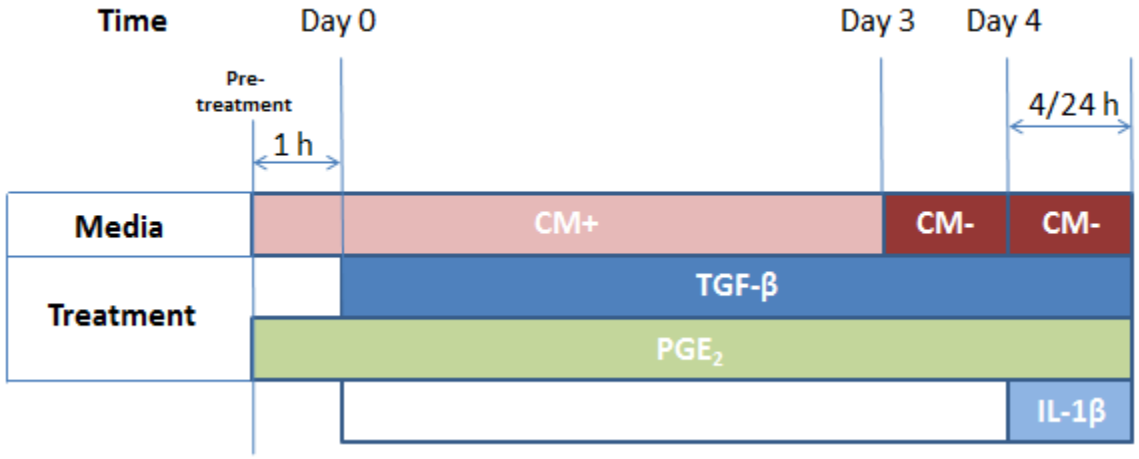


Figure 3-3 Experimental Protocol for PGE_2 and TGF-β experiments

Schematic diagram of the treatment and timelines for the PGE_2 and TGF-β experimental protocol. CM+ = media contain serum, CM- = serum free media.

In order to determine PGE_2 concentration after 3 days of treatment with 1 µM PGE_2 the culture medium was removed and washed twice with CM- before the cells were stimulated with 2 ng/ml IL-1β for 24 hours. The culture medium was collected and stored at -20 ^{o}C until the determination of PGE_2.

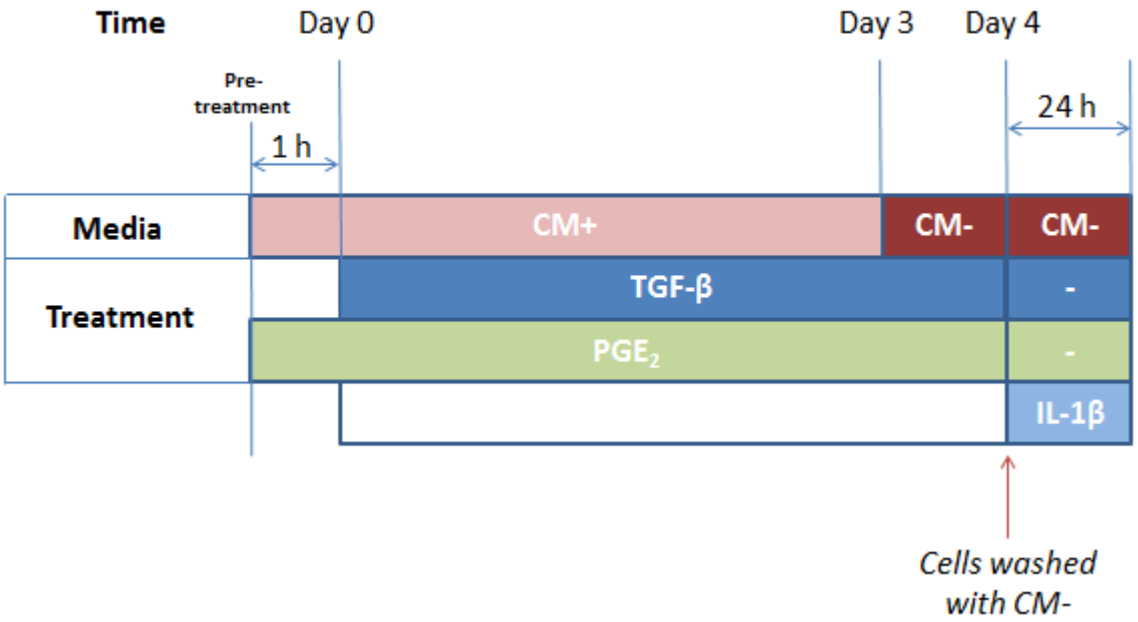


Figure 3-4 Experimental Protocol for measuring PGE_2 production following treatment with PGE_2

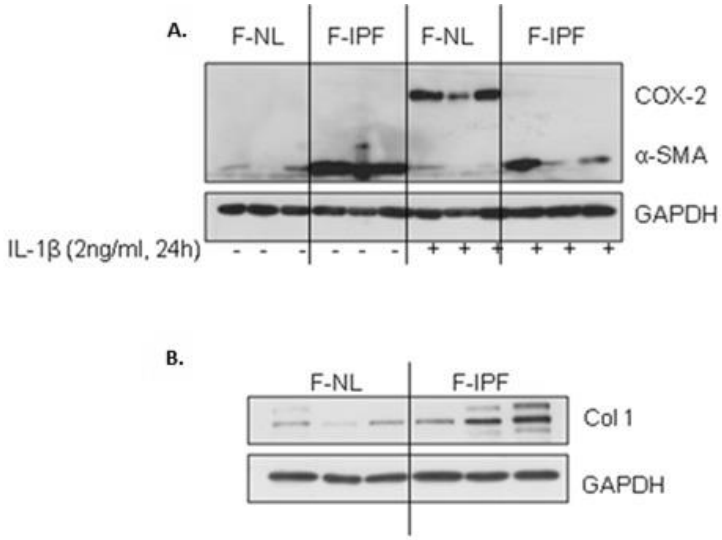
Schematic diagram of the treatment and timelines for measuring PGE_2 production following treatment with PGE_2. CM+ = media contain serum, CM- = serum free media.

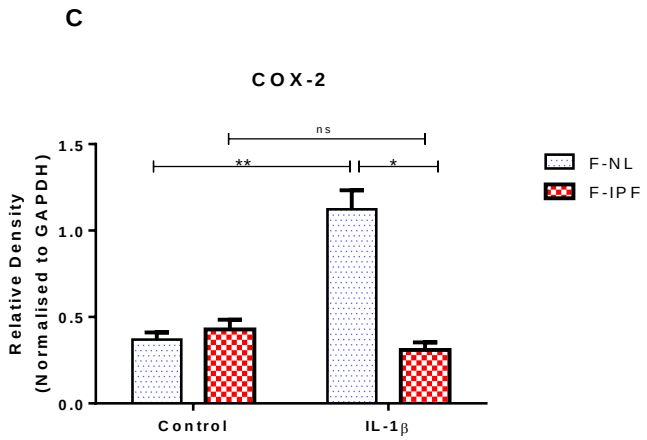
3.4 Results

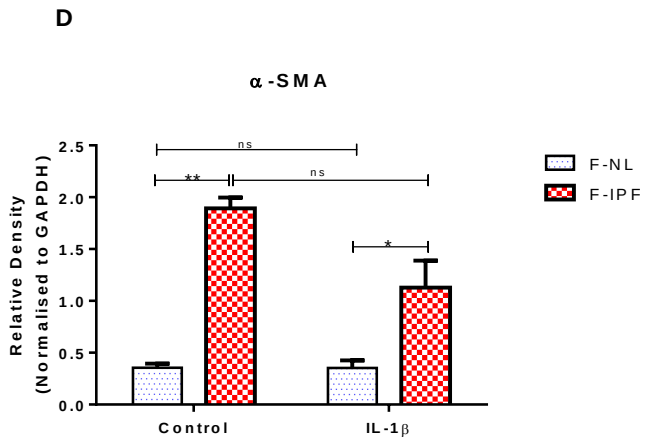
3.4.1 F-IPF have a pro-fibrotic phenotype compared with F-NL

The crucial role that myofibroblasts play in the pathogenesis of IPF is well established. In normal conditions, fibroblasts express little or no α-SMA and have low ECM production. After tissue injury they become activated and differentiate into myofibroblasts and have a pro-fibrotic phenotype as assessed by increased α-SMA and increased collagen production (Hinz et al., 2007c) and repressed COX-2 expression (Wilborn et al., 1995).

We started this study by confirming the phenotype of F-NL and F-IPF and analysed the expression of COX-2, α-SMA, collagen I and PGE_2 production. Previous studies within our laboratory have demonstrated that IL-1β induced COX-2 mRNA and protein expression after 4 hours and 24 hours, respectively (Coward et al., 2009). Therefore, to analyse COX-2 protein expression cells were treated with IL-1β (2 ng/ml) for 24 hours. IL-1β alone had no effect on α-SMA or GAPDH protein expression in either F-NL or F-IPF. Basally, F-NL and F-IPF do not express COX-2, however, following IL-1β stimulation COX-2 is induced in F-NL, but not in F-IPF (Figure 3-5). F-IPF expressed markedly more α-SMA (Figure 3-5) both basally and after stimulation with IL-1β. Furthermore, F-IPF expressed more Col 1 protein basally compared with F-NL (Figure 3-5).


A.
F-NL
F-IPF
F-NL
F-IPF
COX-2
α-SMA
GAPDH
IL-1β (2ng/ml, 24h)
B.
F-NL
F-IPF
Col 1
GAPDH


C
COX-2
Relative Density (Normalised to GAPDH)
ns
**
*
F-NL
F-IPF
Control
IL-1β


D
α-SMA
Relative Density (Normalised to GAPDH)
ns
**
ns
*
F-NL
F-IPF
Control
IL-1β

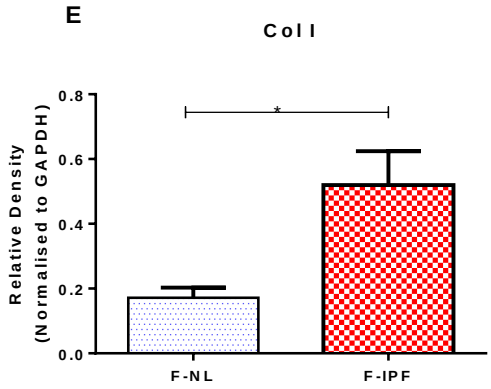

E
Col I
Relative Density (Normalised to GAPDH)
*
F-NL
F-IPF

Figure 3-5 COX-2, α-SMA and Col 1 Protein Expression in F-NL and F-IPF

*Confluent F-NL and F-IPF were serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 hours) prior to the collection of total cell lysate for Western blot analysis of (A) COX-2 and α-SMA and (B) Col 1. This figure includes data from three different cell lines. Relative density was calculated by normalising the density of the COX-2, α-SMA and Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01.*

We next assessed mRNA expression with regards to COX-2, α-SMA and Col 1 expression in F-NL and F-IPF to assess if regulation is transcriptional in order to analyse molecular mechanisms in future studies. Type 1 collagen is a triple helix containing two pro-α-1 chains, encoded by the COL1A1 gene, and one pro-α-2 chain, encoded by the COL1A2 gene, and is the most abundant collagen expressed by myofibroblasts (Zhang et al., 1994). Based on previous experiments within our laboratory (Coward et al., 2009) we treated cells with IL-1β (2 ng/ml) for 4 hours to analyse COX-2 mRNA. Following IL-1β stimulation F-NL had a significant induction of COX-2 mRNA. In contrast, the increase in COX-2 mRNA following IL-1β stimulation was not significant in F-IPF (Figure 3-6). IL-1β-induced COX-2 was slightly decreased in F-IPF compared with F-NL but this was not significant. This may suggest that COX-2 mRNA is reduced in F-IPF compared with F-NL but further experiments would be required to confirm this. There was no difference in expression of α-SMA in F-NL compared with F-IPF (Figure 3-7A) but Col 1 mRNA expression was significantly higher in F-IPF compared with F-NL (Figure 3-7B).

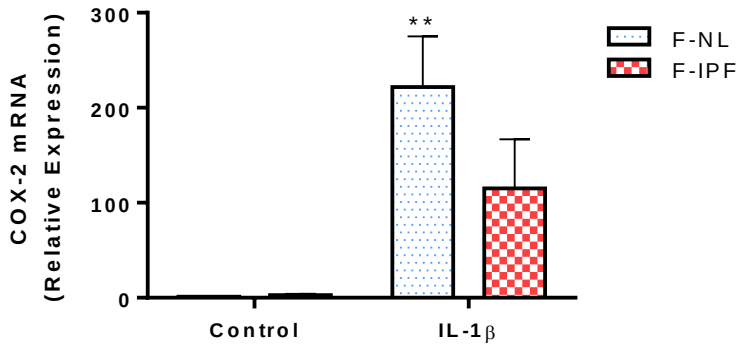


Figure 3-6 COX-2 mRNA Expression in F-NL and F-IPF

*Confluent F-NL and F-IPF were serum-starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for analysis by qPCR. The figure shows the amalgamation of data from separate experiments performed in duplicate of four different cell lines and expressed as mean fold change over untreated control ± SEM. **p<0.01, compared to untreated control.*

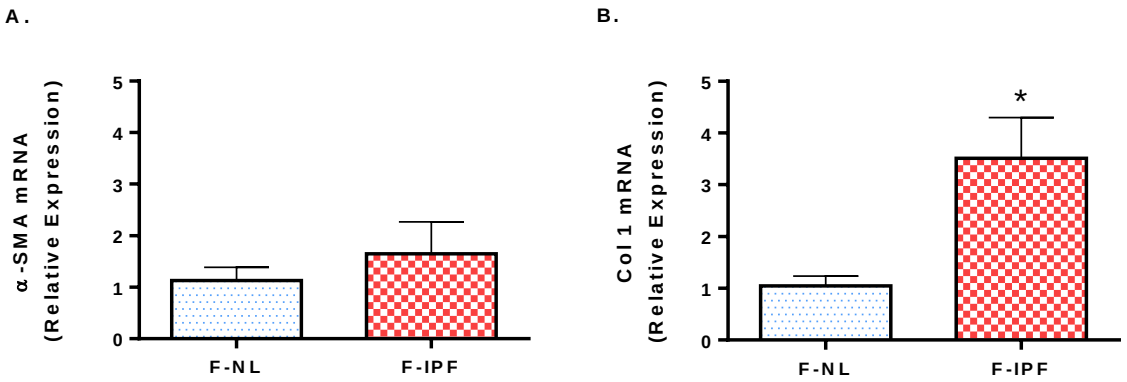


Figure 3-7 α-SMA and Col 1 mRNA Expression in F-NL and F-IPF

*Confluent F-NL and F-IPF were serum-starved for 24 hours prior to the collection of total RNA for analysis by qPCR of (A) α-SMA mRNA and (B) Col 1 mRNA. The figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM. *p<0.05, compared to untreated control.*

In order to confirm if reduced COX-2 expression in F-IPF contributes to the diminished production of PGE_2 we quantified PGE_2 in F-NL and F-IPF (Figure 3-8). Without IL-1β stimulation only a minimal amount of PGE_2 was produced in both F-NL and F-IPF (513.3 ± 28.5 pg x 10^6 cells and 510.8 ± 57.1 pg x 10^6 cells). Following IL-1β stimulation, PGE_2 production increased in F-NL (9371.9 ± 1462.1 pg x 10^6 cells), but F-IPF had significantly less PGE_2 (1384.1 ± 320.8 pg x 10^6 cells) (Figure 3-8). Therefore, following IL-1β stimulation F-IPF are unable to express sufficient COX-2 protein resulting in a diminished capacity to synthesise the anti-fibrotic mediator PGE_2.

Overall, these experiments confirm that F-IPF have a pro-fibrotic phenotype which is consistent with previous observations (Huang et al., 2007; Wilborn et al., 1995). F-IPF have increased α-SMA and Col 1 expression and have a diminished capacity to express COX-2 protein following IL-1β stimulation. Therefore, it is likely that F-IPF consists mainly of myofibroblasts whereas F-NL consist mainly of fibroblasts. In addition, the inability of F-IPF to express COX-2 results in the diminished production of PGE_2. Therefore, the reduction of PGE_2 in myofibroblasts may play an important role in fibroblast to myofibroblast differentiation.

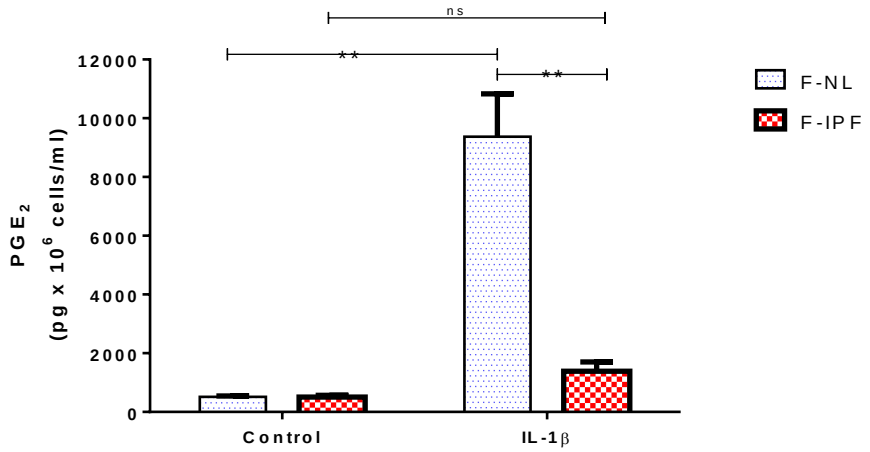


Figure 3-8 PGE_2 Production in F-NL and F-IPF

*F-NL and F-IPF were left unstimulated or stimulated with IL-1ß (2 ng/ml, 24h) and serum starved for 24 hours prior to the collection of cell culture media for analysis of PGE_2 by PGE_2 EIA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and expressed as mean ± SEM. **p<0.01.*

3.4.2 PGE_2 reverses the fibrotic phenotype in F-IPF

The data so far demonstrates that due to the repression of COX-2, F-IPF have a reduced capacity to produce PGE_2, which is a potent anti-fibrotic mediator (Vancheri et al., 2004). Although PGE_2 has been reported to inhibit TGF-β1-induced myofibroblast differentiation the ability of PGE_2 to reverse myofibroblast differentiation and the effect of exogenous PGE_2 on COX-2 expression has not been investigated. Due to this, we investigated the effect of exogenous PGE_2 treatment on COX-2, α-SMA and Col 1 expression and assessed if the lack of PGE_2 contributes to the persistent activation of the myofibroblast phenotype.

We first assessed whether PGE_2 could reverse fibroblast to myofibroblast differentiation as denoted by decreased α-SMA and Col 1. In addition, we investigated the effect of PGE_2 on COX-2 expression to determine if PGE_2 treatment could restore COX-2 expression in F-IPF. In order to determine the optimum treatment time to analyse COX-2 and α-SMA a 5 day time course was performed with PGE_2. PGE_2 treatment alone had no effect on COX-2 expression (Figure 3-9). However, PGE_2 treatment increased IL-1β-induced COX-2 protein expression in F-IPF significantly after day 3 and day 5 of treatment (Figure 3-9). PGE_2 reduced α-SMA protein, both with and without IL-1β stimulation, at day 3 and day 5 (Figure 3-9). Based on these data 3 day PGE_2 treatment was the chosen time point for all further experiments. To confirm the optimum concentration of PGE_2 for future experiments, F-IPF were treated with 0.1, 1 and 10 µM PGE_2. PGE_2 increased IL-β-induced COX-2 protein expression at 1 and 10 µM and reduced α-SMA protein expression at 1 and 10 µM both with and without IL-1β treatment (Figure 3-10). IL-1β had no effect on α-SMA protein expression following PGE_2 treatment (Figure 3-10). As PGE_2 is known to inhibit fibroblast proliferation (Hetzel et al., 2005; Huang et al., 2007) we chose to use PGE_2 at 1 µM for all subsequent experiments to minimise the effect on proliferation during the 3 day treatment period. The effect of PGE_2 on Col 1 was also determined. Unlike α-SMA expression, 1 µM PGE_2 treatment did not effect Col 1 protein

expression in F-IPF (Figure 3-11). Therefore, PGE_2 treatment is able to reverse fibroblast to myofibroblast phenotype by reducing α-SMA expression which is associated with increased expression of IL-1β-induced COX-2.

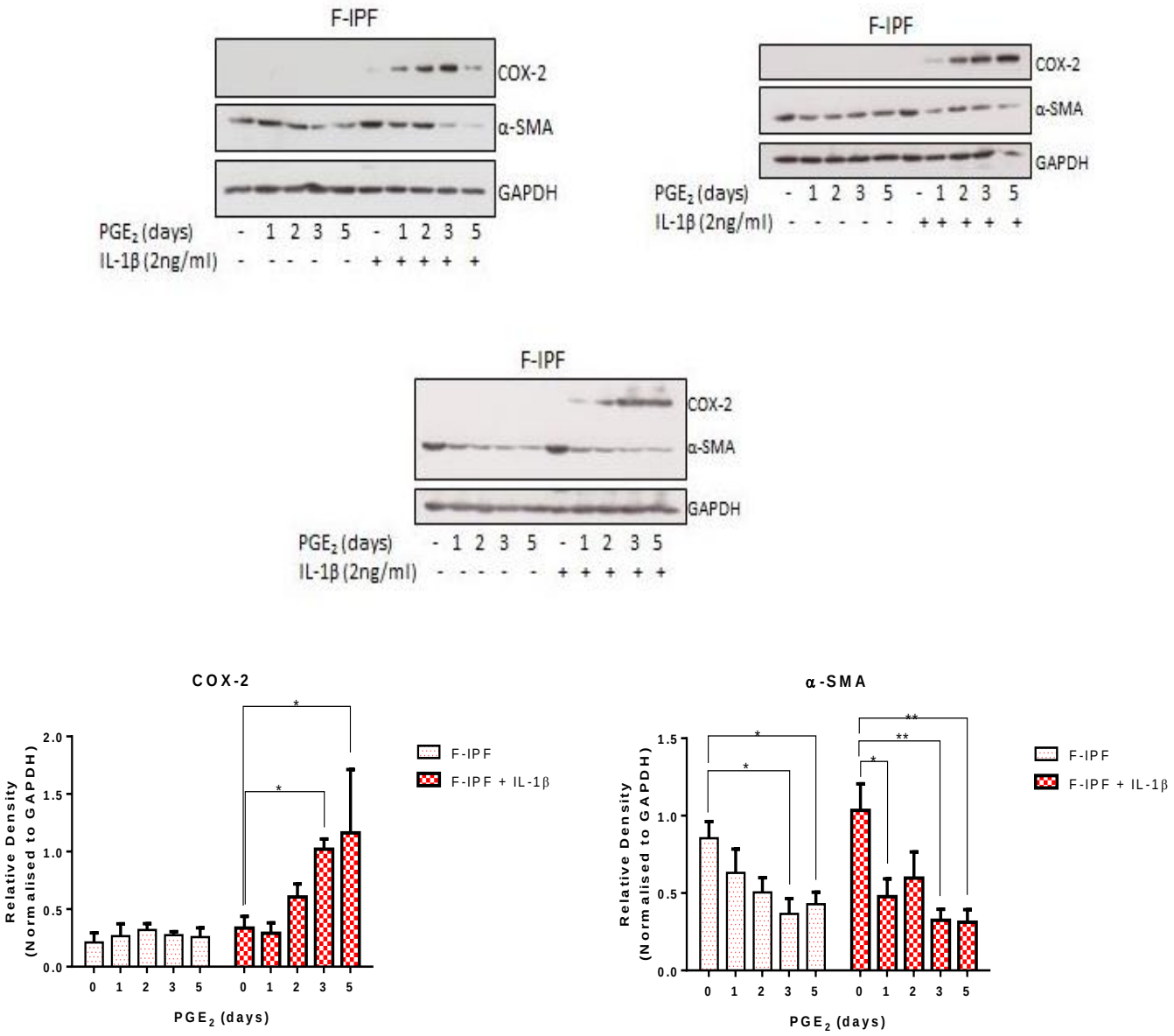


Figure 3-9 Effect of PGE_2 on COX-2 and α-SMA Protein Expression in F-IPF (time course)

*F-IPF were treated with PGE_2 (1 μM) for 0, 1, 2, 3 and 5 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/m, 24 hours) prior to collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01.*

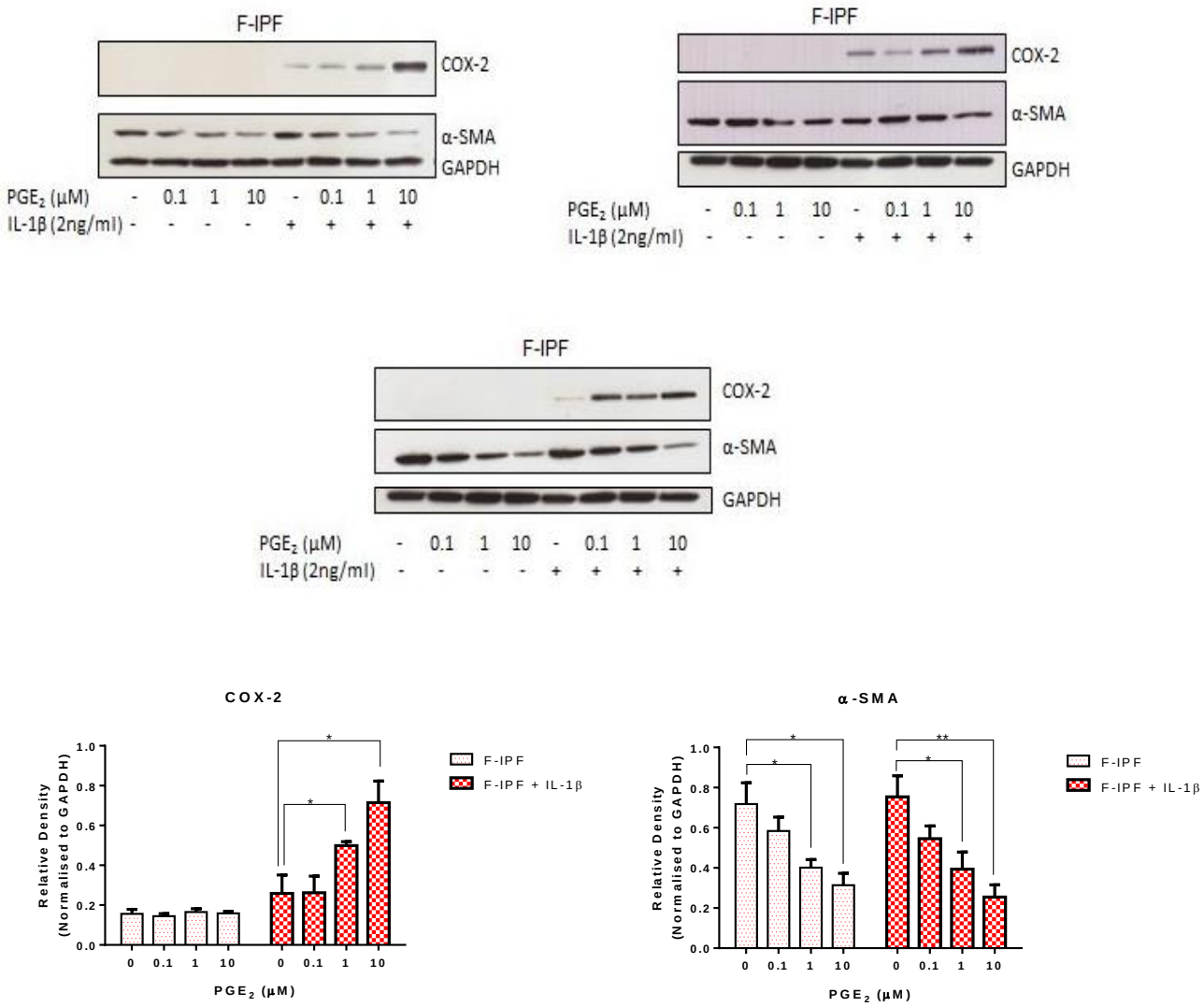


Figure 3-10 Effect of PGE_2 on COX-2 and α-SMA Protein Expression in F-IPF (concentration-response)

*F-IPF were treated with 0.1 μM, 1 μM and 10 μM PGE_2 for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 hours) prior to collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01*

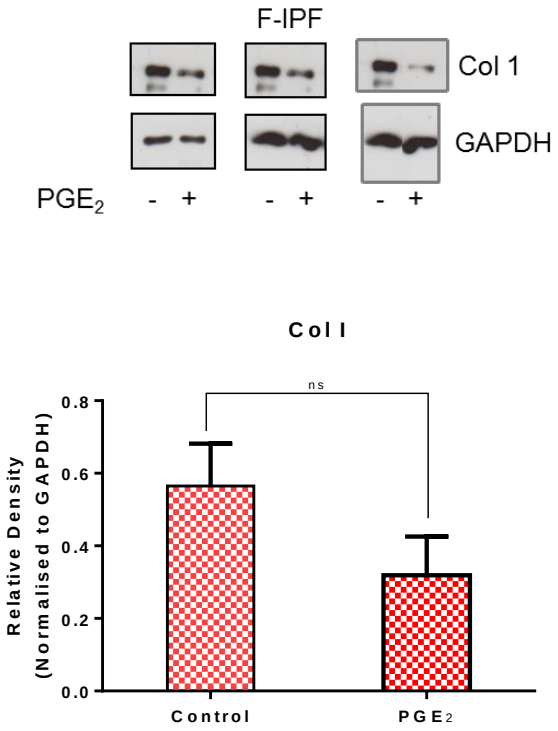


Figure 3-11 Effect of PGE_2 on Collagen 1 Protein Expression in F-IPF

*F-IPF were treated with PGE_2 (1 µM, 3d) and serum starved for 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01*

To confirm if the increased COX-2 expression was functional and resulted in the production of PGE_2 we determined PGE_2 concentration in F-IPF following pre-treatment with PGE_2 (Figure 3-12). 3 day PGE_2 treatment followed by IL-1β stimulation resulted in a significant increase in PGE_2 production (7171.3 ± 1631.4 pg x 10^6 cells/ml) compared with IL-1β only (1614.0 ± 774.8 pg x 10^6 cells/ml) and PGE_2 only (2109.5 ± 739.6 pg x 10^6 cells/ml). Therefore, exogenous PGE_2 treatment caused an increase in IL-1β-induced COX-2 protein expression resulting in increased endogenous PGE_2 production. This suggests that exogenous PGE_2 treatment may compensates for the lack of endogenous PGE_2 in F-IPF.

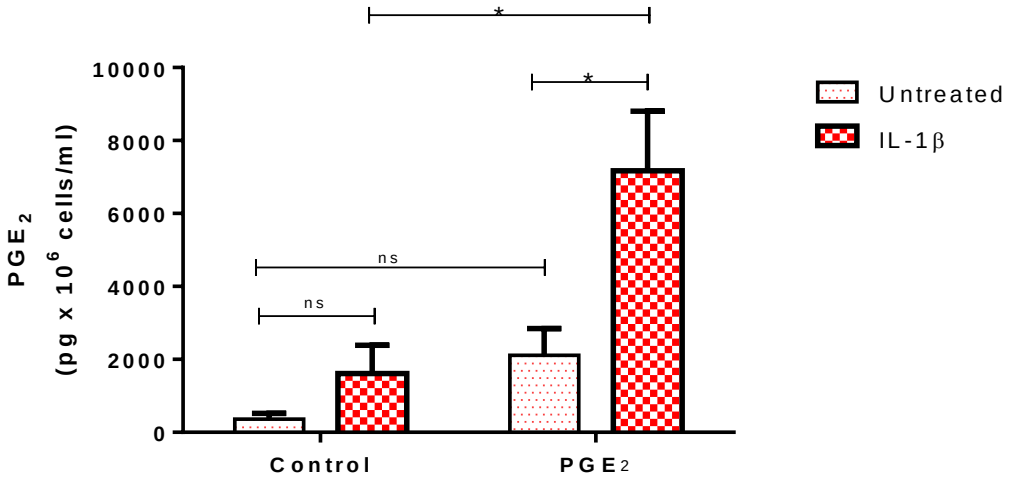


Figure 3-12 Effect of PGE_2 treatment on endogenous PGE_2 production in F-IPF

*F-IPF were pre-treated with PGE_2 (1 μM, 3d), washed with serum free media, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of cell culture media for analysis by PGE_2 EIA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean ± SEM. *p<0.05.*

We next determined if the effects of PGE_2 on COX-2 expression, and myofibroblast to fibroblast differentiation, is due to transcriptional or post-translational regulation. qPCR was performed to measure changes in mRNA expression. In F-IPF, PGE_2 treatment had no effect on COX-2 mRNA following PGE_2 treatment alone or with IL-1β stimulation (Figure 3-13). PGE_2 treatment also had no effect on α-SMA mRNA (Figure 3-14A). However, Col 1 mRNA was significantly decreased following PGE_2 treatment (Figure 3-14B). The mRNA data suggests that PGE_2 treatment may regulate Col 1 via transcriptional regulation but COX-2 and α-SMA protein expression may be due to translational modifications.

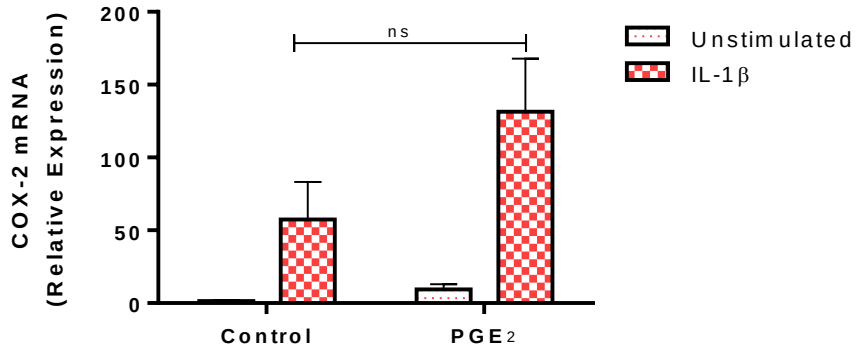


Figure 3-13 Effect of PGE_2 on COX-2 mRNA in F-IPF

F-IPF were treated with PGE_2 (1 μM, 3 days), serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4 hours) prior to the collection of total RNA for qPCR analysis. This figure shows the amalgamation of data from separate experiments performed in duplicate of four different cell lines and is expressed as mean fold change over untreated control ±SEM.

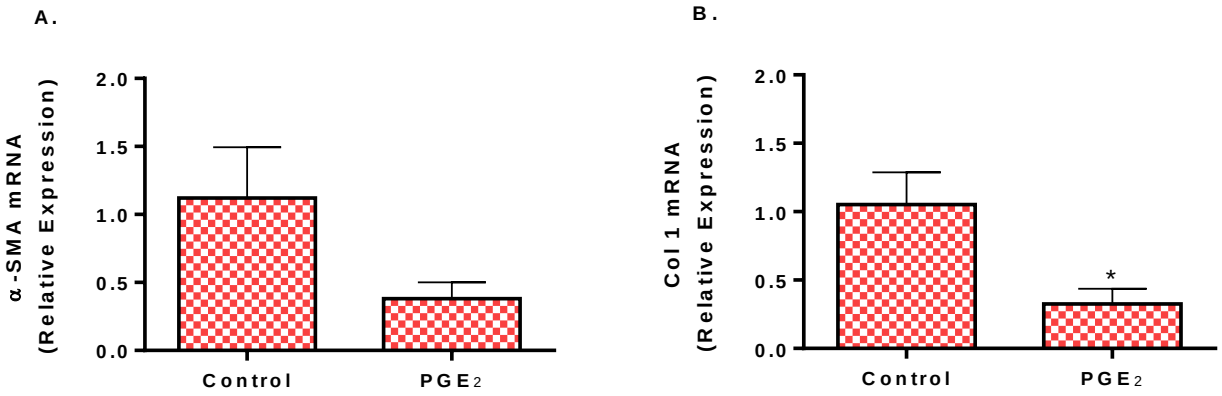


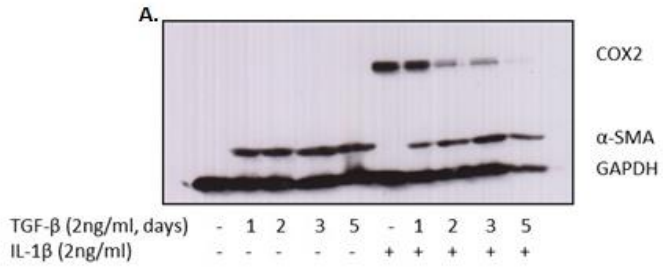
Figure 3-14 Effect of PGE_2 on α-SMA and Col 1 mRNA in F-IPF

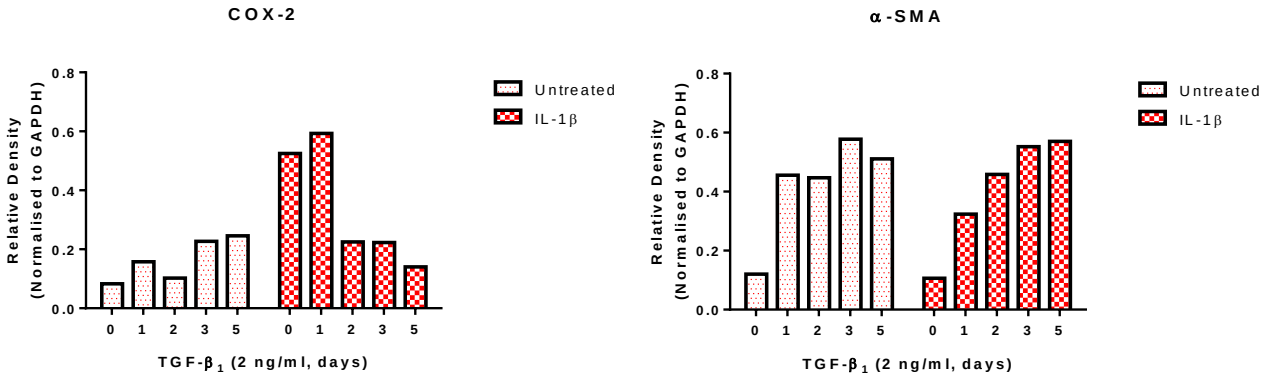
*F-IPF were serum starved for 24 hours and treated with PGE_2 (1 μM, 24 hours) and prior to the collection of total RNA for qPCR analysis of (A) α-SMA mRNA and (B) Col 1 mRNA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ±SEM. *p<0.05, compared with untreated control.*

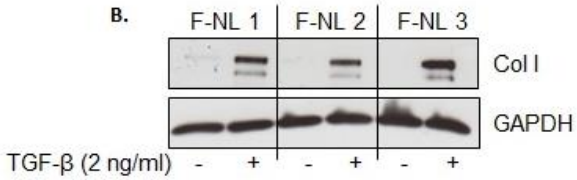
3.4.3 TGF-β1 promotes a pro-fibrotic phenotype in F-NL

We have demonstrated that F-IPF cells have a pro-fibrotic phenotype which may be due to their inability to produce the anti-fibrotic mediator PGE_2 and exogenous PGE_2 treatment reversed fibroblast to myofibroblast differentiation. However, whether or not PGE_2 treatment would be able to prevent TGF-β1-induced fibroblast to myofibroblast differentiation required further investigation.

It is already documented that TGF-β1 is a potent inducer of fibroblast to myofibroblast differentiation both *in vivo* and *in vitro* (Scotton and Chambers, 2007). We hypothesised that TGF-β1-treated F-NL would mimic the pro-fibrotic phenotype of F-IPF and thus could be used as an *in vitro* model of fibroblast to myofibroblast differentiation. In addition, we wanted to determine if the loss of COX-2 expression was associated with fibroblast to myofibroblast differentiation. F-NL were treated with TGF-β1 (2 ng/ml) for 0, 1, 2, 3 and 5 days, with or without IL-1β activation (2 ng/ml, 24h). TGF-β1 treatment resulted in the loss of COX-2 expression in a time dependent manner (Figure 3-15A). COX-2 expression was markedly reduced at day 2 of TGF-β1 treatment and almost completely repressed at day 5. The loss of COX-2 expression was associated with increased α-SMA (Figure 3-15A) suggesting that the loss of COX-2 is associated with fibroblast to myofibroblast differentiation. F-NL expressed little or no α-SMA at baseline, day 1 TGF-β1 treatment increased α-SMA protein expression and expression peaked at day 3 (Figure 3-15A). Based on these data, 3 day TGF-β1 treatment was the chosen time point for all future experiments. TGF-β1 treatment also caused a significant increase in Col 1 protein in F-NL (Figure 3-15B).


A.
COX2
α-SMA
GAPDH
TGF-β (2ng/ml, days) - 1 2 3 5 - 1 2 3 5
IL-1β (2ng/ml) - - - - - + + + + +


COX-2
Relative Density (Normalised to GAPDH)
0.8
0.6
0.4
0.2
0.0
0 1 2 3 5 0 1 2 3 5
TGF-β1 (2 ng/ml, days)
Untreated
IL-1β
α-SMA
Relative Density (Normalised to GAPDH)
0.8
0.6
0.4
0.2
0.0
0 1 2 3 5 0 1 2 3 5
TGF-β1 (2 ng/ml, days)
Untreated
IL-1β


B.
F-NL 1
F-NL 2
F-NL 3
Col I
GAPDH
TGF-β (2 ng/ml) - + - + - +

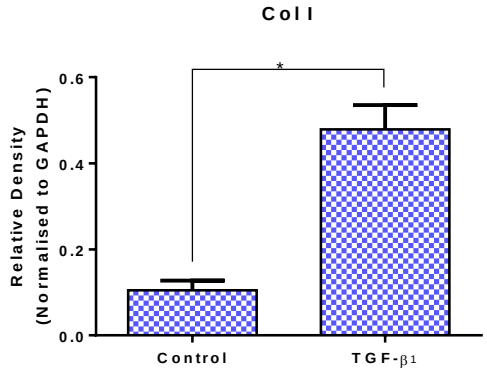

Col I
*
Relative Density (Normalised to GAPDH)
0.6
0.4
0.2
0.0
Control
TGF-β1

Figure 3-15 Effect of TGF-β1 on COX-2, α-SMA and Col 1 Protein in F-NL

*F-NL were treated with TGF-β1 (2 ng/ml) for 0, 1, 2, 3 and 5 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 hours) prior to the collection of total cell lysate for Western blot analysis. This figure was performed in one cell line and as such no statistical analysis was performed. (B) F-NL were treated with TGF-β1 (2 ng/ml, 3 days) and serum starved for 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows data from three different cell lines. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05.*

To confirm that the loss of COX-2 leads to reduced PGE_2 production we also determined the amount of PGE_2 in TGF-β1-treated F-NL. IL-1β treatment resulted in a significant increase of PGE_2 in F-NL (Figure 3-16). In contrast, TGF-β treatment reduced IL-1β induced PGE_2 significantly compared with IL-1β control (Figure 3-16). Thus, TGF-β1-treated F-NL mimicked the pro-fibrotic phenotype of F-IPF (Figure 3-8) and we concluded that this was a valid *in vitro* model of fibroblast to myofibroblast differentiation. Collectively, these experiments demonstrated that the loss of COX-2 is associated with fibroblast to myofibroblast differentiation.

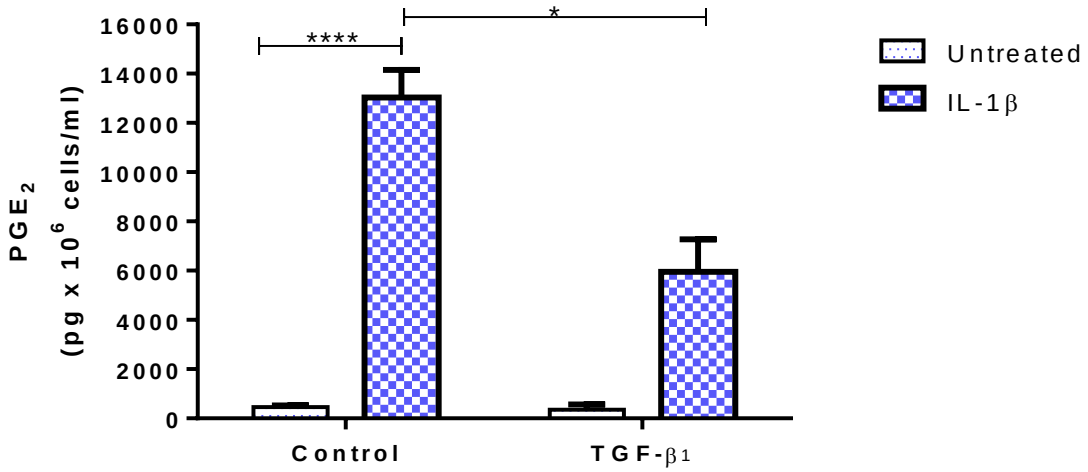
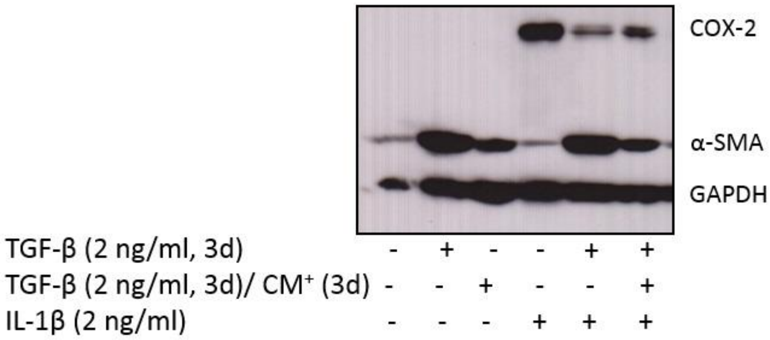
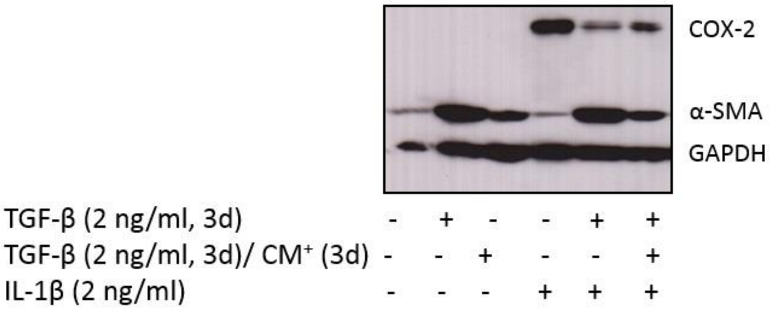


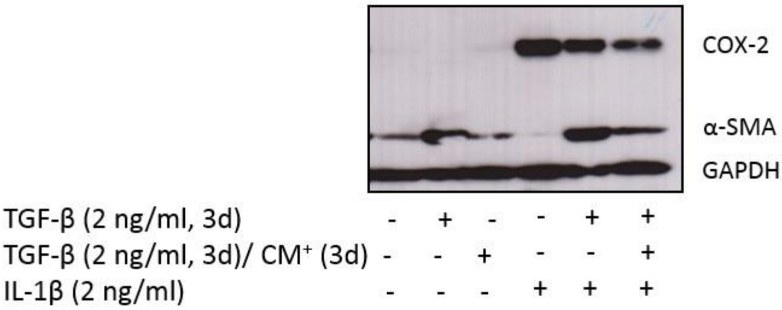
Figure 3-16 Effect of TGF-β1 on endogenous PGE_2 production in F-NL

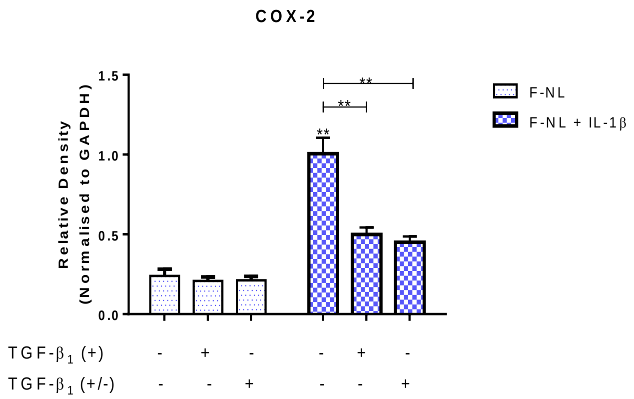
F-NL were pre-treated with TGF-β1 (2 ng/ml, 3d), washed with serum free media, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of cell culture media for analysis by PGE_2 EIA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean ± SEM.

Previous studies have indicated that that the effects of TGF-β1 are permanent (Garrison et al., 2013; Sam et al., 2006). Treatment of IMR-90, a foetal lung fibroblast cell line, with TGF-β1 for 24 hours increased α-SMA expression that persisted for 5 days (Garrison et al., 2013). To determine whether our model of fibroblast to myofibroblast differentiation resulted in a stable differentiated state F-NL were treated with TGF-β1 (2 ng/ml) for 3 days and grown for a further 5 days without repeated dosing (Figure 3-17). TGF-β1 treatment significantly reduced IL-1β-induced COX-2 expression in F-NL and COX-2 remained reduced even after TGF-β1 treatment had been removed for three days (Figure 3-17). TGF-β1 treatment resulted in a significant increase of α-SMA which decreased after the removal of TGF-β1 for 3 days (Figure 3-17). TGF-β1 treatment with IL-1β stimulation increased α-SMA significantly. Following the removal of TGF-β1, α-SMA was still increased compared with IL-1β only but α-SMA had decreased compared with the 3 day TGF-β1 treatment. Therefore, this experiment demonstrates that reduced COX-2 expression persists for at least 3 days after the removal of TGF-β1.


COX-2
α-SMA
GAPDH
TGF-β (2 ng/ml, 3d) - + - - + +
TGF-β (2 ng/ml, 3d)/ CM+ (3d) - - + - - +
IL-1β (2 ng/ml) - - - + + +


COX-2
α-SMA
GAPDH
TGF-β (2 ng/ml, 3d) - + - - + +
TGF-β (2 ng/ml, 3d)/ CM+ (3d) - - + - - +
IL-1β (2 ng/ml) - - - + + +


COX-2
α-SMA
GAPDH
TGF-β (2 ng/ml, 3d) - + - - + +
TGF-β (2 ng/ml, 3d)/ CM+ (3d) - - + - - +
IL-1β (2 ng/ml) - - - + + +


COX-2
Relative Density
(Normalised to GAPDH)
1.5
1.0
0.5
0.0
**
**
**
F-NL
F-NL + IL-1β
TGF-β1 (+) - + - - + -
TGF-β1 (+/-) - - + - - +

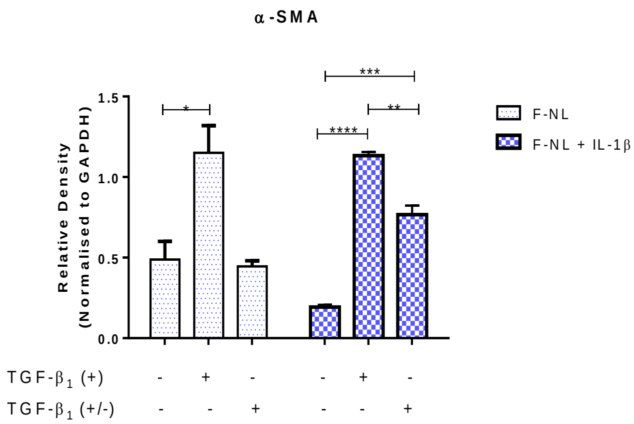

α-SMA
Relative Density
(Normalised to GAPDH)
1.5
1.0
0.5
0.0
*
***
**
****
F-NL
F-NL + IL-1β
TGF-β1 (+) - + - - + -
TGF-β1 (+/-) - - + - - +

Figure 3-17 Long Term Effects of TGF-β1 in F-NL

*F-NL were treated with TGF-β1 (2 ng/ml, 3d) alone or pre-treated with TGF-β1 (2 ng/ml, 3d) and grown for a further 3 days in media (CM+) without repeated dosing. F-NL were then serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml) for 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 compared with untreated control.*

To determine if the effects of TGF-β1 on COX-2 protein expression and fibroblast to myofibroblast differentiation are transcriptionally regulated qPCR was performed. The mRNA data reflected the same trends as seen with the protein data. TGF-β1 treatment resulted in a significant decrease of COX-2 mRNA (Figure 3-18). In order to determine the optimum treatment time to quantify α-SMA and Col 1 mRNA expression in F-NL a time course was performed (Figure 3-19). F-NL were treated with TGF-β1 between 0 and 48 hours. TGF-β1 significantly increased α-SMA at 24 hours which remained significantly elevated at 48 hours (Figure 3-19A). In addition, TGF-β1 treatment caused a significant increase in Col 1 mRNA which peaked at 24 hours and remained significantly elevated at 48 hours (Figure 3-19B). In order to quantify α-SMA and Col 1 mRNA in F-NL 24 hour was the chosen time point for all future experiments.

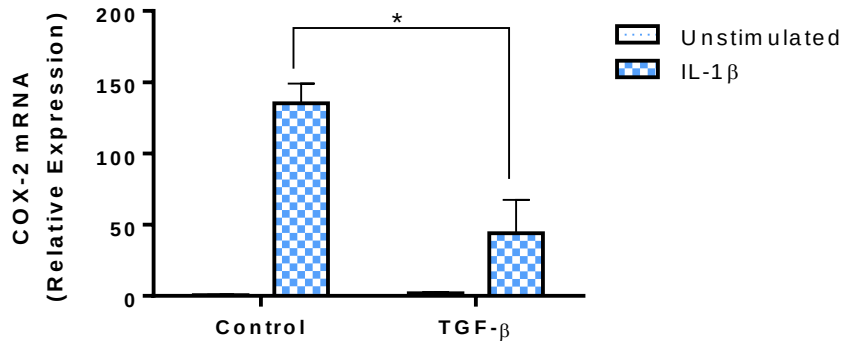


Figure 3-18 Effect of TGF-β1 on COX-2 mRNA in F-NL

*F-NL were treated with TGF-β1 (2 ng/ml, 3 days), serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4 hours) prior to the collection of total RNA for qPCR analysis. This figure shows the amalgamation of data from three separate experiments performed in duplicate of different cell lines and is expressed as the mean fold change over untreated control ±SEM. *p<0.05, compared with IL-1β-treated control.*

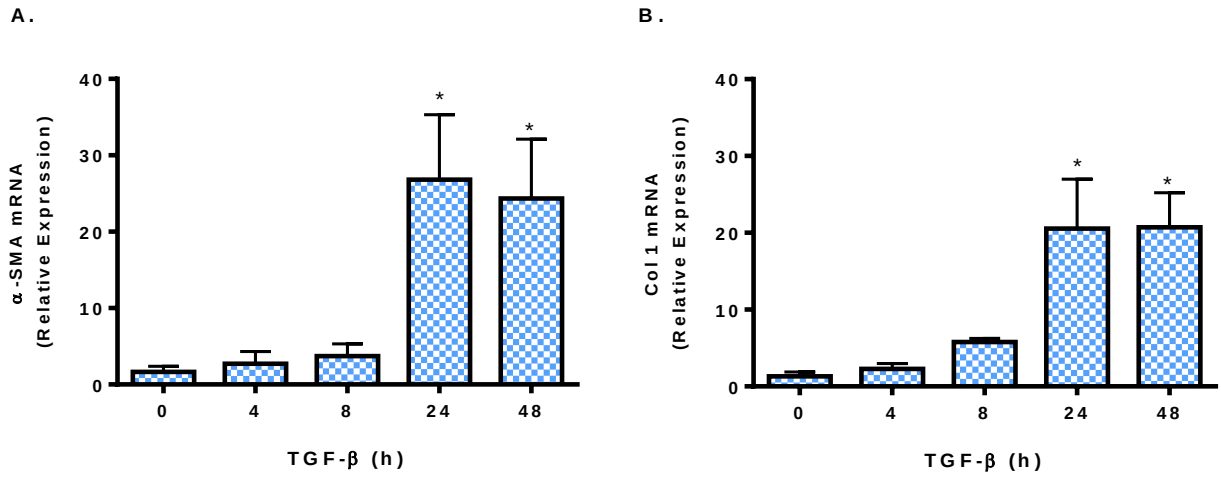


Figure 3-19 Effect of TGF-β1 time course on α-SMA and Col 1 mRNA in F-NL

*F-NL were treated with TGF-β1 (2 ng/ml) for 0, 4, 8, 24 and 48 hours and serum starved for 24 hours prior to the collection of total RNA for qPCR analysis of (A) α-SMA mRNA and (B) Col 1 mRNA. This figure shows the amalgamation of data from three separate experiments performed in duplicate of different cell lines and is expressed as the mean fold change over untreated control ±SEM. *p<0.05, compared with untreated control.*

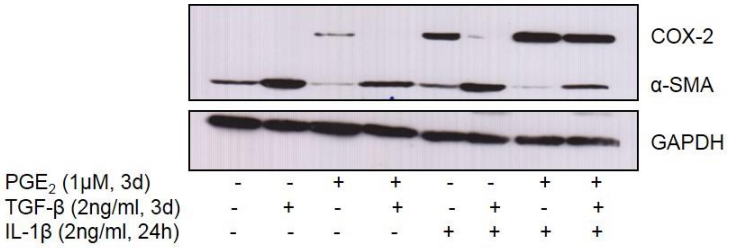
3.4.4 PGE_2 prevents the pro-fibrotic effects of TGF-β1

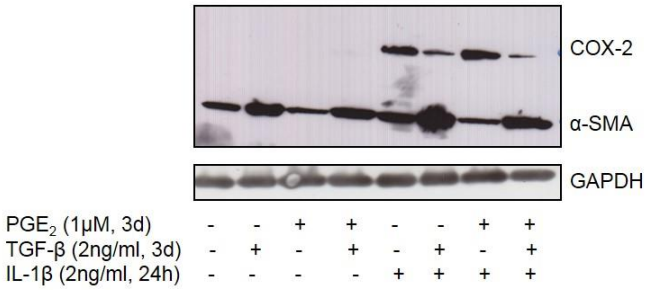
So far we have clearly demonstrated that COX-2 is repressed and subsequent PGE_2 production is reduced following TGF-β1 treatment in F-NL, which may play a key role in fibroblast to myofibroblast differentiation. Exogenous PGE_2 treatment compensated for TGF-β1-induced repression of COX-2 and PGE_2 production and reversed fibroblast to myofibroblast differentiation. Thus, the lack of PGE_2 may cause fibroblasts to have a consistently activated pro-fibrotic phenotype. We next sought to determine if PGE_2 could inhibit TGF-β1-induced repression of COX-2 and PGE_2 production in F-NL and if this would prevent fibroblast to myofibroblast differentiation.

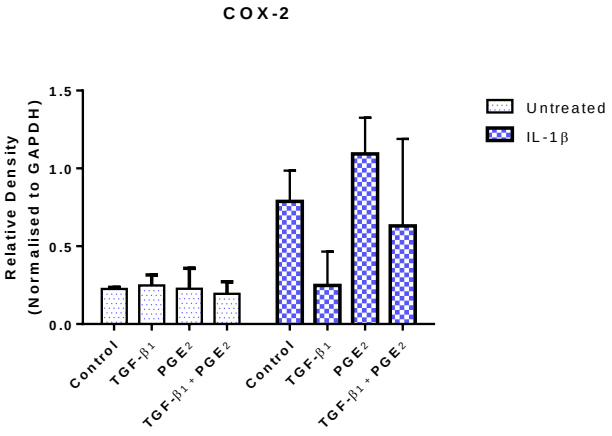
TGF-β1 alone, PGE_2 alone and treatment with TGF-β1 with PGE_2 had no effect on COX-2. PGE_2 with TGF-β had no effect on IL-1β-induced COX-2 expression (Figure 3-20). The effect of PGE_2 and TGF-β treatment had no effect on α-SMA. Our data suggest that PGE_2 prevented TGF-β-induced Col 1 expression but additional experiments are required to confirm this as the experiment was only completed in one cell line (Figure 3-20).

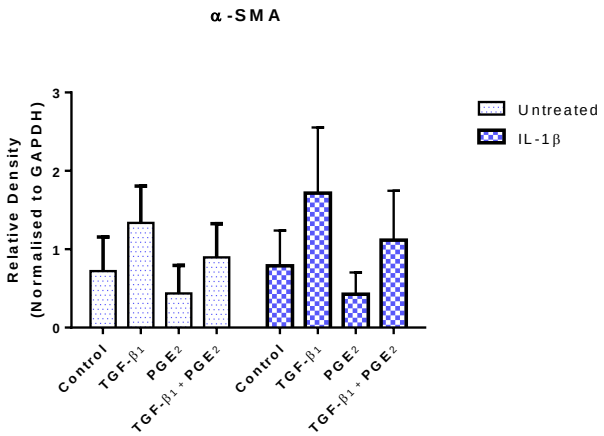
PGE_2 treatment increases IL-1β-induced PGE_2 production whereas TGF-β1 treatment reduced IL-1β-induced PGE_2 (Figure 3-21). In addition, PGE_2 treatment in combination with TGF-β1 prevented TGF-β1 effects on IL-1β-induced PGE_2. Treatment with PGE_2 and TGF-β1 resulted in significantly more PGE_2 production compared with TGF-β1 alone and PGE_2 levels were not significantly different from IL-1β treatment alone (Figure 3-21). Therefore, exogenous PGE_2 is able to prevent the repression PGE_2 production caused by TGF-β1 treatment. In summary, exogenous PGE_2 treatment is not only able to reverse COX-2 repression in F-IPF but is also able to prevent TGF-β1-induced PGE_2 repression in F-NL, most likely through the repression of COX-2. Hence, expression of COX-2 may be able to maintain an anti-fibrotic

phenotype and the loss of COX-2 is associated with a pro-fibrotic phenotype and myofibroblast differentiation.


COX-2
α-SMA
GAPDH
PGE2 (1μM, 3d) - - + + - - + +
TGF-β (2ng/ml, 3d) - + - + - + - +
IL-1β (2ng/ml, 24h) - - - - + + + +


COX-2
α-SMA
GAPDH
PGE2 (1μM, 3d) - - + + - - + +
TGF-β (2ng/ml, 3d) - + - + - + - +
IL-1β (2ng/ml, 24h) - - - - + + + +


COX-2
Relative Density (Normalised to GAPDH)
1.5
1.0
0.5
0.0
Untreated
IL-1β
Control
TGF-β1
PGE2
TGF-β1 + PGE2


α-SMA
Relative Density (Normalised to GAPDH)
3
2
1
0
Untreated
IL-1β
Control
TGF-β1
PGE2
TGF-β1 + PGE2

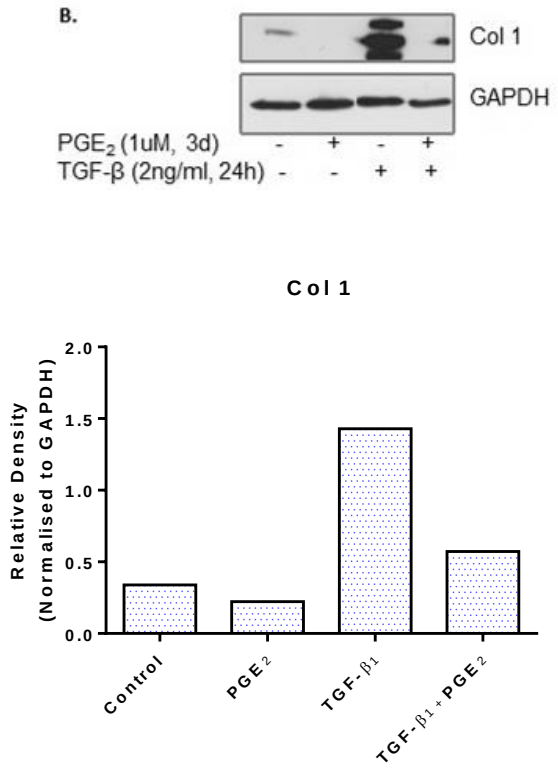


Figure 3-20 Effect of PGE_2 on TGF-β1-induced Fibroblast to Myofibroblast Differentiation

A) F-NL were treated with TGF-β1 (2 ng/ml) alone, PGE_2 (1 μM) alone or TGF-β1 (2 ng/ml) and PGE_2 (1 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml) for 24 hours prior to the collection of total cell lysate for analysis by Western blot of COX-2 and α-SMA protein. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. This figure shows two separate experiments performed in different cell lines as such no statistical analysis was performed. B) F-NL were treated with TGF-β1 (2 ng/ml) alone, PGE_2 (1 μM) alone or TGF-β1 (2 ng/ml) and PGE_2 (1 μM) for 3 days and serum starved for 24 hours prior to the collection of total cell lysate for analysis by Western blot of Col 1 protein. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH. This figure was performed in one cell line and as such no statistical analysis was performed.

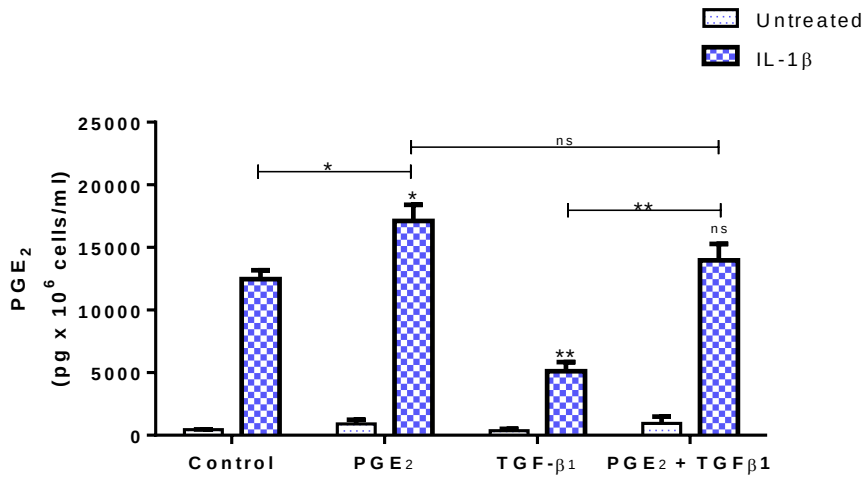


Figure 3-21 Effect of PGE_2 on endogenous PGE_2 production in TGF-β1-treated F-NL

*F-NL were treated with PGE_2 (1 uM) alone, TGF-β1 (2 ng/ml) alone or pre-treated with PGE_2 (1 uM) and TGF-β1 (2 ng/ml) for 3 days, washed with serum free media, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml) prior to the collection of cell culture media for analysis by PGE_2 EIA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed mean ± SEM.*p<0.05, **p<0.01 compared with IL-1β treated cells.*

To establish if PGE_2 can prevent changes in mRNA we also analysed mRNA expression after PGE_2 and TGF-β1 treatment (Figure 3-22 and Figure 3-23). TGF-β1 treatment significantly repressed IL-1β-induced COX-2 mRNA compared with IL-1β treated control. TGF-β1 and PGE_2 treatment increased COX-2 mRNA compared with TGF-β1 alone and therefore prevented the effects of TGF-β1-induced COX-2 mRNA repression (Figure 3-22). PGE_2 had no effect on TGF-β1-induced α-SMA and Col 1 mRNA (Figure 3-23A and Figure 3-23B).

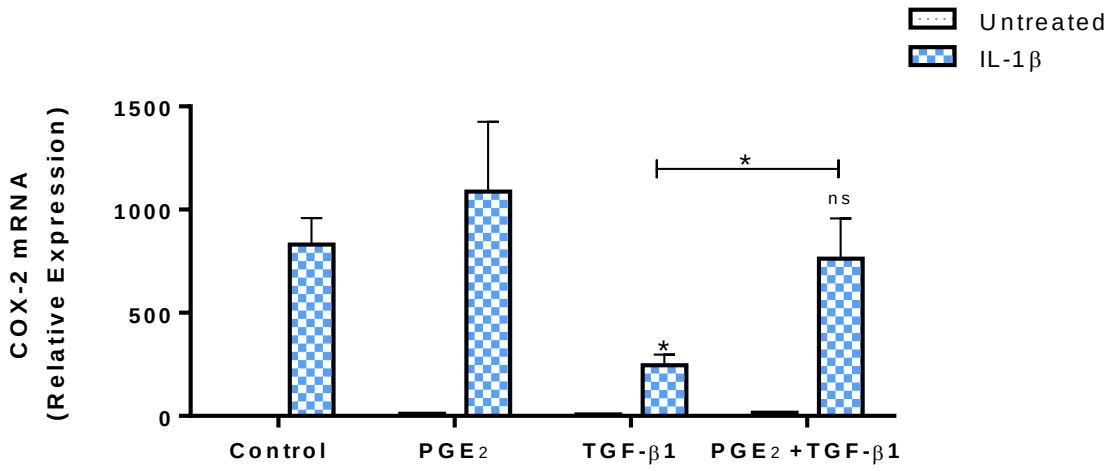


Figure 3-22 Effect of PGE_2 on COX-2 mRNA in TGF-β1-treated F-NL

*F-NL were treated with TGF-β1 (2 ng/ml) alone, PGE_2 (1 μM) alone or pre-treated with PGE_2 (1 μM) and TGF-β1 (2 ng/ml) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for analysis by qPCR. This data is the amalgamation of three separate experiments performed in duplicate of three different cell lines and is expressed as the mean fold change over untreated control ± SEM. *p<0.05 compared with IL-1β treated cells.*

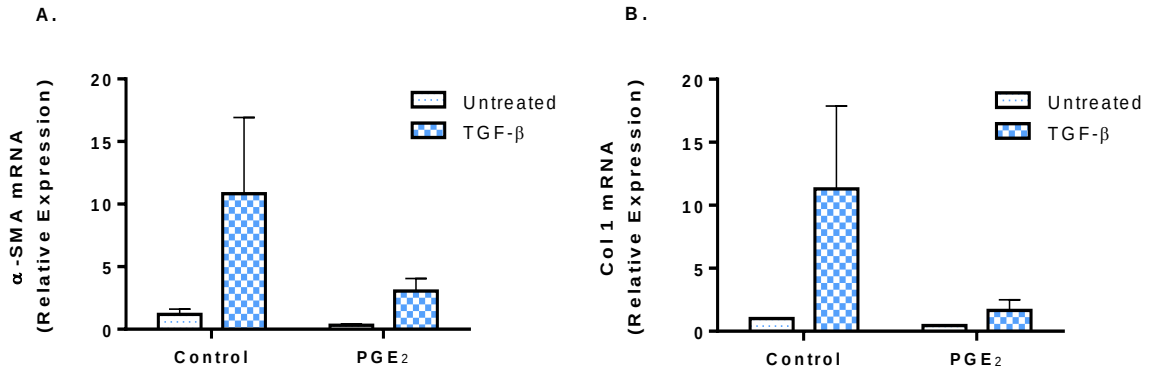


Figure 3-23 Effect of PGE_2 on α-SMA and Col 1 mRNA in TGF-β1 treated F-NL

F-NL were treated with TGF-β1 (2 ng/ml) alone, PGE_2 (1 μM) alone or pre-treated with PGE_2 (1 μM) and TGF-β1 (2 ng/ml) for 3 days and serum starved for 24 hours prior to the collection of total RNA for analysis by qPCR of (A) α-SMA and (B) Col 1. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as the mean fold change over untreated control ± SEM.

3.4.5 The anti-fibrotic effects of PGE_2 are via the EP2 receptor

To determine which EP receptor mediates increased IL-1β COX-2 expression and decreased α-SMA following PGE_2 treatment, F-IPF were treated with selective EP2 and EP4 agonists, ONO-AE1-259 and ONO-AE1-329 respectively. The EP2 specific agonist, ONO-AE1-259, had no effect on IL-1β induced-COX-2 (Figure 3-24). Further experiments confirmed that ONO-AE1-259 had no effect even at higher concentrations (Figure 3-25). There is a trend for the reduction of α-SMA protein expression which is dose dependent (Figure 3-25) however, density analysis data was not significant with the exception of 5 μM concentration which could be an outlier. The EP4 agonist had no effect on either COX-2 or α-SMA (

Figure 3-26 and Figure 3-27). These data suggest that the EP2 receptor may be the receptor responsible for mediating the effects of PGE_2 signalling rather than EP4 but further experiments would be required to confirm this.

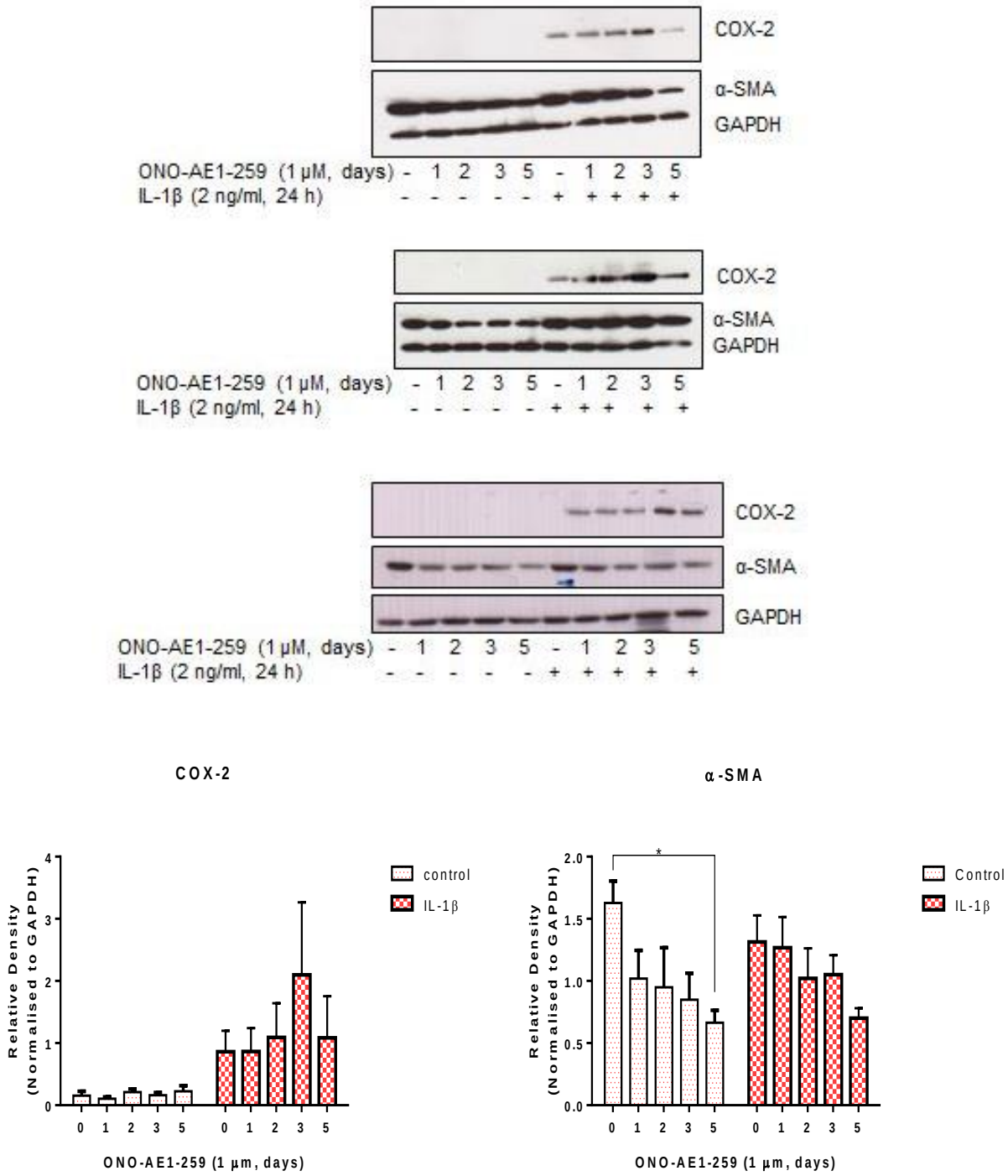


Figure 3-24 Effect of EP2 Agonist (ONO-AE1-259) Time Course on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with ONO-AE1-259 (1 μM) for 0, 1, 2, 3 and 5 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments

performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

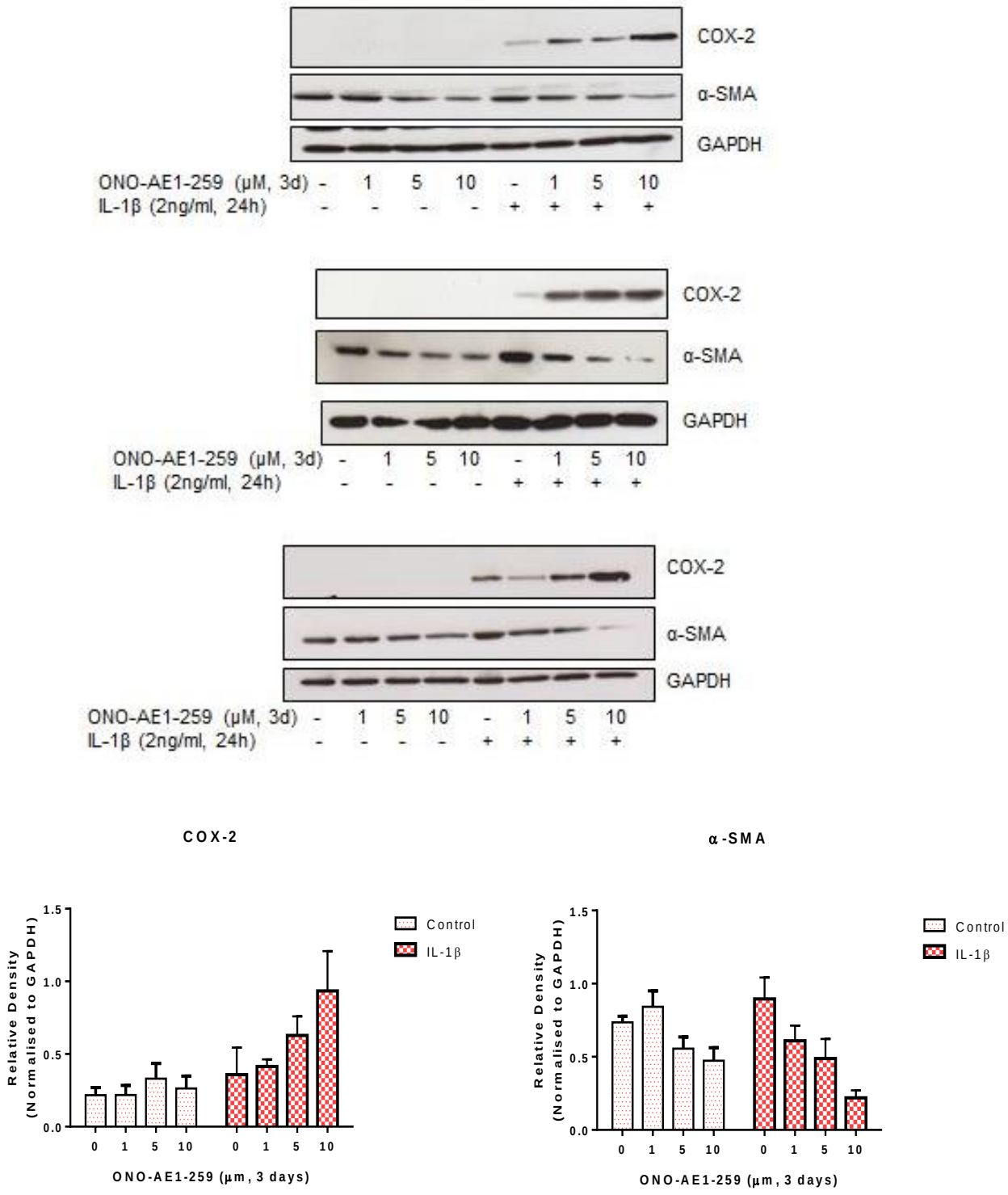


Figure 3-25 Effect of EP2 Agonist (ONO-AE1-259) Dose Response on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with ONO-AE1-259 at 1, 5 and 10 μM for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments

performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

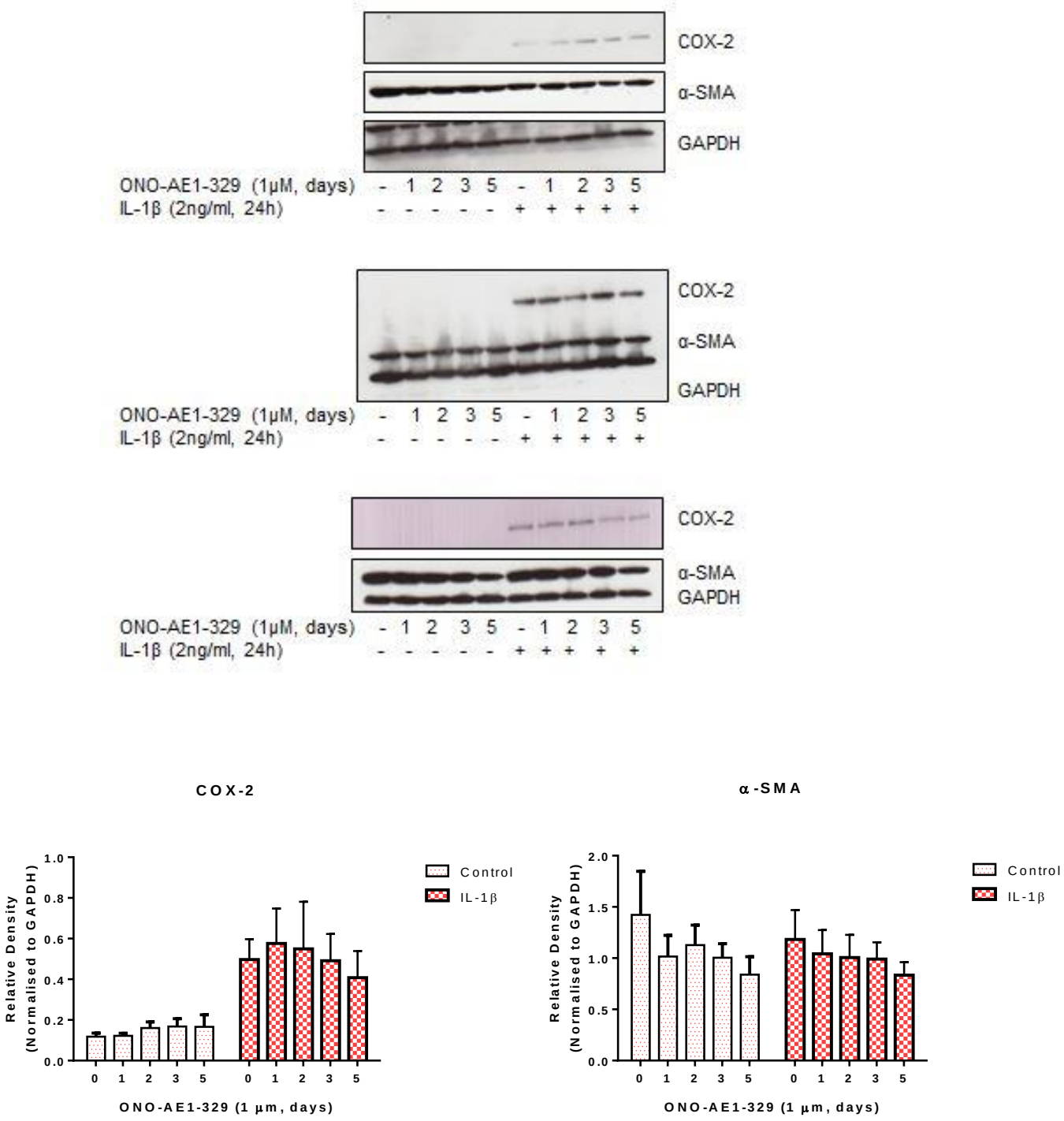


Figure 3-26 Effect of EP4 Agonist (ONO-AE1-329) Time course on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with ONO-AE1-329 (1 μM) for 0, 1, 2, 3 and 5 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments

performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

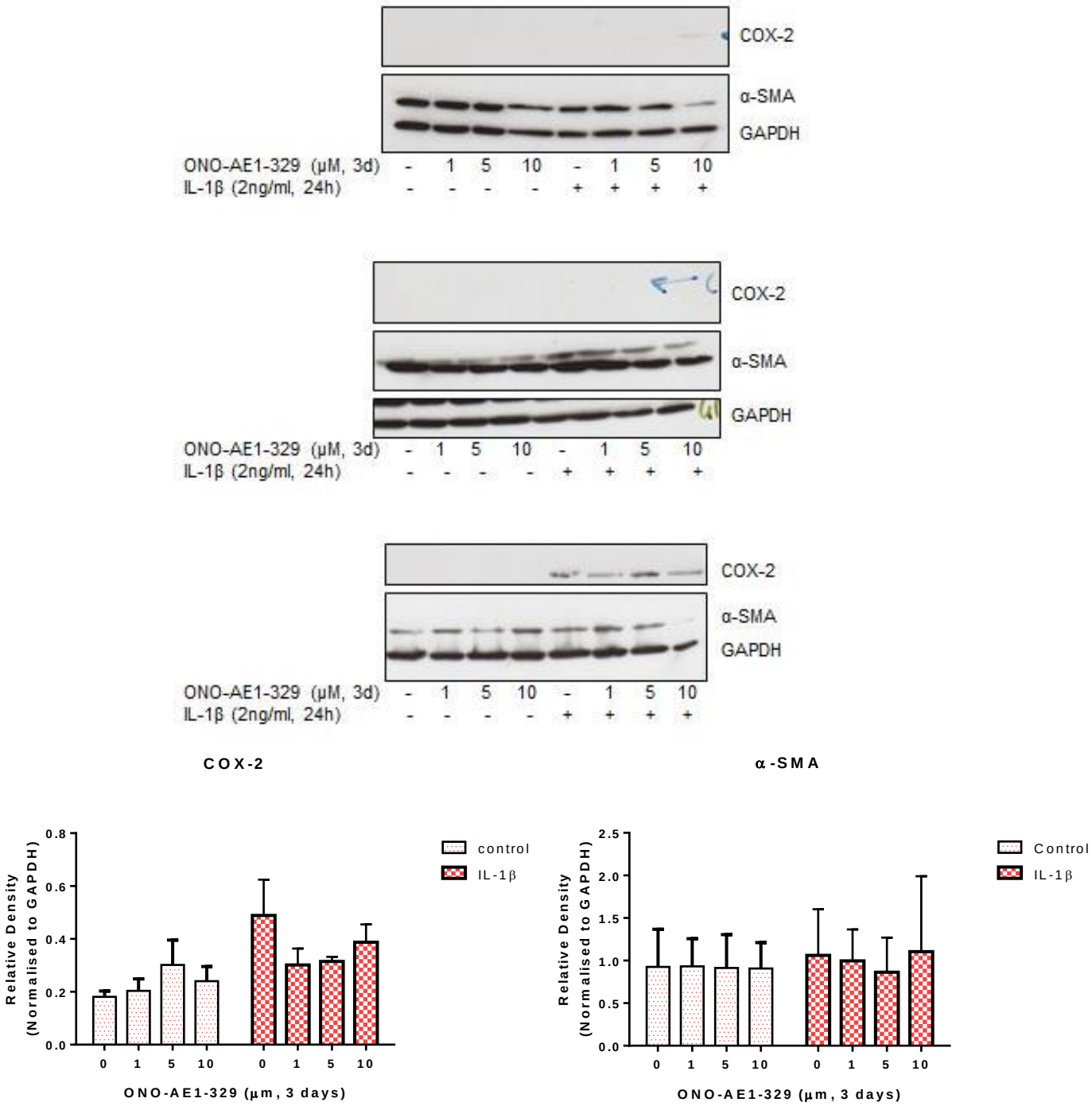


Figure 3-27 Effect of EP4 Agonist (ONO-AE1-329) Dose Response on COX-2 and -SMA Protein Expression in F-IPF

F-IPF were treated with ONO-AE1-329 at 1, 5 and 10 μM for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments

performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

3.5 Discussion

The activation and persistence of myofibroblasts is critical to extracellular matrix production and scarring associated with fibrosis and as such is a key feature in the pathogenesis of IPF and other fibrotic conditions. The pro-fibrotic effects of TGF-β1 and the opposing anti-fibrotic effects of PGE_2 have been well documented, however, the mechanism of TGF-β1-induced fibroblast to myofibroblast differentiation and how PGE_2 can reverse this phenomenon is poorly understood.

This chapter demonstrates the phenotypic differences of F-NL compared with F-IPF. F-IPF have reduced COX-2 expression, reduced PGE_2 production and increased Col 1 expression compared with F-NL. Therefore, F-IPF consists mainly of cells with a pro-fibrotic phenotype. This is supported by data published previously within the laboratory which shows reduced COX-2 mRNA in F-IPF compared with F-NL (Coward et al., 2009). The data in this thesis and previously published data from our laboratory, suggests that diminished COX-2 production in F-IPF compared with F-NL may be due to defective transcriptional mechanisms resulting in reduced COX-2 protein expression and reduced PGE_2 production. F-IPF have increased α-SMA protein compared with F-NL but mRNA expression is similar between F-NL and F-IPF. In addition, F-IPF have increased Col 1 protein expression and increased Col 1 mRNA expression compared with F-NL. These data suggest that Col 1 expression is regulated via transcriptional mechanisms where as α-SMA expression may be regulated via post-transcriptional mechanisms or due to differences in mRNA or protein degradation rates. The effect of IL-1β treatment on Col 1 protein was not assessed during this study. However, previous studies have demonstrated that IL-1β reduces basal and TGF-β-induced collagen expression in normal lung fibroblasts, partially through increases in PGE_2 (Diaz et al., 1993; Mia et al., 2014).

We have also demonstrated that TGF-β1 treated F-NL mimic the phenotype of F-IPF and as such represent an *in vitro* model to study TGF-β1-induced fibroblast to myofibroblast differentiation. This experiment was only completed in one cell line and to ensure this result is significant it should be repeated in additional cell lines. However, our data are supported by several other studies that have used TGF-β1 to induce fibroblast to myofibroblast differentiation in different cell lines (Garrison et al., 2013; Thomas et al., 2007; Togo et al., 2009). Although TGF-β1 is known to promote fibroblast to myofibroblast differentiation the effect of TGF-β1 on COX-2 expression has not yet been investigated. Our data suggests that the loss of COX-2 is associated with a pro-fibrotic phenotype and myofibroblast differentiation.

We show that exogenous PGE_2 is able to reverse fibroblast to myofibroblast differentiation due its ability to induce COX-2 and promote synthesis of endogenous PGE_2. The data suggests that the loss of COX-2 and PGE_2 may play a key role in promoting TGF-β1-induced fibroblast to myofibroblast differentiation. Our results also suggests that PGE_2 treatment may compensate for the lack of COX-2 even in the presence of TGF-β1, however, further experiments are required to confirm this observation as the experiment was only completed in two cell lines. PGE_2 can also be synthesised due to the expression of COX-1 which is constitutively expressed in many cells including fibroblasts. COX-1 expression was not measured in this study so it is possible that some of the PGE_2 production was via COX-1. Previous studies from our laboratory, using the same primary cells, demonstrated there was no difference in COX-1 expression in F-NL compared with F-IPF and COX-1 expression did not change following IL-1β or TGF-β1 treatment (Coward et al., 2009).Therefore, it seems likely that the increased PGE_2 expression seen in this experimental model is due to increased IL-1β-induced COX-2. Although not considered in our study, it is noteworthy that other mitogens such as FGF and PDGF are able to reverse fibroblast to myofibroblast differentiation and are also capable of inducing COX-2 expression (Scotton and Chambers, 2007). This

supports our hypothesis that increasing COX-2 expression and endogenous PGE_2 production promotes and maintains an anti-fibrotic phenotype in fibroblasts.

PGE_2 treatment increased COX-2 production allowing the synthesis of endogenous PGE_2 and simultaneously reversing fibroblast to myofibroblast differentiation as denoted by decreased α-SMA. To verify that the effect of PGE_2 represented true reversal of myofibroblast differentiation, and was not limited to α-SMA, we also analysed Col 1 expression, the predominant matrix protein that is upregulated in myofibroblasts. Treatment with PGE_2 reduced Col 1 mRNA but did not decrease Col 1 protein. There are several other studies which demonstrate a reduction in Col 1 following PGE_2 treatment (Fine et al., 1989; Garrison et al., 2013; Huang et al., 2007). Huang and colleagues (2008) have previously demonstrated that fibroblasts derived from IPF patients exhibit a variable degree of resistance to the anti-fibrotic actions of PGE_2 in respect to Col 1 expression and proliferation. In contrast, none of the F-IPF cell lines used in this study were seen to be resistant to PGE_2 treatment with regards to α-SMA and COX-2 expression. Fibroblasts used by Huang and colleagues (2008) showed no difference in collagen production compared with normal controls. Usual interstitial pneumonia (UIP) fibroblasts were treated with PGE_2 for 18 hours. Control fibroblast exhibited a dose dependent inhibition of Col 1 with a maximal response at 500 nM. In contrast, UIP fibroblasts showed inhibition only at 1000 nM. In this study, we treated F-IPF with 1 μM PGE_2 for 3 days, a reduction in Col 1 may have been seen after a shorter time period, as such a time course and dose response with PGE_2 treatment should be completed to confirm the effect of PGE_2 on Col 1 protein expression. Furthermore, the effect of IL-1β on Col 1 expression was not investigated. It may be that Col 1 would have been reduced with PGE_2 and IL-1β treatment due to increased COX-2 expression.

PGE_2 treatment reversed fibroblast to myofibroblast differentiation but may also be able to prevent TGF-β1-induced COX-2 repression, reduction of endogenous PGE_2 production and fibroblast to myofibroblast differentiation. During TGF-β1-induced fibroblast to myofibroblast differentiation PGE_2 production is reduced. This is likely due to a reduction of COX-2 but further experiments are required to confirm this. PGE_2 may be able to inhibit TGF-β1-induced effects but this experiment was only completed in two cell lines and the results demonstrated an incomplete effect.

Our data also demonstrated that phenotypic changes caused by TGF-β1 treatment were sustained even after the removal of TGF-β1. In our system, TGF-β1-induced repression of COX-2 was maintained for up to 5 days after the removal of TGF-β1. In addition, α-SMA expression remained elevated up to 5 days after the removal of TGF-β1. This is in contrast to the results by Vaughan et al. (2000) who studied human myofibroblasts derived from the patients with Dupuytren's disease, a contractive condition of the hand caused by fibrosis in the surrounding connective tissue. In this study removal of TGF-β1 resulted in reduced expression of α-SMA. However, our data is supported by Garrison et al. (2013) who demonstrated that TGF-β1-treated IMR-90 foetal lung fibroblasts had increased α-SMA which persisted through to 5 days after the removal of TGF-β1. The fibroblast response to TGF-β1 may therefore be dependent on the source of origin or due to the differences in growth medium used. Our data suggests that fibroblast to myofibroblast differentiation results in terminally differentiated myofibroblasts in our experimental protocol.

We investigated which EP receptor was responsible for PGE_2 signalling using EP-selective agonists, both of these compounds are full agonists, ONO-AE1-259 is a selective EP2 agonist with a binding affinity of 0.08 μM and ONO-AE1-329 is a selective EP4 agonist with a binding affinity of 0.0097 μM in CHO cells (Suzawa et al., 2000). Neither the EP2 agonist nor the EP4

agonist induce COX-2 or inhibited α-SMA. Based on previously published data, evidence suggests that the EP2 receptor is the predominant receptor mediating the effects of PGE_2 (Huang et al., 2007; White, 2008). To confirm this observation and ensure the effects were not due to the inefficiacy of the agonists, these experiments should be repeated using selective receptor inhibitors or knock out cells.

In summary, PGE_2 is a key anti-fibrotic mediator and PGE_2 promotes an anti-fibrotic phenotype. The loss of PGE_2, due to COX-2 repression, is associated with fibroblast to myofibroblast differentiation. These data suggest that COX-2/PGE_2 is protective against fibroblast to myofibroblast differentiation and that exogenous PGE_2 treatment can compensate for the endogenous loss of PGE_2. If EP2 is the predominant receptor by which PGE_2 signals it is possible that other pharmacological agents or endogenous mediators that activate the EP2/cAMP pathway may offer a potentially attractive target for therapeutic drug development in IPF.

4 THE EFFECT OF THE CAMP ELEVATING AGENTS ON FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION

4.1 Introduction

PGE_2 signals via four distinct EP receptors (EP1 – EP4). The EP receptors are a family of G protein-coupled receptors. G proteins are membrane bound receptors that have several downstream substrates. EP1 signals through $G\alpha_q$, to increase Ca^{2+}; EP2 and EP4 signal via $G\alpha_s$, to increase cAMP and EP3 predominantly signals via $G\alpha_i$, to decrease cAMP (Breyer et al., 2001).

The anti-fibrotic effects of PGE_2 on COX-2, α-SMA and Col 1 are mediated via the EP2 receptor which is the most abundant out of the four EP receptors expressed in human lung fibroblasts (Huang et al., 2008b). Stimulation of the EP2 receptor activates adenylyl cyclase, which in turn catalyses the conversion of ATP into cAMP. Therefore, cAMP could be a key regulator of COX-2 expression and fibroblast to myofibroblast differentiation. This is further supported by previous studies in WI-38 cells, human foetal lung fibroblasts, and normal human lung fibroblasts in which agents that increase cAMP production inhibited various fibroblast functions including proliferation and collagen synthesis (Huang et al., 2007; Liu et al., 2004). Furthermore, administration of prostacyclin, a prostanoid that increases cAMP via the IP receptor, or aminophylline, which increases cAMP via inhibition of phosphodiesterases, attenuates bleomycin-induced pulmonary fibrosis in mice (Lindenschmidt and Witschi, 1985; Murakami et al., 2006). Thus, agents that increase cAMP production are anti-fibrotic and may have therapeutic potential. The effect of cAMP elevating agents on COX-2 induction and PGE_2 synthesis has not yet been investigated. The signalling events downstream of cAMP responsible for inhibiting fibroblast functions, particularly in fibroblasts derived from IPF patients, are poorly understood. One of the benefits of studying cAMP elevating agents is that

several cAMP elevating agents are already administered clinically and as such have known properties, acceptable safety profiles and reasonable costs. For example, β_2-agonists are regularly used in the treatment of bronchial asthma, in addition, Roflumilast, a phosphodiesterase inhibitor, is used for the treatment of COPD. Whether or not the effect of these drugs is confined to bronchodilation or whether they can exert additional anti-fibrotic effects requires further investigation. PGE_2 is commonly considered a pro-inflammatory mediator and is actively involved in the pathogenesis of periodontitis, rheumatoid arthritis and cancer growth. Therefore, if cAMP elevating agents that are already used clinically, such as β_2-agonists, can mimic the anti-fibrotic effects of PGE_2 but without eliciting proinflammatory side effects this would be an extremely promising therapeutic strategy for patients with IPF.

Understanding the signalling pathway downstream of cAMP may also provide novel therapeutic targets. PKA is the traditional effector of cAMP and is responsible for a myriad of cell-type specific effects, such as, cell growth and proliferation (Stork and Schmitt, 2002). However, more recently Epac has been identified as a novel cAMP effector (de Rooij et al., 1998). Signalling through PKA and Epac have been shown to elicit separate (Wang et al., 2006), synergistic (Christensen et al., 2003) or even antagonist (Mei et al., 2002) effects on cellular function. The role of PKA and Epac in lung fibroblasts has received little attention and the role of PKA and Epac in regulating COX-2 expression is unknown.

4.2 Aims

Within this chapter we sought to confirm if PGE_2 induced cAMP via the EP2 receptor in lung fibroblasts. We also wanted to investigate the effect of other cAMP elevating agents, such as the β_2-agonists, Formoterol (Form) and Salmeterol (Salme), and Forskolin (FSK), a direct adenylyl cyclase activator, on COX-2 expression, PGE_2 production and their ability to both prevent and reverse fibroblast to myofibroblast differentiation with regards to α-SMA and Col 1 expression. To further support that cAMP is involved in the regulation of COX-2 we assessed the effect of Roflumilast, a phosphodiesterase 4 inhibitor, in F-IPF. In addition, we investigated the signalling transduction pathway downstream of cAMP using selective PKA and Epac agonists to determine if COX-2 expression is controlled by PKA and/or Epac activation.

4.3 Experimental Protocol

F-NL and F-IPF were cultured to confluence in 6 well culture plates, medium was changed every 48 hours and confluent cells were growth arrested in serum free medium for 24 hours prior to all experiments. To measure COX-2 protein and mRNA expression cells were treated with 2 ng/ml IL-1β for 24 hours and 4 hours, respectively. After 3 days of treatment, the culture medium was collected and stored at -20 ^{o}C until the determination of $PGE_{2,}$ or cells were subject to protein extraction for Western blot analysis as described in Chapter 2. To test the inhibition of various compounds on TGF-β1-induced fibroblast to myofibroblast differentiation, F-NL were pre-treated for 1 hour with cAMP elevating agents, Form, a long acting β_2-agonist, and FSK, a direct adenylyl cyclase activator, prior to the addition of 2 ng/ml TGF-β1 for 3 days.

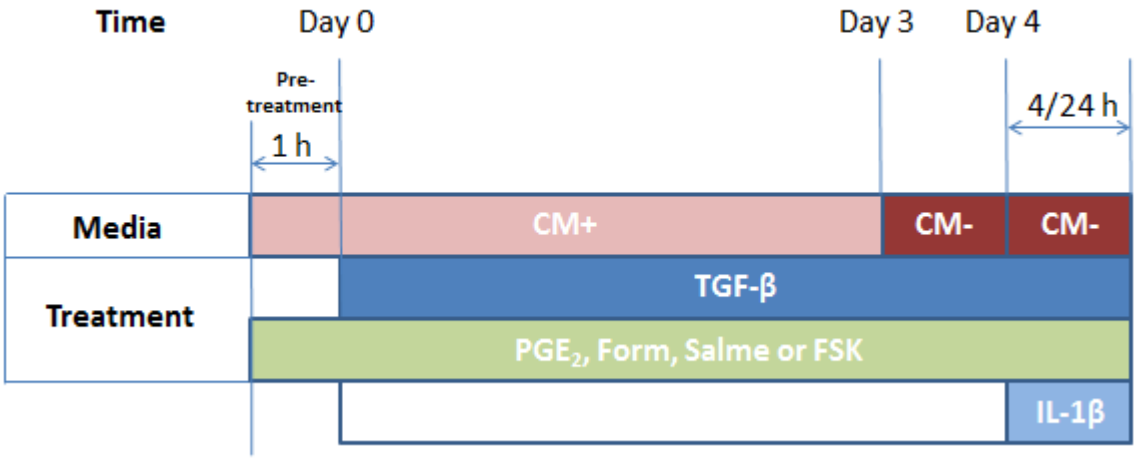


Figure 4-1 Experimental Protocol to test if cAMP elevating agents (PGE_2, Form, Salme and FSK) can prevent pro-fibrotic effects of TGF-β.

Schematic diagram of the timelines used for experimental protocol showing pre-treatment, treatment IL-1ß stimulation and whether serum (CM^+) or serum free (CM^-) media was used.

To determine cAMP production cells were cultured to confluence in 12 well culture plates, growth arrested in serum free medium for 24 hours and pre-treated with 1 μM IBMX for 30

minutes prior to all experiments. To determine which EP receptor mediates PGE_2 signalling EP2 and EP4 selective antagonists were incubated at the indicated concentrations for 30 minutes in the presence of IBMX prior to the addition of PGE_2 for 30 minutes. In order to determine the effect of Roflumilast cells were pre-treated with 1 µM Roflumilast, without IBMX, for 30 minutes, prior to the addition of PGE_2, Form and FSK for 30 minutes. After treatment at the indicated times the culture media was collected and cAMP was determined as described previously in Chapter 2.

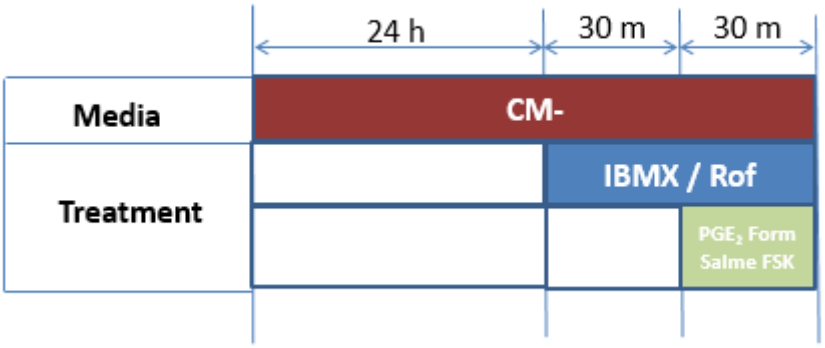


Figure 4-2 Experimental Protocol to test the effect of PGE_2, Form, Salme and FSK on cAMP Production.

Schematic diagram of the timelines used for experimental protocol showing pre-treatment, treatment IL-1ß stimulation and whether serum (CM^+) or serum free (CM^-) media was used.

4.4 Results

4.4.1 cAMP Production is comparable in F-NL and F-IPF

Previous studies in foetal lung fibroblasts and normal lung fibroblasts have demonstrated that PGE_2 inhibits proliferation and collagen production via EP2 activation and increased cAMP (Huang et al., 2007; Kolodsick et al., 2003; Liu et al., 2004). Whether PGE_2 signals via EP2 and cAMP, or via another EP receptor, to regulate COX-2 expression is unknown. Based on previous studies and results already discussed in this thesis we hypothesised that PGE_2-induced COX-2 expression would correlate with cAMP production.

To confirm if PGE_2 treatment increases cAMP production, and to determine if there is any difference in cAMP production between F-NL and F-IPF, we measured cAMP following PGE_2 treatment (Figure 4-3). PGE_2 was used at the same concentration that was effective at inducing COX-2 protein (1 µM) (Figure 3-10). PGE_2 treatment increased cAMP production in F-NL and F-IPF and cAMP levels peaked at 30 minutes. Therefore, 30 minutes treatment was used for all subsequent experiments. Across all time points tested there was no significant difference in cAMP production between F-NL and F-PF. This suggests that although F-IPF have repressed COX-2 and diminished PGE_2 production this is not due to a defective EP2/cAMP signalling cascade.

We next confirmed if formoterol (Form), a long acting β_2-agonist, and forskolin (FSK), a direct adenylyl cyclase activator, were also able to induce cAMP in F-NL and F-IPF. Both Form and FSK significantly increased cAMP in F-NL and F-IPF (Figure 4-4). Form and FSK treatment resulted in markedly less cAMP production compared with PGE_2 treatment and FSK induced the least amount of cAMP. The cAMP increase, following all three treatments, was similar in F-NL and F-IPF further confirming our suggestion that the lack of PGE_2 in F-IPF is not due to

a defective EP2/cAMP signalling pathway in F-IPF and instead that the lack of PGE_2 contributes to the pro-fibrotic phenotype.

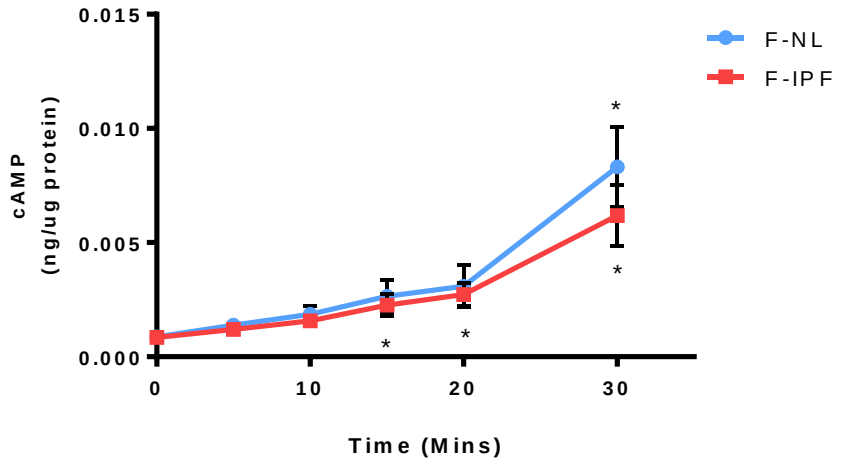


Figure 4-3 Effect of PGE_2 on cAMP Production in F-NL and F-IPF (Time Course).

*Confluent F-NL and F-IPF were serum-starved for 24 hours and treated with IBMX (1 mM) and PGE_2 (1 µM) for 0, 5, 10, 15, 20 and 30 minutes. To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from separate experiments performed in duplicate of four different cell lines and is expressed as mean ±SEM. *p<0.05 compared with untreated control.*

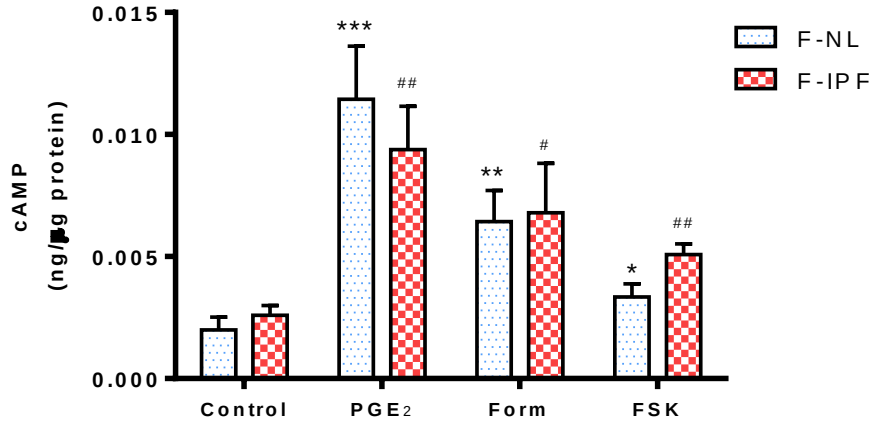


Figure 4-4 Effect of PGE_2, Form and FSK on cAMP Production in F-NL and F-IPF

*Confluent F-NL and F-IPF were serum starved for 24 hours and treated for 30 minutes with either PGE_2 (1 µM), Form (10 µM) or FSK (10 µM). To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.005 compared to F-NL control; #p<0.05, ##p<0.01, compared to F-IPF control.*

4.4.2 cAMP Elevating Agents Reverse the Fibrotic Phenotype in F-IPF

To determine if increased cAMP mimicked the effects of PGE_2 we investigated whether other cAMP elevating agents were also able to induce COX-2 expression in F-IPF. Salmeterol (Salme), a long acting β_2-agonist, Form and FSK increased IL-1β induced-COX-2 expression in F-IPF (Figure 4-5, Figure 4-6, and Figure 4-7 respectively). Form, Salme and FSK had no effect on α-SMA. Collagen expression was significantly reduced following treatment with Form and FSK (Figure 4-8). These data imply that PGE_2 increases IL-1β-induced COX-2 expression via increases in cAMP.

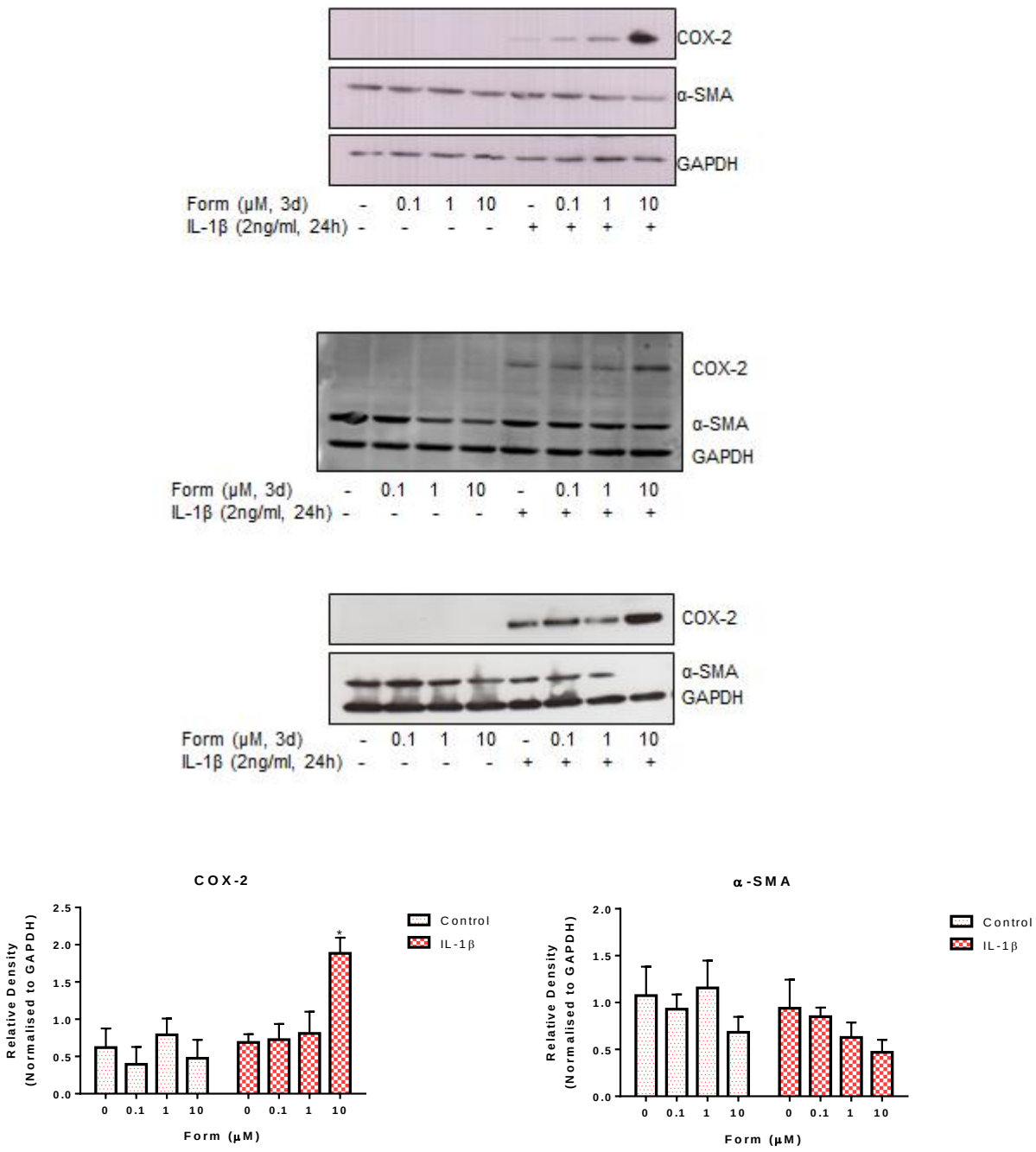


Figure 4-5 Effect of Form on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with Formoterol (Form) for 3 days at 0.1, 1 and 10 μM, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows data from three separate experiments performed in different cell lines. Relative density was calculated by normalising

*the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with IL-1β-treated control.*

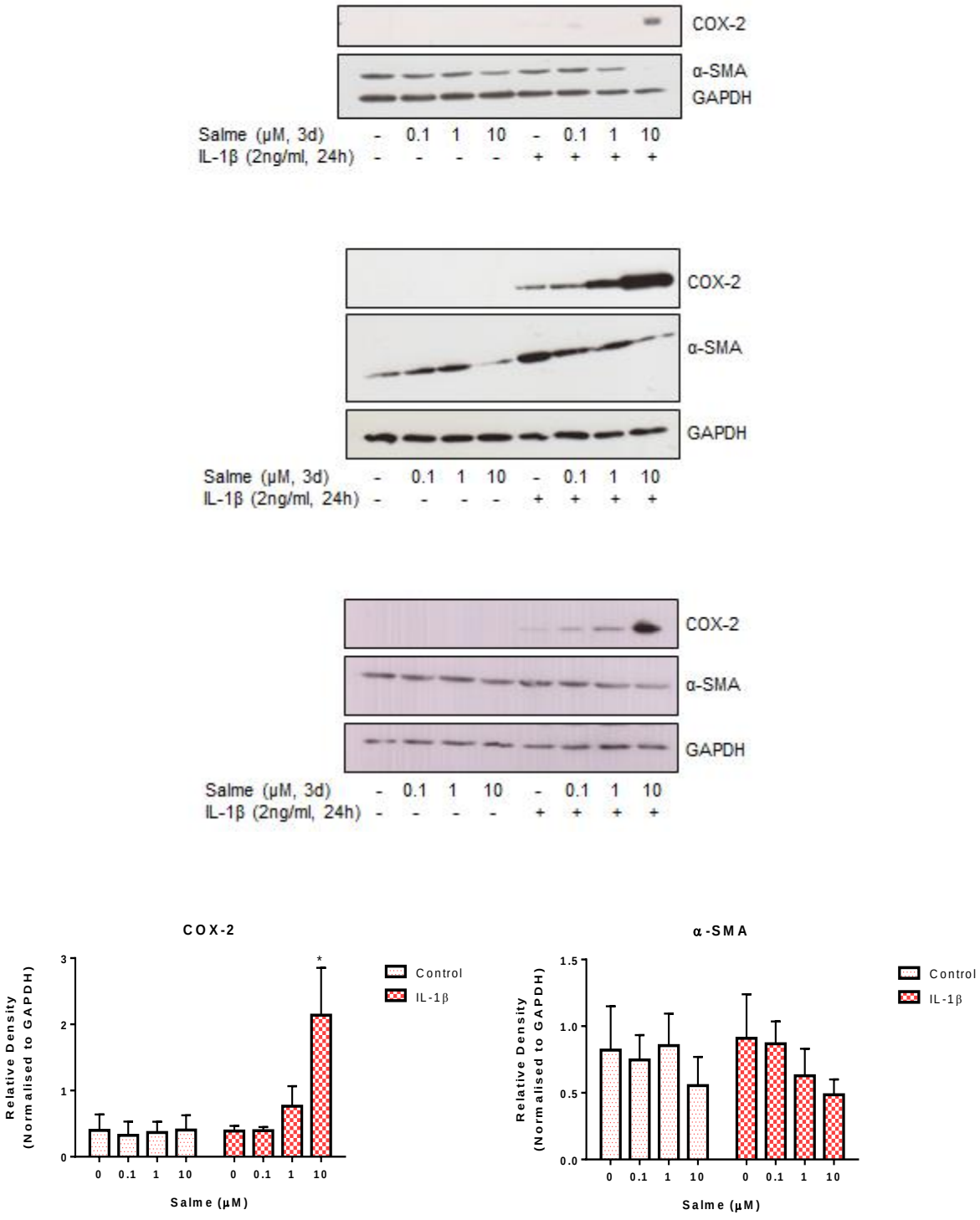


Figure 4-6 Effect of Salme on COX-2 and α-SMA Protein Expression F-IPF

F-IPF were treated with Salmeterol (Salme) for 3 days at 0.1, 1 and 10 μM, serum starved for 24 hours and left unstimulated or stimulated with IL-1ß (2 ng/ml, 24h) prior to the collection of

*total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with IL-1β-treated control*

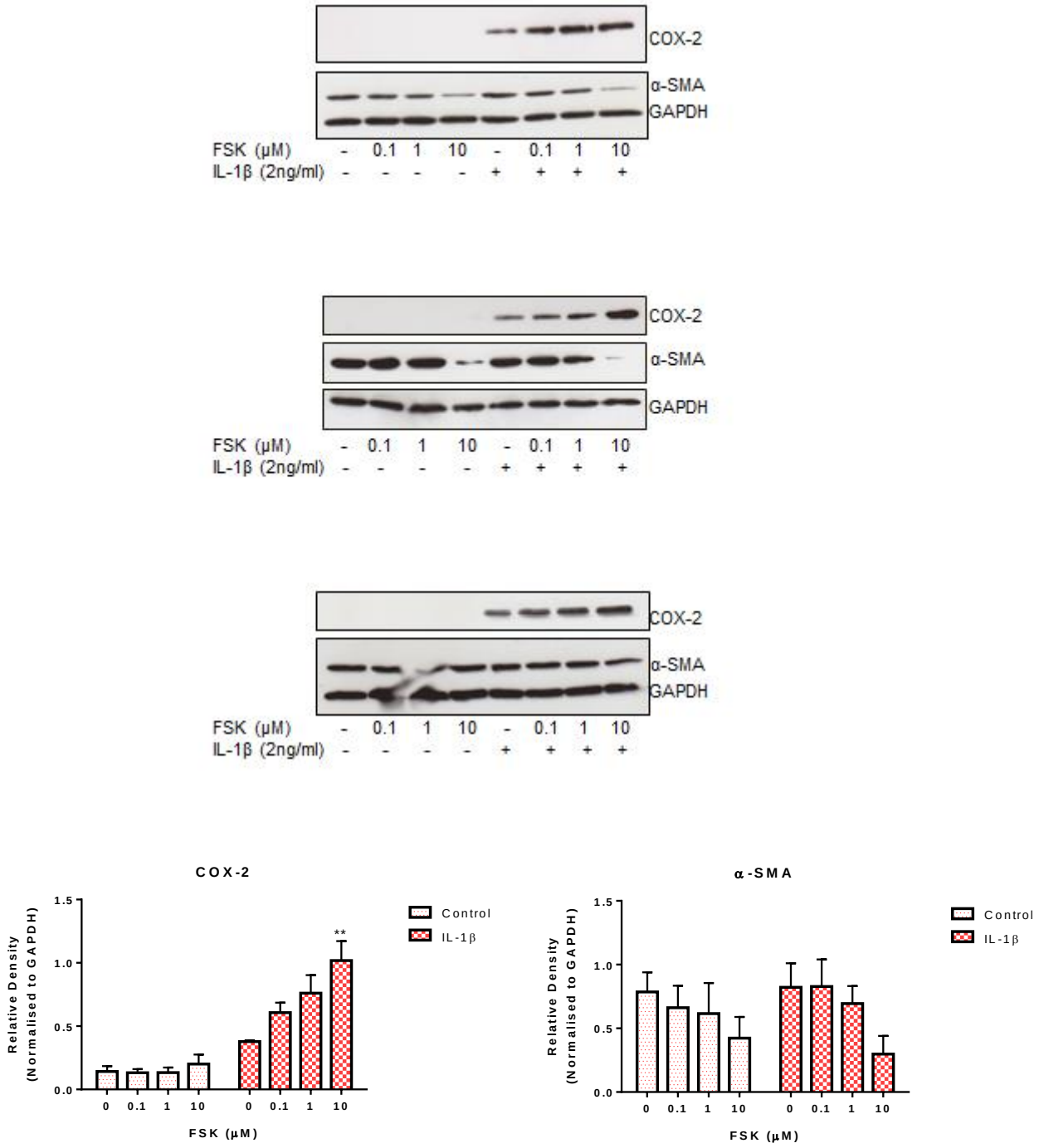


Figure 4-7 Effect of FSK on COX-2 and α-SMA Expression in F-IPF

F-IPF were treated with Forskolin (FSK) for 3 days at 0.1, 1 and 10 μM, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments

*performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. **p<0.01 compared with IL-1β-treated control.*

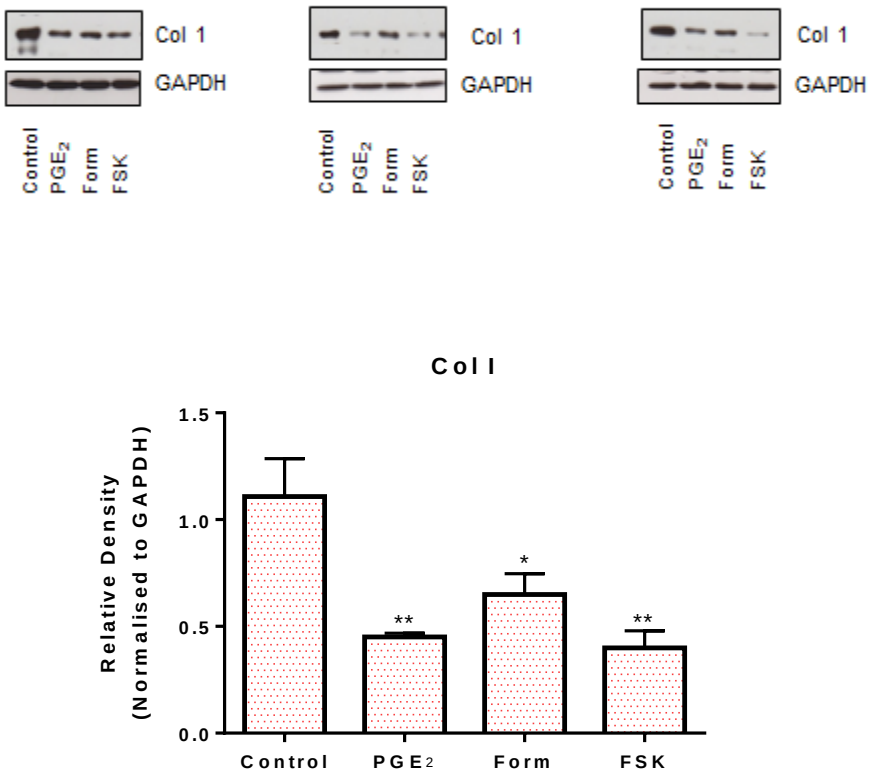


Figure 4-8 Effect of PGE_2, Form and FSK on Col 1 Protein Expression in F-IPF

*F-IPF were treated with either PGE_2, Form or FSK (10 µM, 3d) and serum starved for 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. **p<0.01 compared with control.*

Finally, we wanted to confirm that Form- and FSK-induced COX-2 expression was functional and corresponded to increased PGE_2 production. Form and FSK increased IL-1β-induced PGE_2 production in F-IPF compared with IL-1β alone (Figure 4-9). Collectively, these data confirm that Form and FSK increase cAMP, resulting in increased IL-1β-induced COX-2 expression and subsequent PGE_2 production. Interestingly, FSK treatment resulted in the production of the most PGE_2 (Figure 4-9) but induced the least amount of cAMP (Figure 4-4). This suggests that the levels of cAMP do not always correspond to the functional read out.

Overall, these data support our findings from chapter 3 and demonstrate that the anti-fibrotic effects of PGE_2 are via increased cAMP production, increased COX-2 expression and subsequent PGE_2 production. Therefore, cAMP elevating agents mimic the effect of exogenous PGE_2. These data suggest that the use of β_2-agonists, or cAMP elevating agents, may have anti-fibrotic effects *in vivo* and may be an attractive alternative treatment to PGE_2.

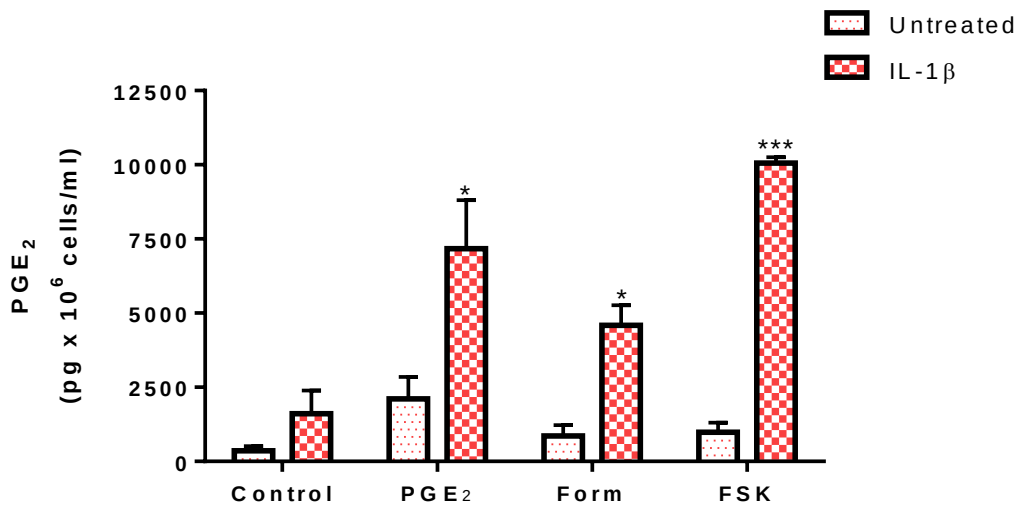


Figure 4-9 Effect of PGE_2, Form and FSK on Endogenous PGE_2 Production in F-IPF

*F-IPF were pre-treated with either PGE_2 (1 μM), Form (10 μM) and FSK (10 μM) for 3 days, washed in serum free media, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of cell culture media for analysis by PGE_2 EIA. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean ± SEM. *p<0.05, ***p<0.005, compared with IL-1β treated control.*

As we have demonstrated that cAMP elevating agents, Form, Salme and FSK, increased IL-1β-induced COX-2 expression we next analysed the effect of cAMP elevating agents on mRNA. For these experiments we used FSK as the cAMP elevating agent, as FSK is a direct adenylyl cyclase inhibitor this will avoid any possible non-cAMP effects caused by Form or Salme. FSK treatment alone and with IL-1β treatment had no effect on COX-2 mRNA expression (Figure 4-10). We next sought to determine if FSK regulated α-SMA and Col 1 mRNA expression. FSK had no effect on α-SMA mRNA expression in F-IPF (Figure 4-11A) but significantly reduced Col 1 mRNA expression to a level comparable with PGE_2 treatment in F-IPF (Figure 4-11B). These data suggest that cAMP elevating agents increase IL-1β-induced COX-2 expression but expression is not regulated via transcriptional mechanisms. In contrast, increases in cAMP may effect the transcriptional regulation Col 1 expression.

Overall, these data demonstrate that PGE_2, via cAMP production, induces COX-2 and subsequent production of endogenous PGE_2. Thus, cAMP elevating agents may be able to compensate for the lack of endogenous PGE_2 in F-IPF, promote an anti-fibrotic phenotype and reverse fibroblast to myofibroblast differentiation.

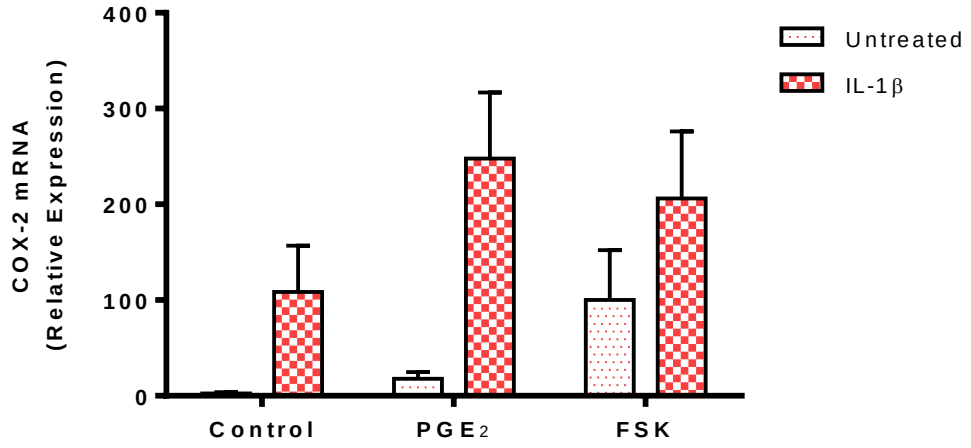


Figure 4-10 Effect of PGE_2 and FSK on COX-2 mRNA Expression in F-IPF

F-IPF were treated with either PGE_2 (1 μM) or FSK (10 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for qPCR analysis. This data is the amalgamation of separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM

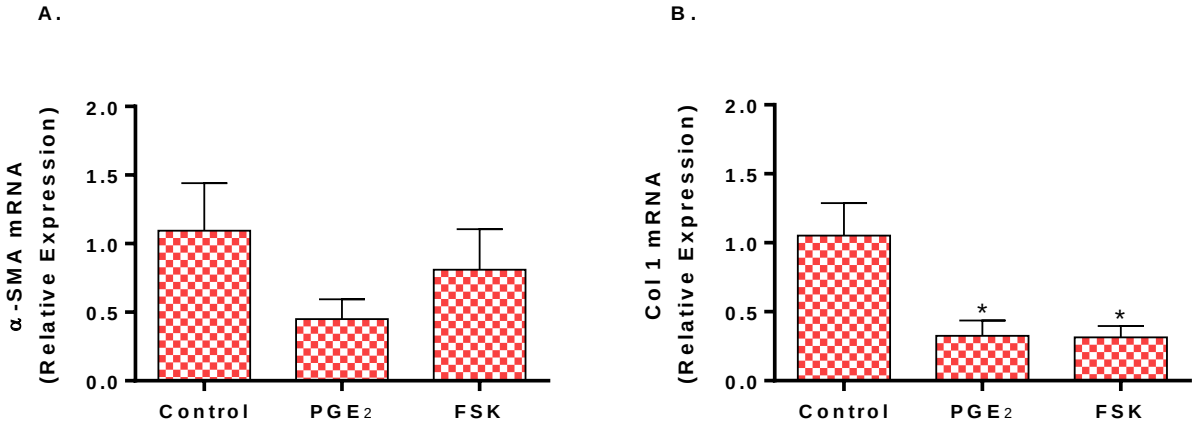


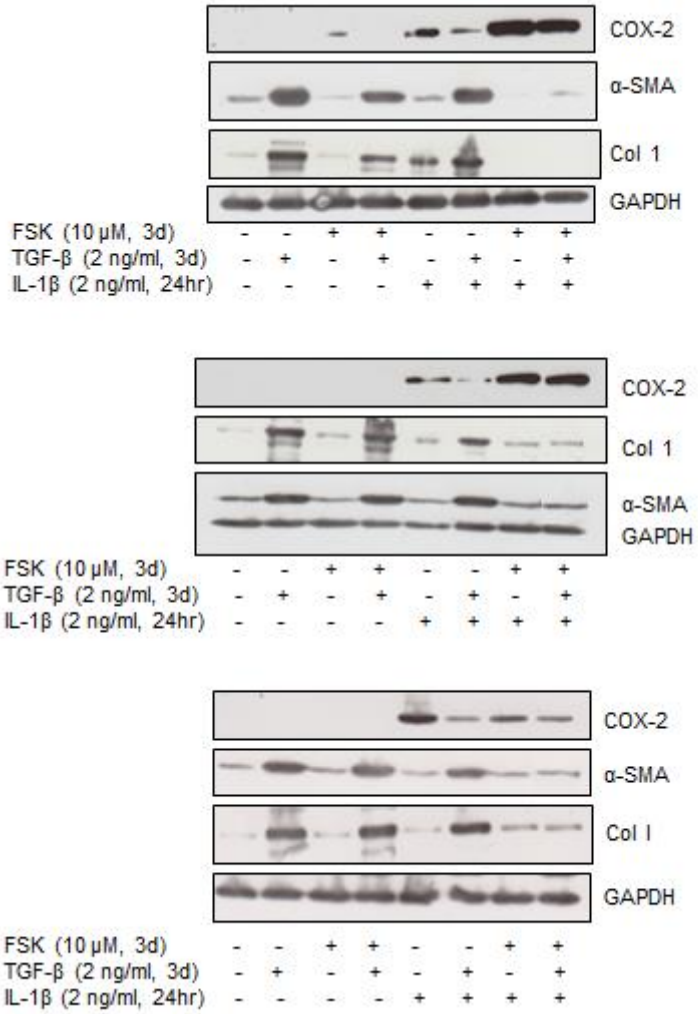
Figure 4-11 Effect of PGE_2 and FSK on α-SMA and Col 1 mRNA in F-IPF

*F-IPF were treated with either PGE_2 (1 μM) or FSK (10 μM) for 24 hours and serum starved for 24 hours prior to the collection of total RNA for qPCR analysis of (A) α-SMA and (B) Col 1. This figure is the amalgamation of separate experiments performed in duplicate of three different cell lines and is expressed as mean ± SEM. *p<0.05, compared to untreated control.*

4.4.3 cAMP Elevating Agents Prevent the Pro-fibrotic Effects of TGF-β1

Our data so far has demonstrated that PGE_2, via increased cAMP production, increases IL-1β-induced COX-2 expression, compensating for the lack of endogenous PGE_2 production and promoting an anti-fibrotic phenotype in F-IPF. Our results suggest that during TGF-β1-induced fibroblast to myofibroblast differentiation COX-2 expression is gradually repressed (Figure 3-15). We next assessed if cAMP elevating agents can prevent the loss of COX-2 during TGF-β1-induced fibroblast to myofibroblast differentiation. FSK treatment alone had no effect on COX-2 or IL-1β-induced COX-2 expression. However, FSK with IL-1β treatment was able to prevent TGF-β-induced reduction of COX-2 (Figure 4-12). Without IL-1β stimulation FSK treatment had no effect on TGF-β1-induced α-SMA and Col 1. With IL-1β stimulation FSK treatment prevented TGF-β1-induced α-SMA and Col 1 expression (Figure 4-12). This implies that without IL-1β stimulation there is insufficient COX-2 to prevent the pro-fibrotic effects of TGF-β1.

These data suggest that FSK treatment prevents TGF-β1-induced repression of IL-1β-induced COX-2 thus maintaining endogenous PGE_2 levels and preventing fibroblast to myofibroblast differentiation. This further supports our hypothesis that COX-2 expression is necessary to maintain an anti-fibrotic phenotype. We have shown that FSK produced less cAMP compared with PGE_2 (Figure 4-4) but is able to both prevent and reverse fibroblast to myofibroblast differentiation (Figure 4-12). Therefore, the amount of cAMP may not always correlate with the efficiency to promote an anti-fibrotic phenotype.


COX-2
α-SMA
Col 1
GAPDH
FSK (10 μM, 3d) - - + + - - + +
TGF-β (2 ng/ml, 3d) - + - + - + - +
IL-1β (2 ng/ml, 24hr) - - - - + + + +
COX-2
Col 1
α-SMA
GAPDH
FSK (10 μM, 3d) - - + + - - + +
TGF-β (2 ng/ml, 3d) - + - + - + - +
IL-1β (2 ng/ml, 24hr) - - - - + + + +
COX-2
α-SMA
Col I
GAPDH
FSK (10 μM, 3d) - - + + - - + +
TGF-β (2 ng/ml, 3d) - + - + - + - +
IL-1β (2 ng/ml, 24hr) - - - - + + + +

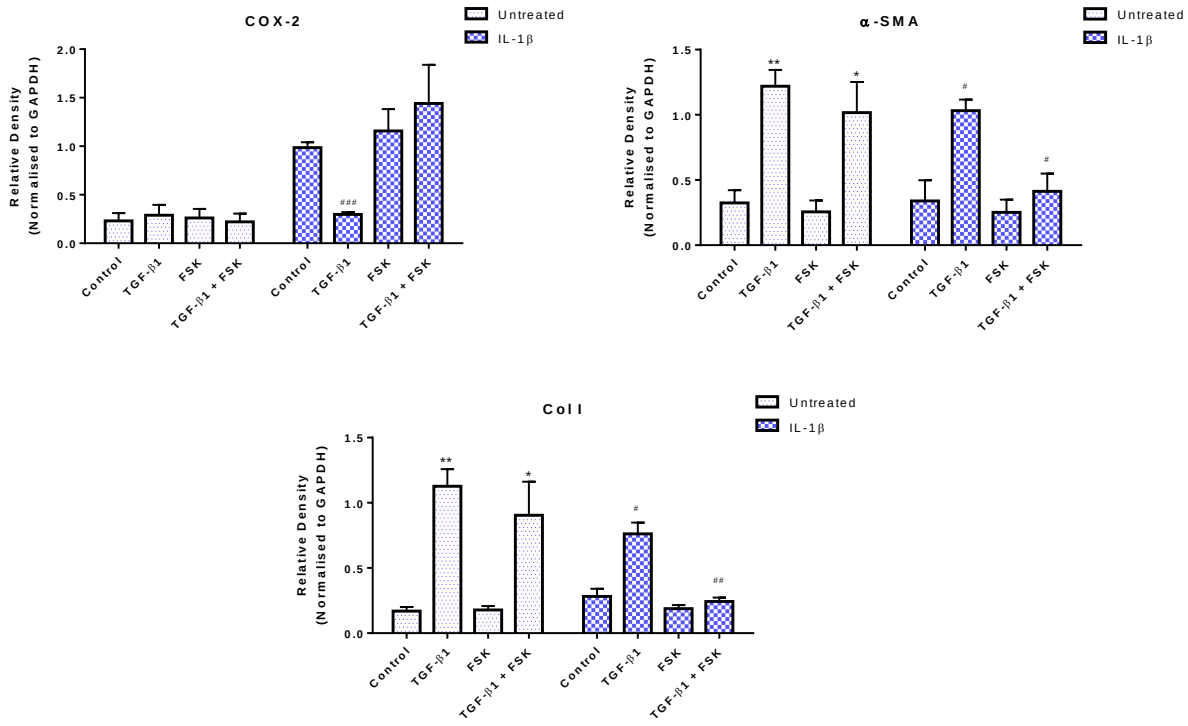


Figure 4-12 Effect of FSK on TGF-β1-induced Fibroblast to Myofibroblast Differentiation

*F-NL were treated with TGF-β1 (2 ng/ml) alone, FSK (10 μM) alone or TGF-β1 (2 ng/ml) and FSK (10 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24h) prior to the collection of total cell lysate for analysis by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2, α-SMA and Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01 compared with untreated control, #p<0.05, ##p<0.01, compared with IL-1β-treated control.*

To determine if changes in COX-2, α-SMA and Col 1 protein were mirrored by changes in mRNA, real-time RT-PCR was also performed. FSK treatment alone had no effect on COX-2 mRNA or IL-1β-induced COX-2 mRNA. FSK and TGF-β1 treatment increased IL-1β-induced COX-2 compared with TGF-β1 alone. These data demonstrate that FSK can prevent TGF-β-induced COX-2 repression (Figure 4-13). FSK treatment also reduced α-SMA and Col 1 mRNA expression compared with control. Furthermore, FSK treatment prevented TGF-β1-induced α-SMA mRNA expression (Figure 4-14A) and prevented TGF-β1-induced Col 1 mRNA (Figure 4-14B).

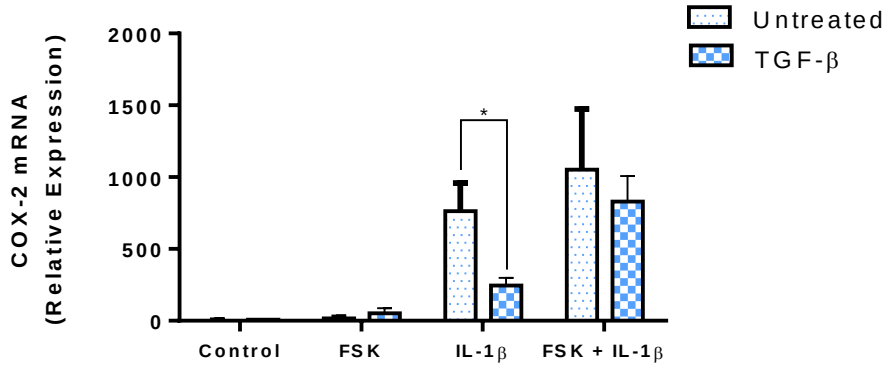


Figure 4-13 Effect of FSK on COX-2 mRNA Expression in TGF-β1-treated F-NL

*F-NL were treated with TGF-β1 (2 ng/ml) alone, FSK alone (10 μM), or TGF-β1 (2 ng/ml) and FSK (10 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for analysis by qPCR. This data is the amalgamation of three separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM. *p<0.05, compared with IL-1β treated control.*

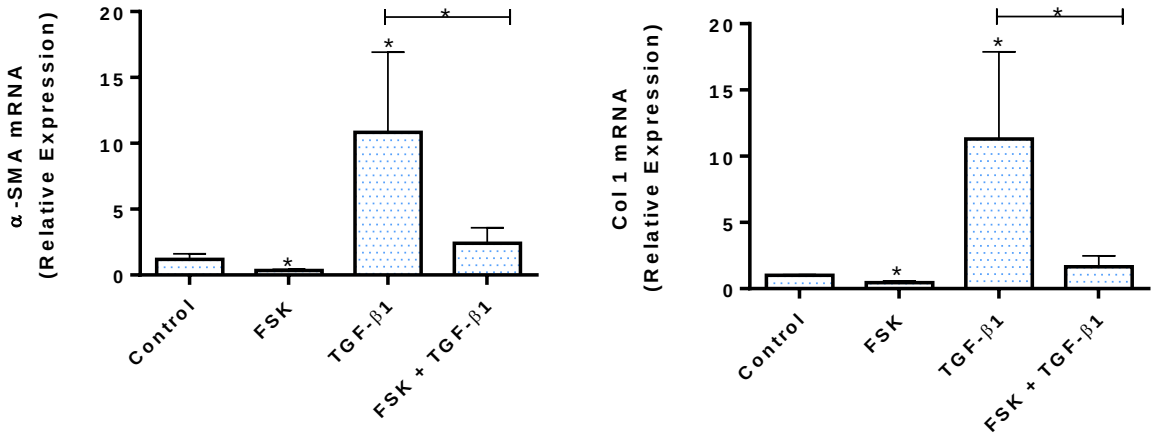


Figure 4-14 Effect of FSK on α-SMA and Col 1 mRNA in TGF-β1-treated F-NL

*F-NL were treated with TGF-β1 (2 ng/ml) alone, FSK alone (10 μM) and TGF-β1 (2 ng/ml) and FSK (10 μM) for 24 hours and serum starved for 24 hours prior to the collection of total cell lysate for analysis by qPCR of (A) α-SMA and (B) Col 1. This data is the amalgamation of three separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM. *p<0.05, compared with untreated control.*

4.4.4 The Anti-fibrotic Effects of PGE_2 are via the EP2 Receptor

The biological effects of PGE_2 are mediated via four EP receptors. Two of these receptors, EP2 and EP4, activate adenylyl cyclase resulting in increased cAMP production. Our experimental results demonstrate that PGE_2 increases cAMP production in F-IPF and cAMP levels were similar in both F-NL and F-IPF (Figure 4-3 and Figure 4-4). We next confirmed if PGE_2 signals via the EP2 or EP4 receptor to increase levels of cAMP and examined the ability of selective EP2 and EP4 antagonists to inhibit PGE_2-induced cAMP production. F-NL and F-IPF were treated with AH6809, an EP2 receptor antagonist, and ONO-AE2-227, a selective EP4 receptor antagonist (Figure 4-15). AH6809 alone had no effect on cAMP production but caused a dose-dependent decrease in PGE_2-induced cAMP production in F-NL and F-IPF (Figure 4-15A and Figure 4-15C, respectively). At 100 µM AH6809 significantly inhibited of PGE_2-induced cAMP production and reduced cAMP levels comparable to basal. In contrast, ONO-AE2-227 had no effect alone on cAMP production and did not inhibit PGE_2-induced cAMP production in F-NL or F-IPF (Figure 4-15B) and Figure 4-15D, respectively). Therefore, as the EP2 antagonist AH6809 inhibited PGE_2-induced cAMP production, but the EP4 antagonist ONO-AE2-227 had no effect, this supports our previous data that EP2, and not EP4, has a dominant role in mediating the effects of PGE_2 on COX-2 expression. Thus, compounds that activate the EP2 receptor should be able to reverse fibroblast to myofibroblast differentiation in F-IPF.

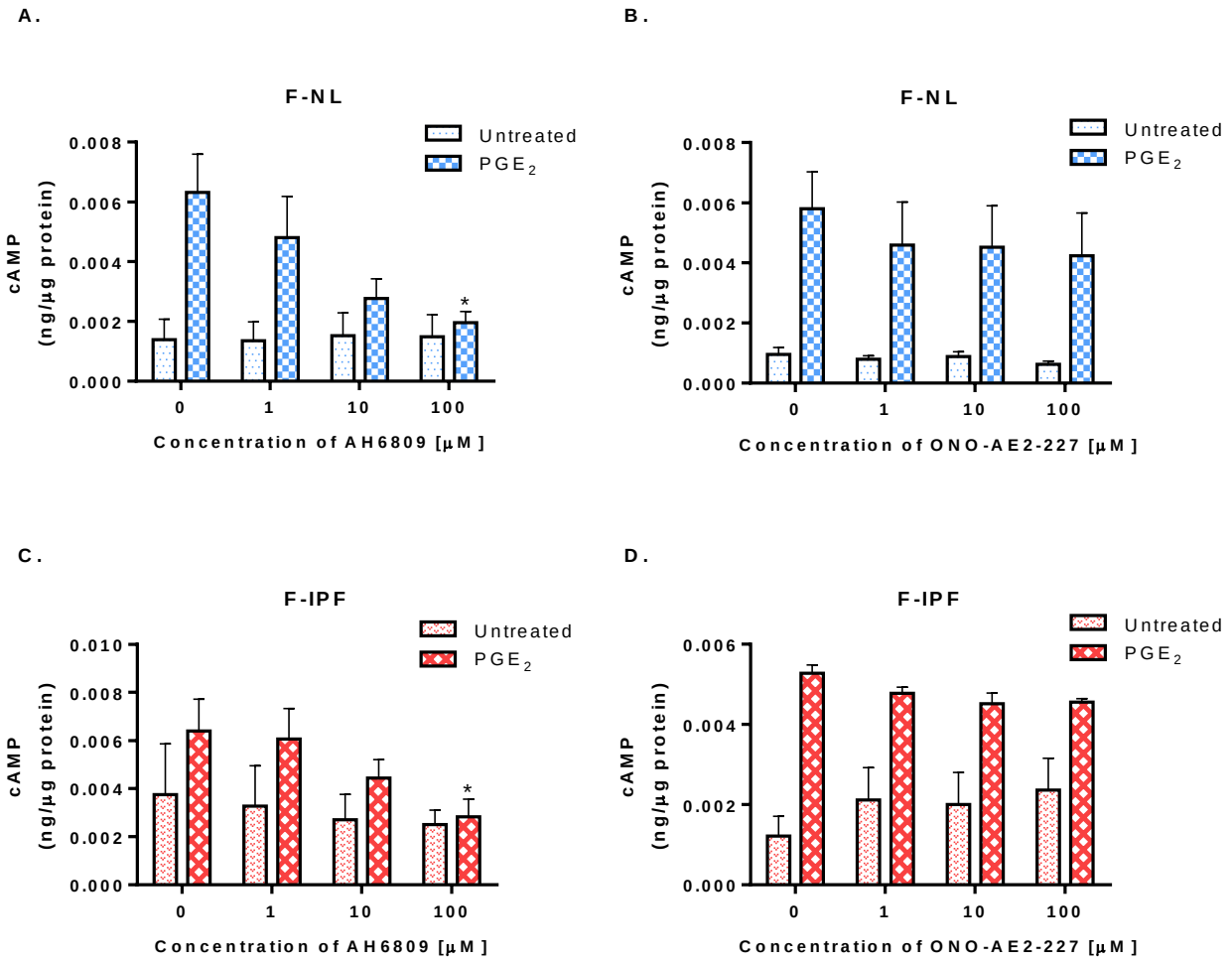


Figure 4-15 Effect of AH6809 and ONO-AE2-227 on PGE_2-induced cAMP Production in F-NL and F-IPF

*F-NL were serum starved for 24 hours and pre-treated with (A) AH6809 or (B) ONO-AE2-227 at 0, 1, 10 and 100 µM for 30 minutes before the addition of PGE_2 (1 µM) for 30 minutes. F-IPF were serum starved for 24 hours and pre-treated with (C) AH6809 or (D) ONO-AE2-227 at 0, 1, 10 and 100 µM for 30 minutes before the addition of PGE_2 (1 µM) for 30 minutes. To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from four separate experiments performed in duplicate of four different cell lines and is expressed as mean ± SEM. *$p<0.05$, compared with PGE_2 treated control.*

4.4.5 EP2 Receptor expression is greater than EP4 expression in both F-NL and F-IPF

The EP2 receptor is the most abundantly expressed EP receptor in normal human lung fibroblasts and therefore it is not surprising that PGE_2 acts via EP2 to mediate its anti-fibrotic effects (Huang et al., 2007) It has been reported that EP2 receptor expression is down regulated in a subset of fibroblasts isolated from patients with usual interstitial pneumonia (Huang et al., 2008a) and in myofibroblasts isolated from mice after bleomycin-induced lung fibrosis (Moore et al., 2005). The loss of EP2 expression following bleomycin treatment resulted in fibroblasts having a blunted cAMP response and the reduced ability of PGE_2 to inhibit proliferation and collagen secretion (Moore et al., 2005). Therefore, not only was PGE_2 production limited but PGE_2 signalling was also impaired. Our data demonstrated that to increase IL1β-induced COX-2, PGE_2 signals via the EP2 receptor and there was no difference in PGE_2-induced cAMP levels between F-NL and F-IPF (Figure 4-3). This suggests that F-NL and F-IPF have a similar level of EP2 receptor expression and that the PGE_2 signalling pathway is not impaired in F-IPF. To confirm this we analysed the expression of EP2 and EP4 in F-NL and F-IPF by immunocytochemistry and calculated mean fluorescence intensity (MFI) to quantify receptor expression. In both F-NL and F-IPF there was significantly more EP2 receptor expression compared with EP4 (Figure 4-16A). Mean fluorescent intensity of the EP2 receptor was not significantly different between F-NL and F-IPF (Figure 4-16B). EP4 fluorescence was too low to quantify. Our immunocytochemistry data allowed direct comparison of EP2 and EP4 expression however, to confirm EP2 and EP4 expression and antibody specificity we next detected EP2 and EP4 receptor expression protein levels via Western blot. Both EP2 and EP4 receptor protein was detected in F-NL and F-IPF (Figure 4-17). Therefore, in contrast to previous studies, we did not detect any difference in EP2 receptor expression or PGE_2 signalling between F-NL and F-IPF. EP2 and EP4 receptor expression is similar in both F-NL and F-IPF, however, our immunocytochemistry results suggest that EP2 receptor expression is greater compared with EP4 expression.

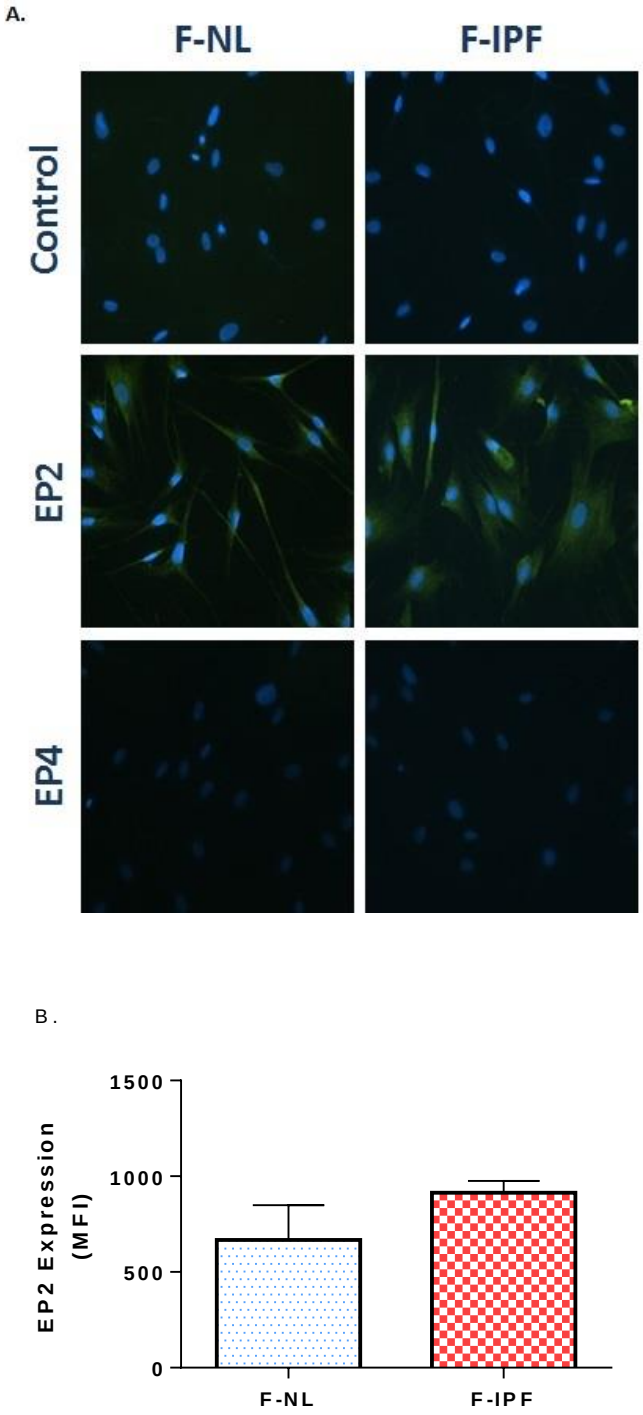


Figure 4-16 EP2 and EP4 Reception Expression and Mean Fluorescence Intensity in F-NL and F-IPF

(A) F-NL and F-IPF were grown until 50% confluent and serum starved for 24 hours prior to fixing, stained for the EP2 and EP4 receptor and visualised using a light microscope. As a control, cells were stained with the secondary antibody only and cell nuclei were stained with DAPI (blue). This figure is representative of three separate experiments performed in different

cell lines. (x20 magnification). (B) Mean fluorescence Intensity (MFI) was calculated using Nikon NIS Elements image analysis software. This data is the amalgamation of three different cell lines and expressed as mean ± SEM.

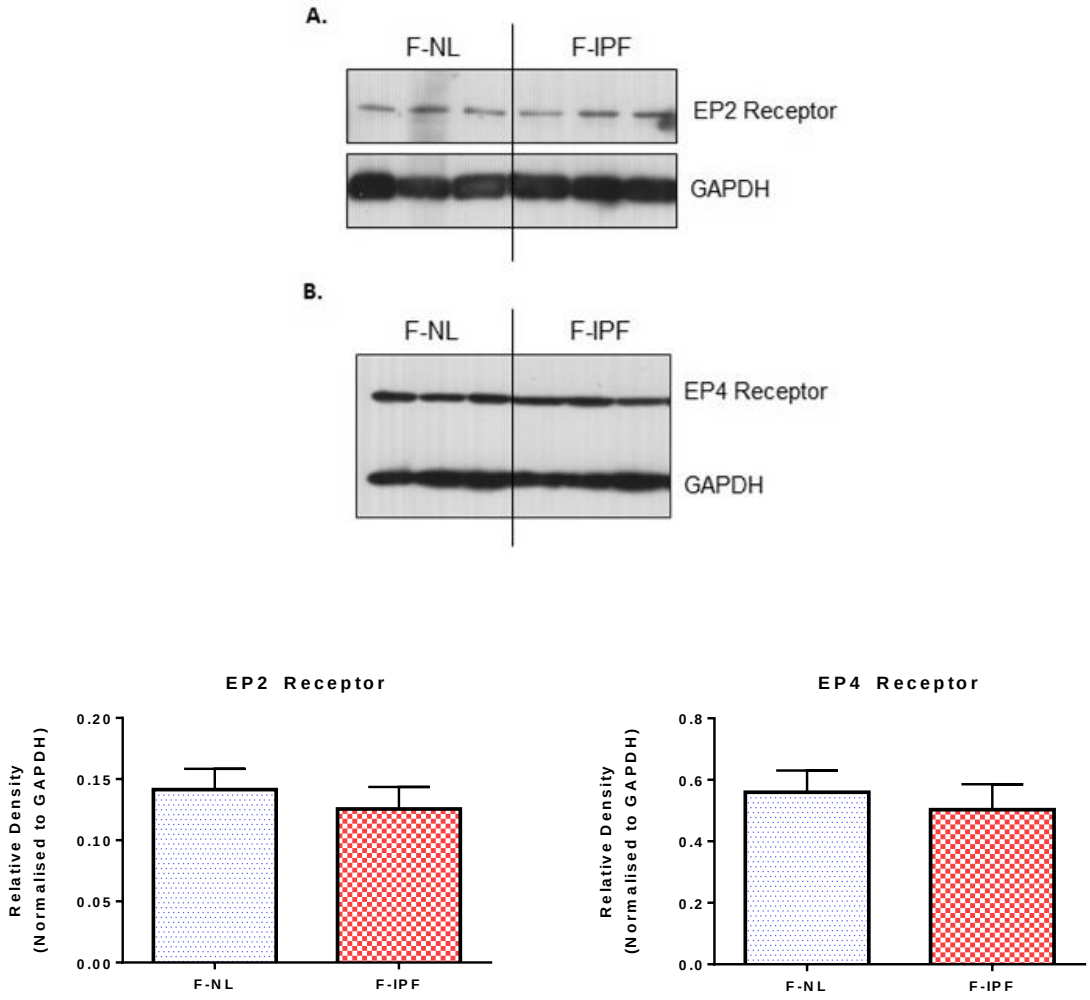


Figure 4-17 EP2 and EP4 Receptor Protein Expression in F-NL and F-IPF

F-NL and F-IPF were serum starved 24 hours prior to the collection of total cell lysate for analysis by Western blot of (A) EP2 receptor and (B) EP4 receptor expression. This figure shows data from three different cell lines. Relative density was calculated by normalising the density of the EP2 and EP4 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

4.4.6 The Anti-fibrotic Effects of PGE_2 are Mediated by PKA but not Epac Activation

We next sought to determine which cAMP effector, PKA or Epac, is involved in regulating COX-2 expression. F-IPF were treated with varying concentrations of the selective PKA agonist, 6-Bnz-cAMP, or the selective Epac agonist, 8-pCPT-2'O-Me-cAMP, and the effect on COX-2 and α-SMA protein expression was determined by Western blot (Figure 4-18 and Figure 4-19). The concentration of cAMP analogues used were shown to have specificity for PKA or Epac based on previous studies (Christensen et al., 2003). The PKA agonist increased IL-1β-induced COX-2 expression at 50 μM (Figure 4-18). The PKA agonist had no effect on α-SMA expression at either 10 μM, 25 μM or 50μM (Figure 4-18). Treatment with the Epac agonist had no effect on COX-2 or α-SMA at all concentrations tested (Figure 4-19). Overall, PKA was able to significantly increase IL-1β-induced COX-2 expression but had no effect on α-SMA expression. In contrast, the Epac agonist had no effect on COX-2 expression or α-SMA expression. This data suggests that PKA may have a role regulating COX-2 expression but Epac does not. The fact that PGE_2 is able to increase IL-1β-induced COX-2 expression and reduce α-SMA suggests that PGE_2 may activate PKA and alternative pathway(s) to exert its anti-fibrotic effects.

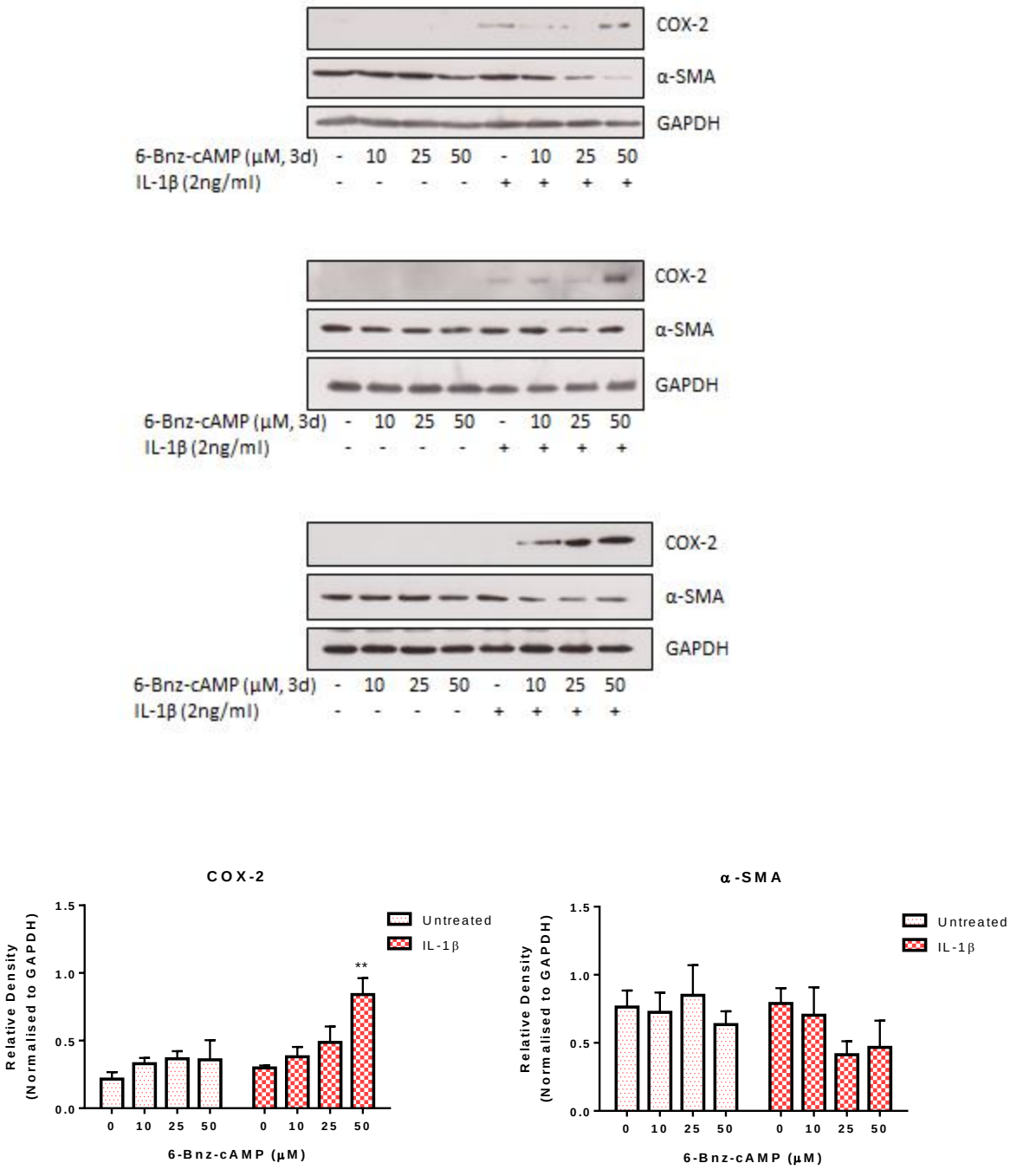


Figure 4-18 Effect of PKA Agonist, 6-Bnz-cAMP, on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with 6-Bnz-cAMP for 3 days at 0, 10, 25 and 50 μM, serum starved for 24 hours and left unstimulated or stimulated with 2 ng/ml IL-1β for 24 hours prior to the collection of total cell lysate for analysis by Western blot. This figure shows three separate experiments

*performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. **p<0.01 compared with IL-1β-treated control.*

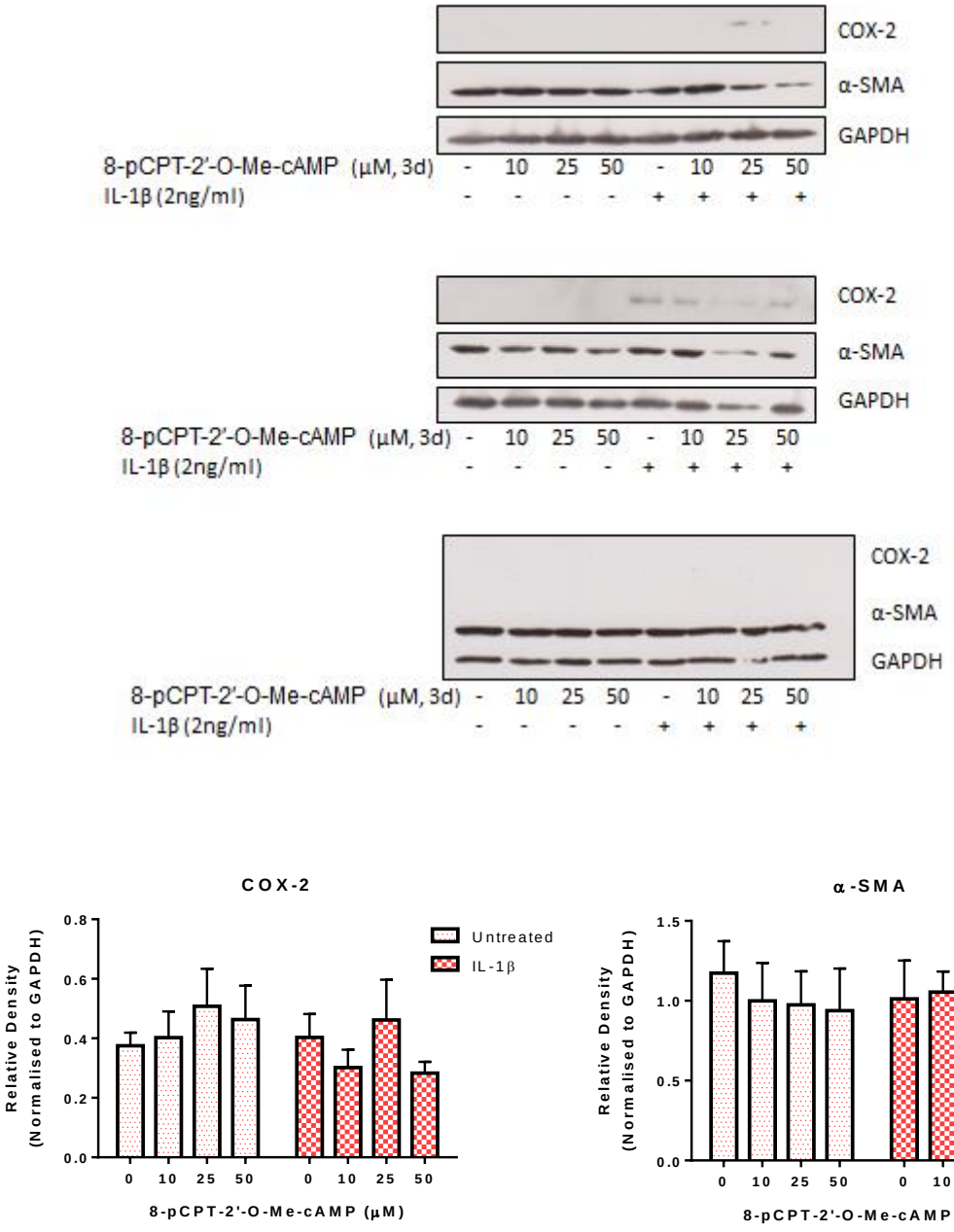


Figure 4-19 Effect of Epac agonist, 8-pCT-2'O-Me-cAMP, on COX-2 and -SMA Protein Expression in F-IPF

F-IPF were treated with 8-pCT-2'O-Me-cAMP for 3 days at 0, 10, 25 and 50 μM, serum starved for 24 hours and left unstimulated or stimulated with 2 ng/ml IL-1β for 24 hours prior to the collection of total cell lysate for analysis by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising

the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

4.4.7 Effect of Roflumilast on PGE_2-induced Fibroblast to Myofibroblast Differentiation

Our results so far, using PGE_2 and cAMP elevating agents, have demonstrated that cAMP is critical for COX-2 expression and subsequent PGE_2 production to maintain an anti-fibrotic phenotype, and prevent and reverse fibroblast to myofibroblast differentiation. cAMP levels are tightly controlled by adenylyl cyclases, which catalyse cAMP production, and phosphodiesterases (PDE), which catalyse cAMP degradation. There are several isoforms of PDEs that degrade either cAMP or cGMP or both. PDE4 is the most commonly expressed isoform responsible for the degradation of cAMP (Lugnier, 2006). As such we wanted to determine the effect of Roflumilast on COX-2 expression and fibroblast to myofibroblast differentiation. We hypothesised that inhibition of PDE4, by Roflumilast, would prevent cAMP degradation and further enhance the effects of cAMP elevating agents, such as PGE_2, on COX-2 expression and the reversal of fibroblast to myofibroblast differentiation in F-IPF. There was no significant difference of IL-1β-induced COX-2 expression between PGE_2 treatment alone and PGE_2 and Roflumilast treatment. Therefore, Roflumilast treatment had no effect alone or in combination with PGE_2 on IL-1β-induced COX-2 expression in F-IPF (Figure 4-20). Furthermore, Roflumilast had no effect on α-SMA protein expression (Figure 4-20). In order to understand why Roflumilast did not effect PGE_2-induced COX-2 expression we assessed the effect of Roflumilast on cAMP production in F-IPF. Roflumilast treatment alone had no effect on basal cAMP levels and did not increase cAMP production after treatment with PGE_2, Form or FSK (Figure 4-21). The fact that cAMP production did not increase following treatment with various cAMP elevating agents suggests that the lack of effect by Roflumilast was not specific to PGE_2 treatment. This result suggests that PDE4 is unlikely to be the main phosphodiesterase isoform involved in cAMP degradation in F-IPF. It would be interesting to investigate if other phosphodiesterase isoforms could increase cAMP alone or potentiate the effect of other cAMP elevating agents.

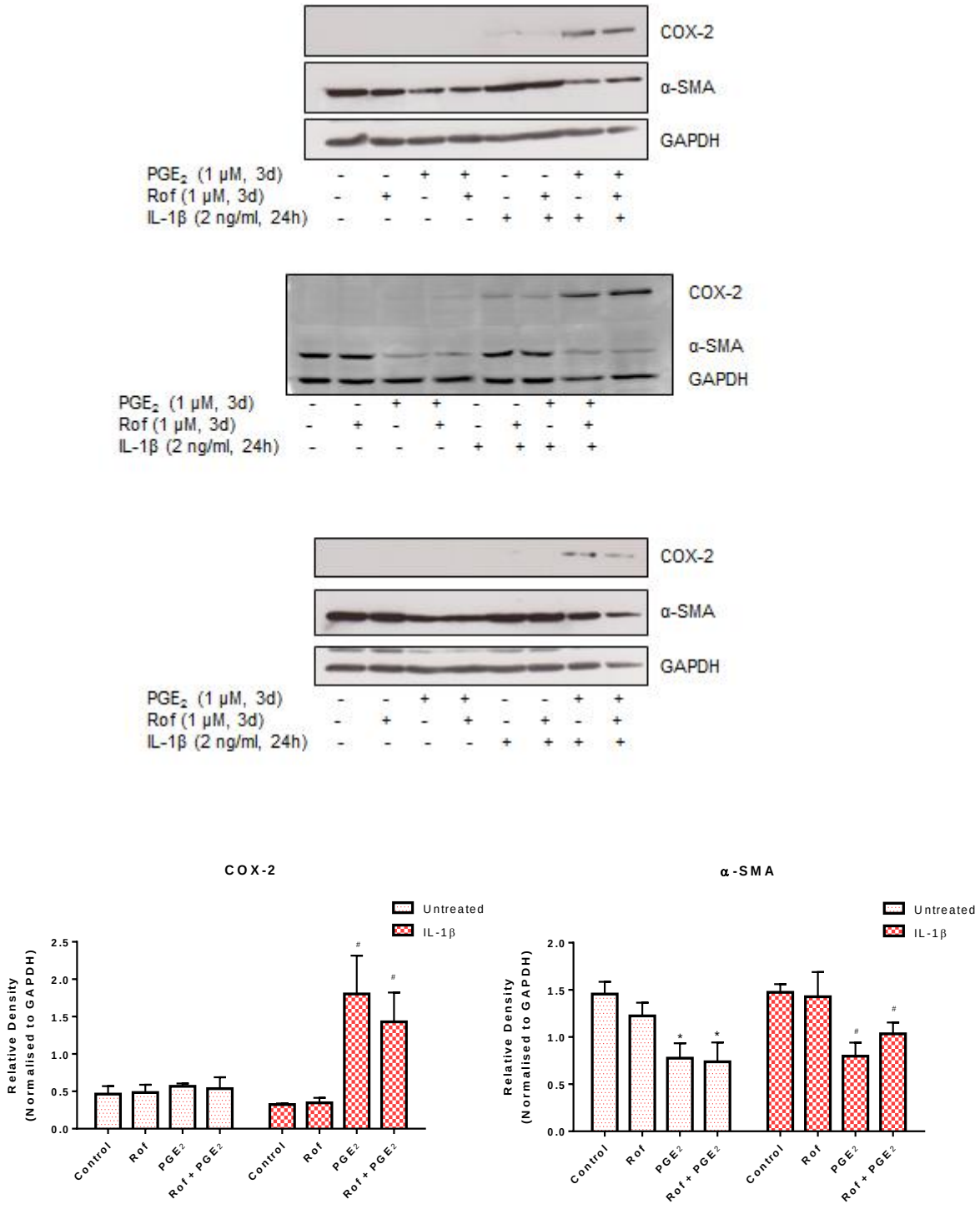


Figure 4-20 Effect of Roflumilast on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with PGE_2 (1 μM, 3d) with or without Roflumilast (Rof) (1 μM, 3d), serum starved for 24 hours and left unstimulated or stimulated with IL-1ß (2 ng/ml, 24h) prior to the

*collection of total cell lysate and analysis by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared to untreated control, #p<0.05 compared with IL-1β treated control.*

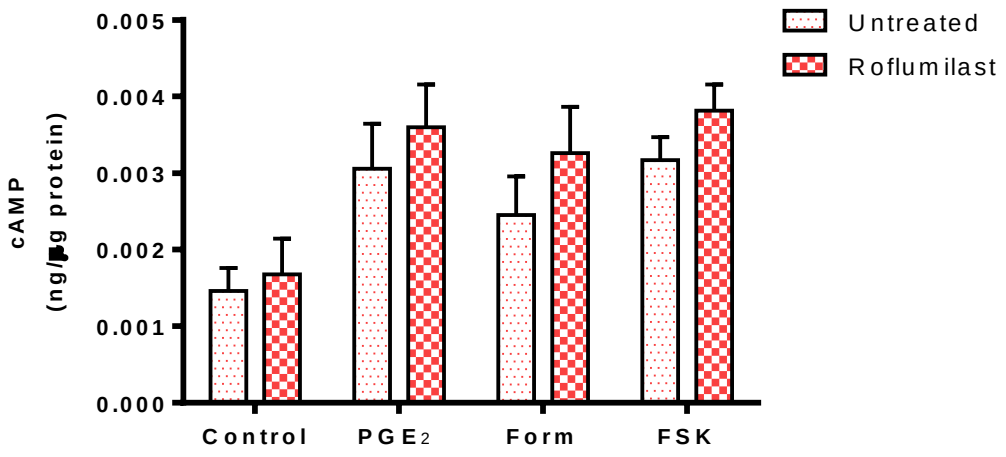


Figure 4-21 Effect of Roflumilast on cAMP Production in F-IPF

F-IPF were serum starved for 24 hours and treated for 30 minutes with either PGE_2 (1 µM), Form (10 µM) or FSK (10 µM) with or without Roflumilast (1 µM, 30mins). To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from separate experiments performed in duplicate of four different cell lines and expressed as mean ± SEM.

4.5 Discussion

For the first time we provide evidence that cAMP elevating agents can compensate for the loss of endogenous PGE_2 and maintain an anti-fibrotic phenotype due to their ability to increase IL-1β-induced COX-2 expression and subsequent PGE_2 production. We have also demonstrated that PGE_2 is likely to signal via the EP2 receptor to increase cAMP. PKA, the downstream cAMP mediator, is likely to regulate COX-2 expression and PGE_2 signalling.

This chapter suggests that the effect of PGE_2 on COX-2, α-SMA and Col 1 expression is via activation of the EP2 receptor and increased cAMP production. Although data using selective EP2 and EP4 agonists did not show any effect on COX-2 or α-SMA, as discussed in chapter 2, PGE_2-induced cAMP was significantly inhibited by the EP2 antagonist, but not the EP4 antagonist, in both F-NL and F-IPF. These data supports the dominant role of EP2 in regulating the effects of PGE_2 and fibroblast to myofibroblast differentiation. The immunohistochemistry data suggests that there is a paucity of EP4 receptor expression in both F-NL and F-IPF. However, as a positive control was not used in this experiment we are unable to confirm this as the EP4 antibody may have not been suitable for immunohistochemistry analysis. Expression of both EP2 and EP4 receptor was confirmed by Western blot analysis. The Western blot data demonstrated that there was no difference in receptor expression between F-NL and F-IPF. Our antagonist data suggests that EP2 is the dominant receptor involved in PGE_2 signalling and this is supported by data from other studies. Huang and colleagues (2007) demonstrated that relative mRNA expression for EP2 was 160-fold greater than EP4. In addition, studies using IMR-90 cells, a human foetal lung fibroblast cell line, have demonstrated significantly higher EP2 expression compared with EP4 at both mRNA and protein levels (Choung et al., 1998; Kolodsick et al., 2003). Furthermore, our data is supported by other studies demonstrating that PGE_2 acts mainly via the EP2 receptor to mediate fibroblast to myofibroblast differentiation (Kolodsick et al., 2003), collagen production

(Huang et al., 2007) and proliferation (Liu et al., 2004). These studies suggest that EP2 mediates many of the inhibitory actions of PGE_2 and thus these biological responses share a common pathway. The importance of EP2 is further supported by observations that EP2 knockout mice show an increased susceptibility to bleomycin-induced fibrosis (Moore et al., 2005).

Interestingly, Huang and colleagues (2008a) demonstrated that a small subset of fibroblasts isolated from fibrotic lung, exhibited resistance to the inhibitory effects of PGE_2 with regards to proliferation and collagen production which was in part ascertained to diminished EP2 receptor expression. In addition, a small number of cell lines had reduced PKA expression which also contributed to impaired PGE_2 responsiveness (Huang et al., 2008a). In contrast to this study, both F-NL and F-IPF had similar EP2 receptor expression and responded to PGE_2 in a similar manner with regards to cAMP production. These differences could be accountable to the different PGE_2 concentrations used. Huang and colleagues (2008) used a lower concentration of PGE_2, which may explain why PGE_2 resistance was not evident in F-IPF used in our study. In support of Huang's (2008) study, it has been demonstrated that fibroblasts isolated from a mice model of bleomycin-induced pulmonary fibrosis have down-regulated EP2 receptor expression leading to blunted PGE_2-induced cAMP compared with control fibroblasts (Moore et al., 2005). It is possible that following fibrotic challenge the initial increase in PGE_2 may contribute to the down regulation of the EP2 receptor. As PGE_2 is already reduced in patients with IPF this may explain why we did not see any difference in EP2 receptor expression in F-IPF. To confirm this EP2 receptor expression should be quantified following PGE_2 treatment. In addition, to confirm EP2 and EP4 receptor expression and differences between F-NL and F-IPF flow cytometry analysis of receptor expression should be completed.

The ability of PGE_2, EP2 agonists and other cAMP-elevating agents, such as FSK, to induce COX-2 and increase PGE_2 production in F-IPF highlights the therapeutic potential of pharmacologic agents that increase cAMP. Although administration of exogenous PGE_2 and inhalation of liposomal PGE_2 has been shown to prevent bleomycin-induced pulmonary fibrosis (Dackor et al., 2011; Ivanova et al., 2013) the effect of alternative cAMP elevating agents, other than PGE_2, have not been studied in animal models or IPF patients. As formoterol, a long actin β_2-agonist, and roflumilast, a PDE4 inhibitor, are cAMP elevating agents already in clinical use, we assessed their ability to induce COX-2 in F-IPF and their effect on fibroblast to myofibroblast differentiation.

Form, Salme and FSK increased IL-1β-induced COX-2 expression in F-IPF. Form and FSK also significantly reduced Col 1 expression and therefore promoted an anti-fibrotic phenotype. Density analysis confirmed that Form, Salme and FSK had no significant effect on α-SMA. Potentially, these data suggest that regulation of α-SMA expression may not be via the cAMP pathway. However, despite density analysis showing that Form, Salme and FSK had no significant effect on α-SMA, most individual Western blots showed a visible reduction in α-SMA with the combination of IL-1β and Form, Salme and FSK compared with IL-1β alone. The percentage reduction of α-SMA following IL-1β treatment with 10 μM Form, Salme and FSK is 48.9%, 57.4% and 55.9% respectively, compared with IL-1β alone (data not shown). The data therefore suggest that Form, Salme and FSK may be able to decrease α-SMA expression via induction of IL-1β-induced COX-2 and the cAMP pathway. Further experiments with additional cell lines are needed to confirm this observation.

Treatment with Form and FSK also increased endogenous PGE_2 production. Interestingly, FSK had a greater effect on PGE_2 production compared with Form. This could be explained by the fact that β_2-adrenergic receptors undergo rapid desensitisation following treatment with

agonists (Hu et al., 2008; Lefkowitz et al., 1990), whereas, the EP2 receptor does not (Regan, 2003). In fact, it has been reported that the loss of airway smooth muscle cell sensitivity to β_2-adrenergic receptor agonists is due to the induction of COX-2 (Pang et al., 1998a, b). Therefore, long term treatment with Form may have a reduced effect on COX-2 due to receptor sensitisation. The amount of cAMP produced varied depending on the agonist used. PGE_2 had the strongest effect on cAMP production whereas FSK-induced cAMP was markedly less than both PGE_2 and Form. Therefore, the level of cAMP produced may not always correlate with cell function. cAMP regulation and production is not a linear pathway and various studies have demonstrated that cAMP signalling is compartmentalised within the cell. Brunton and colleagues (1981) demonstrated that whilst various receptors stimulated cAMP production in cardiac myocytes, stimulation of the different receptors resulted in different physiological outcomes. Further experimentation illustrated that certain receptors were linked to specific adenylyl cyclases and physical compartmentation of PKA resulted in only a subset of PKA substrates being phosphorylated in response to a specific receptor stimulation (Hayes and Brunton, 1982).

In order to avoid any non-CAMP effects of β_2-agonists and avoid receptor desensitisation, we used FSK as the cAMP elevating agent to measure changes in mRNA expression of COX-2, α-SMA and Col 1. FSK treatment had no effect on COX-2 or IL-1β-induced COX-2 mRNA expression. In addition, FSK treatment did not effect α-SMA mRNA expression. However, FSK treatment significantly reduced Col 1 mRNA expression. These data suggest that Col 1 may be regulated via transcriptional mechanisms whereas COX-2 and α-SMA are regulated via post-translational mechanisms.

We also assessed the effect of FSK on preventing fibroblast to myofibroblast differentiation in TGF-β1-treated F-NL. FSK and IL-1β treatment restored TGF-β1-repressed COX-2

expression and reduced α-SMA and Col 1. Thus, FSK was able to prevent fibroblast to myofibroblast differentiation. In addition, FSK prevented TGF-β1 repression of IL-1β-induced COX-2 and TGF-β1-induced α-SMA and Col 1 at both the protein and mRNA level. In comparison, FSK did not significantly increase IL-1β-induced COX-2 mRNA expression in F-IPF; however there was a trend for COX-2 mRNA to increase and α-SMA to decrease following PGE_2 and FSK treatment. Further experiments to increase the number of cell lines tested may confirm the effect of PGE_2 and FSK on COX-2 and α-SMA mRNA in F-IPF. Furthermore, experiments investigating the effect of FSK on α-SMA and Col 1 mRNA did not include IL-1β treatment. Therefore, IL-1β may have a key role in regulating α-SMA and Col 1 mRNA following treatment with PGE_2 and FSK.

The anti-fibrotic effects of roflumilast have been previously demonstrated *in vitro* in lung fibroblasts. Roflumilast treatment inhibited TGF-β1-induced gel contraction on collagen gels and TGF-β1-induced chemotaxis in both foetal and primary human normal lung fibroblasts (Togo et al., 2009). Another study demonstrated that treatment with roflumilast in combination with the β_2-agonist indacaterol reduced expression of pro-fibrotic mediators such as ET-1 and CTGF in a normal human lung fibroblast cell line (Tannheimer et al., 2012). More recently, knockdown of PDE4B and PDE4D subtypes were shown to inhibit TGF-β1-induced fibroblast to myofibroblast differentiation in a normal human lung fibroblast cell line (Selige et al., 2011). Previous studies have focussed on the anti-fibrotic effects of roflumilast in normal lung fibroblasts and commercially available cell lines. This is the first time that the effect of roflumilast has been assessed in fibroblasts derived from IPF patients. In contrast to the aforementioned studies and our own hypothesis, roflumilast had no effect on PGE_2-induced COX-2 expression or fibroblast to myofibroblast differentiation. In fact, roflumilast had no effect on cAMP production after treatment with PGE_2, Form or FSK. In support of previous studies roflumilast increased cAMP production in F-NL after treatment with PGE_2, Form and FSK (data not shown). Furthermore, roflumilast also increased IL-1β-induced COX-2 but had no effect

on α-SMA (data not shown). One explanation for these results could be that PDE4 is differentially regulated in F-NL compared with F-IPF and PDE4 may be down regulated in F-IPF. Thus the expression of PDE isoforms may change during fibroblast to myofibroblast differentiation. Alternatively, the PDE4 isoform may not be the predominant isoform catalysing cAMP degradation. Interestingly, a study identified the PDE isoforms expressed in primary normal human fibroblasts and demonstrated that the main PDE isoforms expressed were PDE5 (hydrolysis of cGMP), PDE1 (hydrolysis of cGMP and cAMP) and to a small extent PDE4 (Dunkern et al., 2007). In addition, cGMP hydrolysis, PDE5-activity and PDE5 protein expression was significantly reduced following TGF-β1–induced fibroblast to myofibroblast differentiation (Dunkern et al., 2007). Further studies investigating the expression of PDE isoforms in F-IPF and F-NL would be needed to fully investigate the therapeutic potential of phosphodiesterases. However, based on our results and the study conducted by Dunkern et al (2007) the inhibition of phosphodiesterases may have little effect if they are down regulated in F-IPF.

It is well documented that the inhibitory effects of PGE_2 on fibroblasts are mediated through the common second messenger cAMP, however, how PGE_2/cAMP mediates multiple fibroblast functions has never been elucidated. Although PKA is the classic effector of cAMP other effectors, such as Epac, are increasingly recognised (Montminy, 1997). No prior reports have differentiated which downstream effectors of cAMP are responsible for the induction of COX-2 by PGE_2 or involved in regulating fibroblast to myofibroblast differentiation. We sought to determine if PKA, Epac or both were involved in regulating COX-2 expression in F-IPF. IL-1β-induced COX-2 increased following treatment with the PKA agonist, 6-Bnz-cAMP, at the highest concentration but there was no effect using the Epac agonist, 8-pCTP-2'O-Me-cAMP, at any of the concentrations tested. Neither PKA activation nor Epac activation had an effect on α-SMA expression. Although not measured in our experiments it would be worth investigating the effects of PKA and Epac on Col 1 expression. The anti-fibrotic role of PKA

and Epac in F-IPF is supported by a study demonstrating that inhibition of Epac, but not PKA, inhibited fibroblast proliferation whereas inhibition of PKA, but not Epac, inhibited fibroblast collagen production (Huang et al., 2008b). Therefore, it seems likely that PGE_2 signals via both PKA and Epac to finely regulate fibroblast to myofibroblast differentiation but PKA activation is predominantly involved in COX-2 regulation. It has also been documented that Epac is down regulated in TGF-β1-treated rat cardiac fibroblasts (Yokoyama et al., 2008). Based on this observation it is possible that Epac could also be downregulated in F-IPF. Therefore, it would be worthwhile to determine expression levels of Epac in F-NL, F-IPF and TGF-β1-treated F-NL.

In summary, these data demonstrate that PGE_2 increases IL-1β-induced COX-2 resulting in increased endogenous PGE_2 production which promotes an anti-fibrotic phenotype. This occurs mainly via the EP2 receptor and is a cAMP-dependent process. Downstream of cAMP, our data and other studies suggest that the activation of PKA may be necessary to mediate the effects of PGE_2.

5 ROLE OF RAS SIGNALLING PATHWAY IN COX-2 EXPRESSION AND FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION

5.1 Introduction

The Ras family consists of GTP-binding proteins that relay signals from receptor tyrosine kinases to the nucleus and are responsible for regulating a diverse spectrum of intracellular processes including cellular proliferation, differentiation and apoptosis (Downward, 2003). Ras proteins are expressed in almost all adult and foetal tissues acting as molecular switches and activating the signal transduction of downstream pathways. Ras proteins are attached to the inner surface of the plasma membrane where they are activated by cell surface receptors to induce the conversion of inactive, GDP bound Ras, to active, GTP bound Ras. Active Ras acts as an important signalling branch point as it activates several signalling pathways through a number of different effectors and signalling pathways such as Raf/MAPK kinase and PI3K/Akt.

Previous studies have identified a role for Ras in several fibrotic conditions. Ras activity has been shown to be increased in liver fibrosis (Liu et al., 1994; Nonomura et al., 1987) and renal fibrosis (Bechtel et al., 2011). Furthermore, inhibition of Ras, by the Ras antagonist Farnesylthiosalicylic acid (FTS), has been shown to have anti-fibrotic effects in several experimental models of fibrosis. FTS is a unique and potent Ras inhibitor. It is a synthetic derivative of carboxylic acid, which structurally resembles the carboxy-terminal farneslcysteine group common to all Ras proteins. It acts as a functional Ras antagonist by affecting Ras membrane interactions and dislodging the protein from its anchorage domains, facilitating its degradation and thus reducing cellular Ras content and the cells ability to activate it (Haklai et al., 1998; Marom et al., 1995). FTS treatment ameliorated fibrosis in a mouse model of muscular dystrophy (Nevo et al., 2011), inhibited experimentally induced liver fibrosis in rats

(Reif et al., 2004) and ameliorated renal fibrosis in an experimentally induced mouse model (Bechtel et al., 2011). However, the role of Ras or the effect of FTS has not yet been investigated in IPF.

Once Ras is activated, the extracellular signal-regulated kinase (ERK) is a common downstream pathway mediating Ras signalling. In this pathway Ras recruits and activates Raf, which results in the phosphorylation of mitogen-activated protein/ERK kinases-1 and -2 (MEK-1/2) and subsequent phosphorylation of ERK-1/2. In mammals, there are three Raf proteins, A-Raf, B-Raf and Raf-1. Each Raf isoform activates MEK-1/2 but with different intensities (Mercer and Pritchard, 2003). ERK phosphorylates over 70 cytosolic proteins and translocates to the nucleus to control the activity of various transcription factors. Thus, ERK-1/2 can regulate numerous cellular processes such as gene expression, metabolism and morphology (Dumaz and Marais, 2005). Previous studies have reported that the ERK cascade is involved in the regulation of lung inflammation and injury (Yoshida et al., 2002). Inhibition of the ERK pathway reduced TGF-β1 induction in primary mouse fibroblasts (Sullivan et al., 2005) and completely blocked CTGF-induced Col 1 gene expression in human pulmonary fibroblasts (Ponticos et al., 2009). More recently, ERK activation has been shown to be increased in a bleomycin model of lung fibrosis in mice and ERK inhibition prevented bleomycin-induced fibrosis (Galuppo et al., 2011). Therefore, the Ras/Raf/ERK pathway may play an important role in regulating COX-2 expression and fibroblast to myofibroblast differentiation in IPF.

Our data demonstrates that cAMP is a key regulator of COX-2 expression and subsequent PGE_2 production. In addition, PGE_2 and cAMP elevating agents promote an anti-fibrotic phenotype via their ability to induce cAMP production and COX-2 expression. Nonetheless, the signalling mechanisms through which cAMP acts to induce COX-2 remain unknown. As already discussed in chapter 1, TGF-β1 signals via both the canonical Smad signalling

pathway and the non-canonical Smad-independent signalling pathway, but the specific pathways activated by TGF-β1 vary depending on the individual cell type and cellular context (Derynck and Zhang, 2003). Of particular interest, TGF-β1 is able to signal via Ras and activate the Ras/Raf/ERK pathway. TGF-β1 caused transient activation of the MEK/ERK cascade in an intestinal epithelial cell line, IEC4-1 (Hartsough et al., 1996). Depending on the cell type in question, MEK/ERK activation can be necessary for TGF-β1 induced gene transcription (Watanabe et al., 2001) or can enhance TGF-β1-induced gene transcription (Leask and Abraham, 2004).

The fact that cAMP analogues inhibited growth factor-stimulated ERK activation in NIH 3T3 fibroblasts demonstrated the existence of cross talk between the cAMP and Ras/Raf/ERK signalling pathways (Wu et al., 1993). The effect of cAMP elevating agents on ERK activation in other cells has been related to the expression of either Raf-1 or B-Raf (Dumaz and Marais, 2005) however, the relative expression and function of Raf isoforms and their regulation has not been investigated in pulmonary fibroblasts. Recently, Stratton and colleagues demonstrated that iloprost, a prostacyclin derivate, suppressed TGF-β1-induced fibrosis, at least in part, by suppressing the Ras/MEK/ERK pathway, in a PKA-dependent manner in human dermal fibroblasts (Stratton et al., 2002). With regard to our results, and previously published data, we hypothesised that Ras hyperactivity in F-IPF could be responsible for repressed COX-2 expression and the persistence of the pro-fibrotic phenotype in F-IPF. Treatment with PGE_2, or cAMP analogues, could decrease Ras activity, induce COX-2 and reverse fibroblast to myofibroblast differentiation.

5.2 Aims

This chapter aims to determine any differences in Ras activity between F-NL and F-IPF. Using the potent and selective Ras antagonist, FTS, we sought to assess if Ras activity has a regulatory role in COX-2 expression and fibroblast to myofibroblast differentiation. In addition, we further examined one of the downstream signalling pathways of Ras, the ERK-1/2 signalling pathway, and analysed Raf-1, B-Raf and ERK-1/2 expression in both F-NL and F-IPF. We assessed the effect of both acute and chronic treatment of TGF-β1 and PGE_2 on Ras activity and ERK-1/2 expression to determine if TGF-β1 signalled via the Ras/Raf/ERK pathway to repress COX-2 expression and promote fibroblast to myofibroblast differentiation. We also assessed if PGE_2 was able to inhibit the Ras/Raf/ERK pathway in order to increase COX-2 expression and prevent and reverse fibroblast to myofibroblast differentiation.

5.3 Experimental Protocol

F-NL and F-IPF were cultured to confluence in 6 well culture plates and growth arrested in serum free medium for 24 hour prior to all experiments. To measure COX-2 protein and mRNA expression cells were treated with 2 ng/ml IL-1β for 24 hours and 4 hours, respectively. Immediately before each experiment fresh serum free medium containing the compounds to be tested was added. After treatment cells were subject to protein extraction or total RNA isolation for Western blot and qRT-PCR analysis, as describe in Chapter 2. mRNA levels of COX-2, α-SMA, Col 1 and the internal control, β2-microglobulin (β-2M), were determined by quantitative RT-PCR. The results are calculated as the ratio of the gene of interest mRNA and β-2M mRNA and then fold change over untreated control and are expressed as mean ± SEM of separate experiments performed in duplicate.

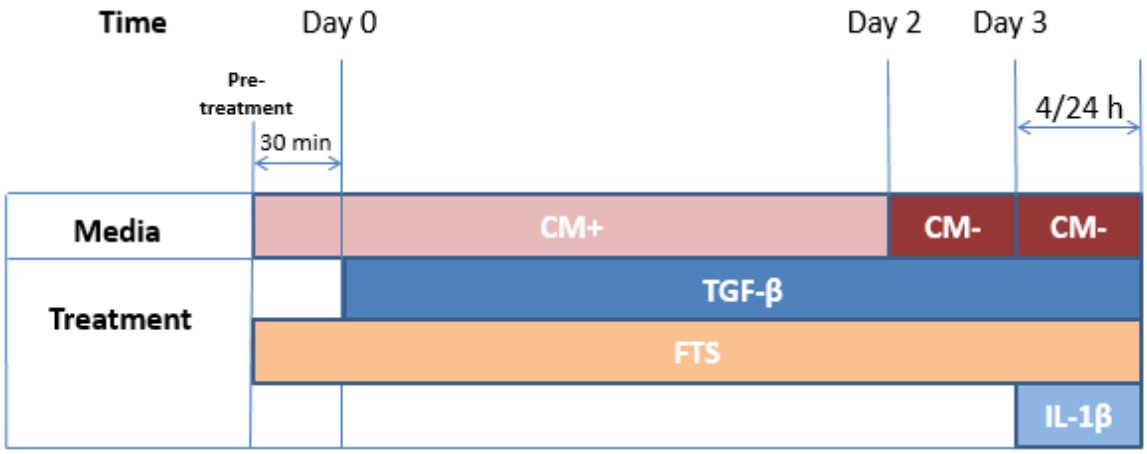


Figure 5-1 Experimental Protocol for effect of FTS and TGF-β on Ras Signalling Pathways

Schematic diagram of the timelines used for experimental protocol showing pre-treatment, treatment, IL-1β stimulation and whether serum (CM^+) or serum free (CM^-) media was used.

To test the effect of FTS on TGF-β1-induced fibroblast to myofibroblast differentiation, F-NL were treated with FTS (5 μM) 30 minutes prior to the addition of TGF-β1 (2 ng/ml) for three days. To determine Ras activity, F-NL and F-IPF were cultured to confluence in T150 cm^2

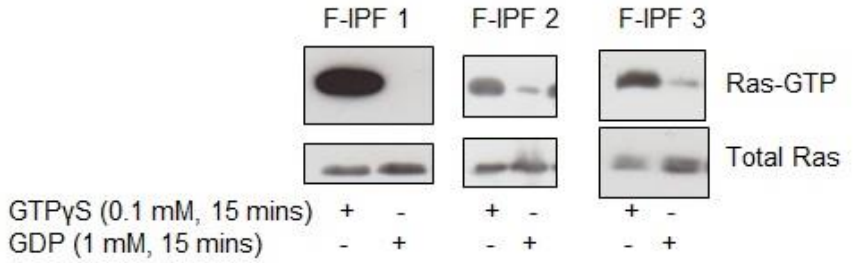
flasks and growth arrested in serum free medium for 24 hours prior to all experiments. In the acute time course experiments cells were treated with FTS (5 μM), TGF-β1 (2 ng/ml) or PGE_2 (1 μM) for 0 – 15 minutes or for 3 days in chronic time course experiments. At the indicated times cells were subject to the GTP Ras pull down assay as described in Chapter 2. To assess any effect of FTS treatment on cAMP production cells were incubated with FTS (5 μM) and IBMX (1 mM) alone or pre-treated for 30 minutes prior to the addition of cAMP elevating compounds. After 30 minutes of treatment the culture media was collected and cAMP production was determined as described in Chapter 2.

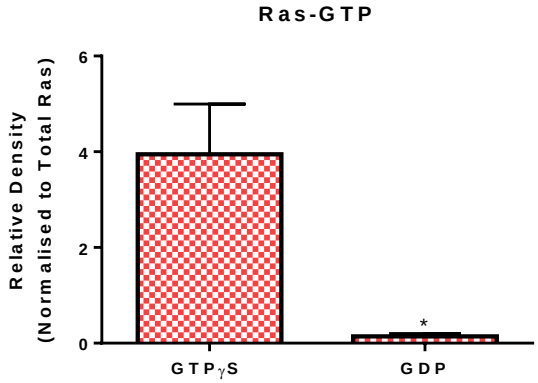
5.4 Results

5.4.1 FTS induces COX-2 in F-IPF and promotes an anti-fibrotic phenotype

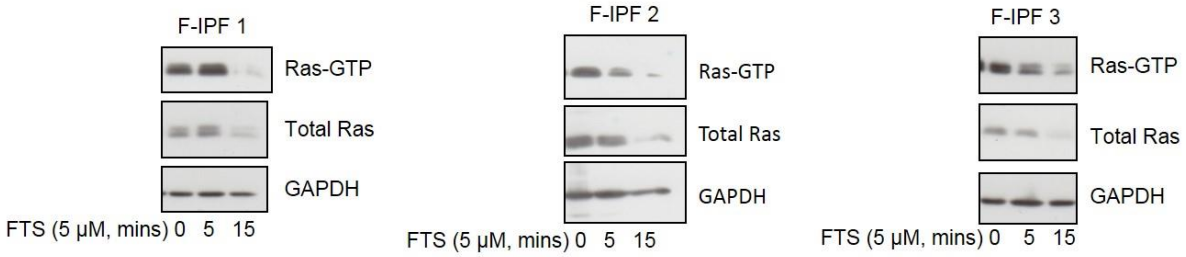
To determine if Ras signalling is involved in regulating COX-2 expression we analysed the effect of FTS on COX-2 expression and fibroblast to myofibroblast differentiation. Initially, we wanted to confirm that FTS does indeed inhibit Ras activity in fibroblasts. As this was a new technique used within our laboratory we also wanted to validate the specificity of the Ras-GTP pull down assay. F-IPF total cell lysates were incubated with GTPγS or GDP to activate or inactivate endogenous GTPases, respectively. The anti-Ras GST antibody detected a strong signal in GTPγS treated lysates, whereas minimal or no signal was detected in GDP treated lysates thus confirming the specificity of the assay for GST-Raf-1 recombinant binding domain (Figure 5-2). In addition, FTS treatment reduced both Ras-GTP and total Ras after 15 minutes of treatment in F-IPF (Figure 5-2). After confirming that the assay specifically detects Ras-GTP and FTS antagonised Ras-GTP we then assessed the effect of Ras inhibition on COX-2 expression and fibroblast to myofibroblast differentiation in F-IPF. FTS alone had no effect on COX-2 expression, however, FTS at 5 μM significantly increased IL-1β-induced COX-2 expression in F-IPF (Figure 5-43). FTS had no effect on α-SMA with or without IL-1β treatment at any of the concentrations tested (Figure 5-43). FTS significantly reduced Col 1 protein expression in F-IPF (Figure 5-4). These results demonstrate that FTS promotes an anti-fibrotic phenotype by increasing IL-1β-induced COX-2 expression and reducing Col 1 expression and therefore Ras signalling may play a role in fibroblast to myofibroblast differentiation.

A)


F-IPF 1
F-IPF 2
F-IPF 3
Ras-GTP
Total Ras
GTPγS (0.1 mM, 15 mins) + - + - + -
GDP (1 mM, 15 mins) - + - + - +


Ras-GTP
Relative Density (Normalised to Total Ras)
6
4
2
0
*
GTPγS
GDP

B)


F-IPF 1
Ras-GTP
Total Ras
GAPDH
FTS (5 μM, mins) 0 5 15
F-IPF 2
Ras-GTP
Total Ras
GAPDH
FTS (5 μM, mins) 0 5 15
F-IPF 3
Ras-GTP
Total Ras
GAPDH
FTS (5 μM, mins) 0 5 15

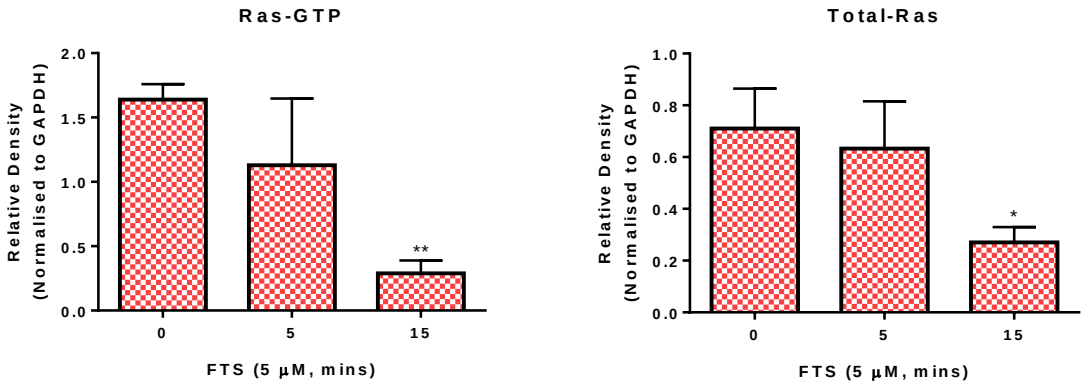


Figure 5-2 Detection of Ras-GTP and Effect of FTS on Ras-GTP in F-IPF

A) *Confluent F-IPF were serum starved for 24 hours prior to the collection of total cell lysate. 500 µg of cell lysate was treated with GTPγS (0.1 mM) or GDP (1 mM) for 15 minutes at 30 ^{o}C prior to performing the GTP pull down assay and analysis by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Ras-GTP bands against that of total-Ras. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with GTPγS treated control. B). Confluent F-IPF were serum starved for 24 hours and treated with FTS (5 µM) for 0, 5 and 15 minutes prior to the collection of total cell lysate. 500 µg of total cell lysate was used in the GTP pull down assay and then analysed by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Ras-GTP and total-Ras bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with untreated control.*

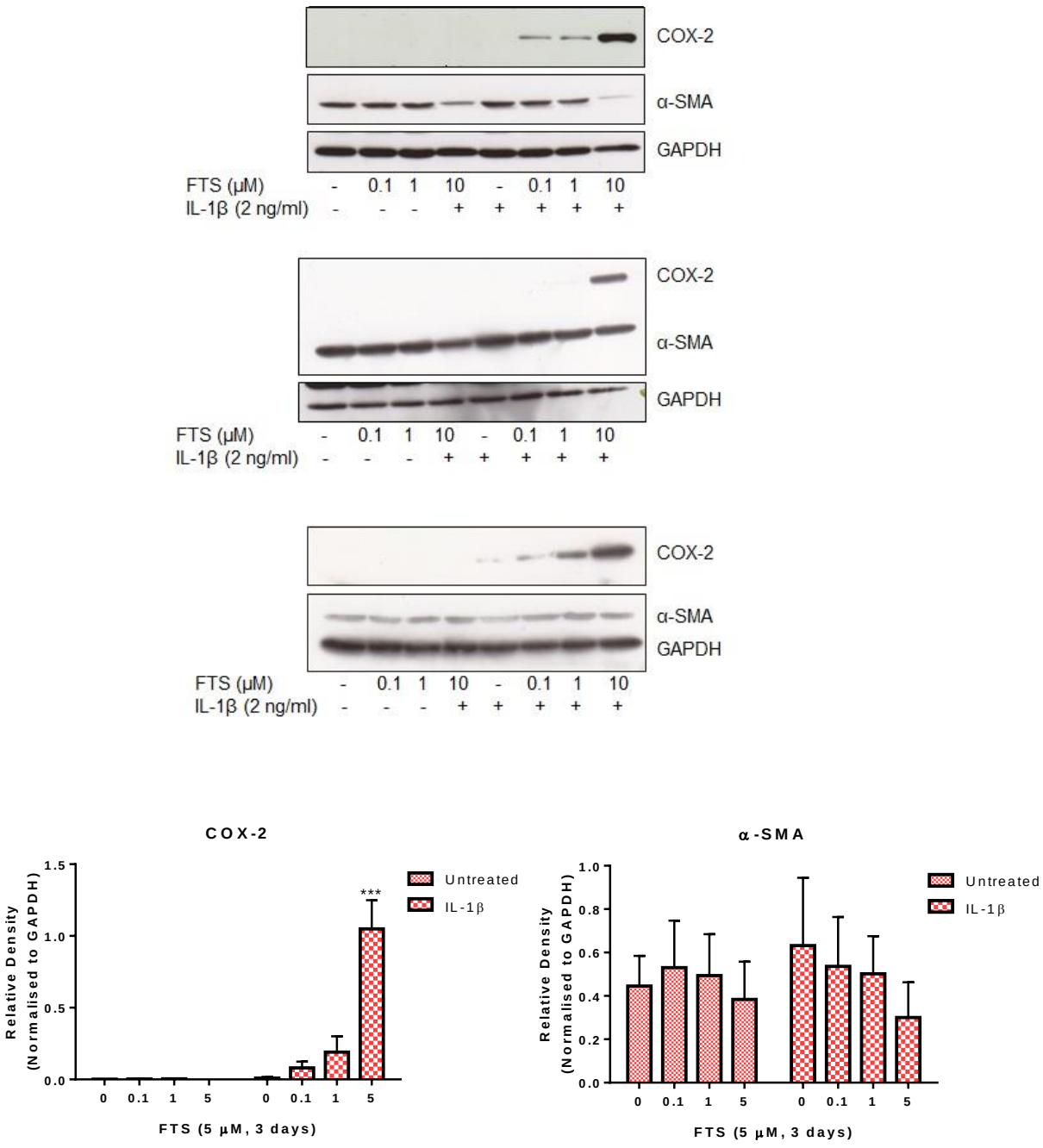


Figure 5-3 Effect of FTS on COX-2 and α-SMA in F-IPF

*Confluent F-IPF were serum starved for 24 hours and treated with FTS at 0.1, 1 and 5 μM for 3 days and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 h) prior to collection of total cell lysate and Western blot analysis of COX-2 and α-SMA. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. ***p<0.005 compared with IL-1β treated control.*

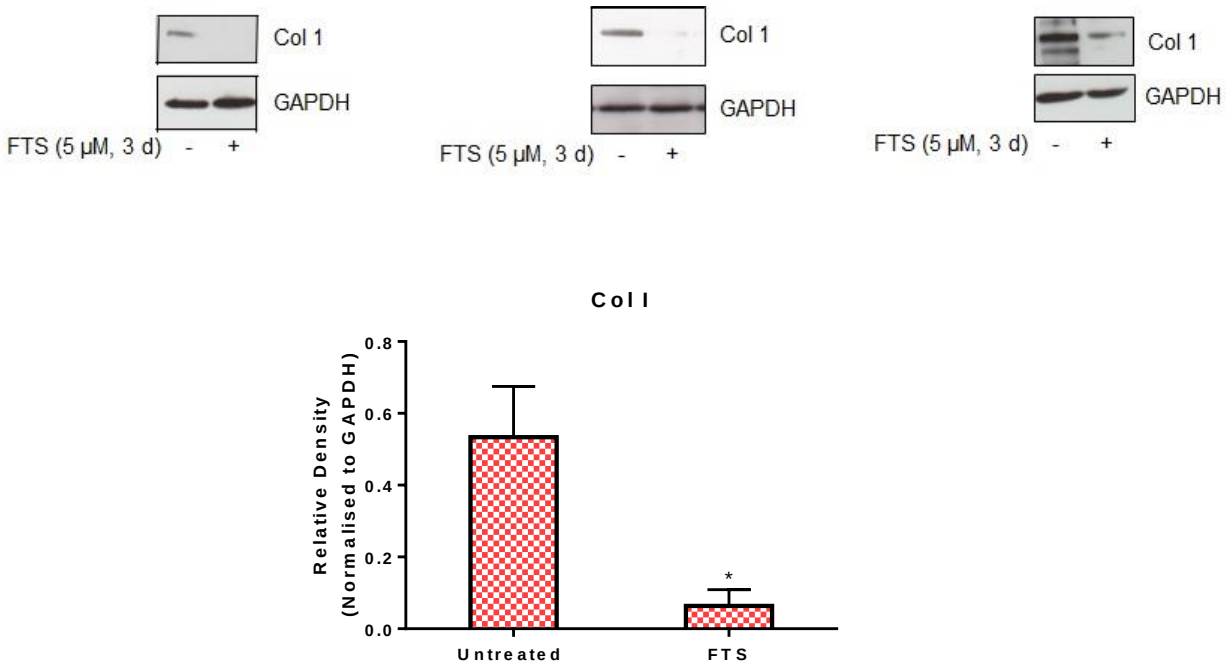


Figure 5-4 Effect of FTS on Col 1 Protein Expression in F-IPF

*Confluent F-IPF were serum starved for 24 hours and treated with FTS (5 µM, 3 days) prior to collection of total cell lysate and Western blot analysis of Col 1. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with untreated control.*

Following on from the protein changes induced by FTS treatment we next sought to determine if FTS regulated COX-2, α-SMA and Col 1 gene transcription and analysed mRNA expression in F-IPF. FTS treatment had no effect on COX-2 or IL-β-induced COX-2 expression (Figure 5-5) and no effect on α-SMA (Figure 5-6A) or Col 1 mRNA expression (Figure 5-6B). The mRNA data suggests FTS is unlikely to regulate COX-2, α-SMA and Col 1 expression via transcriptional regulation and is more likely to increase IL-1β-induced COX-2 and reduce Col 1 via post transcriptional mechanisms.

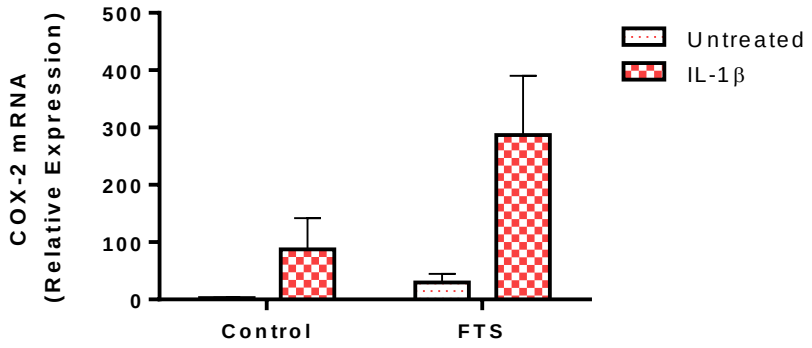


Figure 5-5 Effect of FTS on COX-2 mRNA Expression in F-IPF

Confluent F-IPF were treated with FTS (5 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for qPCR analysis. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM.

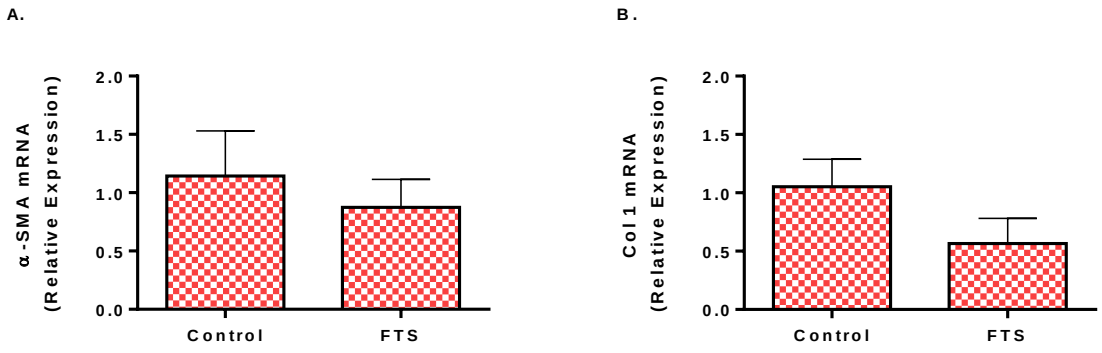


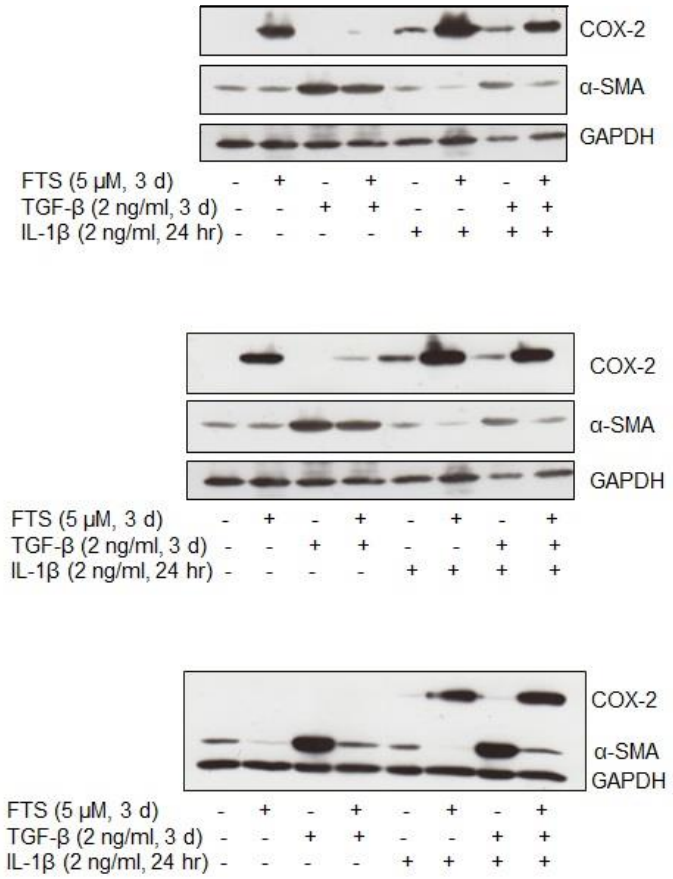
Figure 5-6 Effect of FTS on α-SMA and Col 1 mRNA Expression in F-IPF

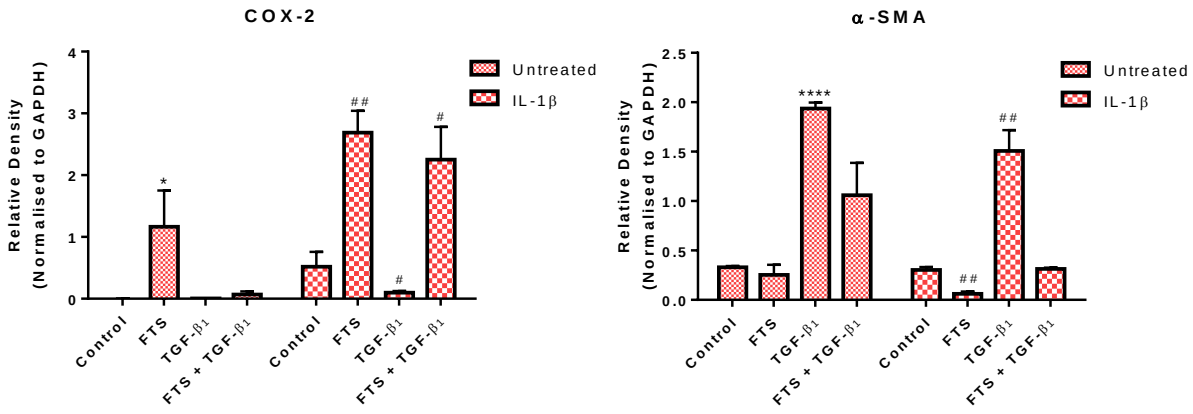
Confluent F-IPF were serum starved for 24 hours and treated with FTS (5 μM) for 24 hours prior to the collection of total RNA for qPCR analysis of (A) α-SMA and (B) Col1. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM.

5.4.2 FTS Prevents TGF-β1-induced repression of COX-2 and fibroblast to myofibroblast differentiation in F-NL

Our results demonstrate that FTS increased IL-1β-induced COX-2 expression and reversed fibroblast to myofibroblast differentiation in F-IPF, thus, FTS mimicked the anti-fibrotic effects of PGE_2. We next assessed if FTS treatment, like PGE_2, would be able to prevent TGF-β1-induced COX-2 repression and fibroblast to myofibroblast differentiation. Interestingly, FTS treatment alone was able to induce COX-2 expression and significantly increased IL-1β-induced COX-2 in F-NL (Figure 5-7). FTS treatment also prevented TGF-β1-induced COX-2 repression and COX-2 was significantly increased compared with IL-1β alone (Figure 5-7). Furthermore, FTS treatment with IL-1β stimulation significantly reduced α-SMA expression and prevented TGF-β1-induced α-SMA (Figure 5-7). FTS alone reduced Col I and data suggests that FTS also prevented TGF-β1-induced Col 1 expression, however this experiment was only completed in one cell line so further experiments are required to confirm this observation (Figure 5-7). Therefore, Ras inhibition is able to prevent TGF-β1-repressed COX-2 expression and inhibit TGF-β1-induced fibroblast to myofibroblast differentiation, suggesting that Ras activity or Ras signalling is necessary for TGF-β1-induced fibroblast to myofibroblast differentiation. These data further support the hypothesis that Ras activity may be necessary to maintain a pro-fibrotic phenotype as seen in F-IPF.

A)


COX-2
α-SMA
GAPDH
FTS (5 μM, 3 d) - + - + - + - +
TGF-β (2 ng/ml, 3 d) - - + + - - + +
IL-1β (2 ng/ml, 24 hr) - - - - + + + +
COX-2
α-SMA
GAPDH
FTS (5 μM, 3 d) - + - + - + - +
TGF-β (2 ng/ml, 3 d) - - + + - - + +
IL-1β (2 ng/ml, 24 hr) - - - - + + + +
COX-2
α-SMA
GAPDH
FTS (5 μM, 3 d) - + - + - + - +
TGF-β (2 ng/ml, 3 d) - - + + - - + +
IL-1β (2 ng/ml, 24 hr) - - - - + + + +


COX-2
Relative Density (Normalised to GAPDH)
Untreated
IL-1β
Control
FTS
TGF-β1
FTS + TGF-β1
α-SMA
Relative Density (Normalised to GAPDH)
Untreated
IL-1β
Control
FTS
TGF-β1
FTS + TGF-β1

B)

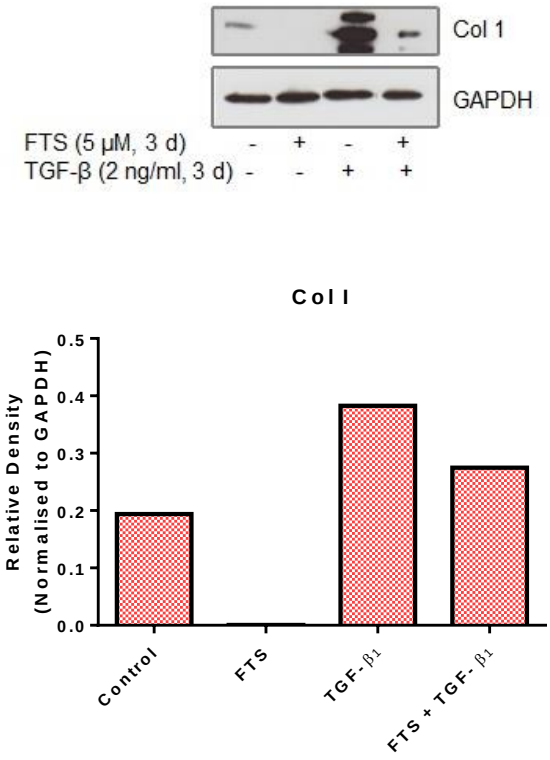


Figure 5-7 Effect of FTS on COX-2, α-SMA and Col 1 Protein in TGF-β1-treated F-NL

A) *Confluent F-NL were treated with TGF-β1 (2 ng/ml) alone, FTS (5 μM) alone or TGF-β1 (2 ng/ml) and FTS (5 μM) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml) for 24 hours prior to the collection of total cell lysate for analysis by Western blot of COX and α-SMA. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. ****p<0.001 compared with untreated control, #p<0.05, ##p<0.01 compared with IL-1β-treated control. B) Confluent F-NL were treated with TGF-β1 (2 ng/ml) alone, FTS (5 μM) alone or TGF-β1 (2 ng/ml) and FTS (5 μM) for 3 days and serum starved for 24 hours prior to the collection of total cell lysate for analysis by Western blot of Col 1. This experiment*

was performed in one cell line only. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH.

To determine if FTS regulates COX-2, α-SMA and Col 1 expression by transcriptional regulation we next assessed mRNA expression in F-NL. FTS treatment alone had no effect on COX-2 mRNA or IL-1β-induced COX-2 mRNA in F-NL. FTS treatment also had no effect on TGF-β1-induced COX-2 mRNA repression (Figure 5-8). In addition, FTS treatment had no effect on TGF-β1-induced α-SMA and Col 1 mRNA expression in F-NL (Figure 5-9). It is therefore likely that FTS regulates COX-2, α-SMA and Col 1 via post-transcriptional mechanisms.

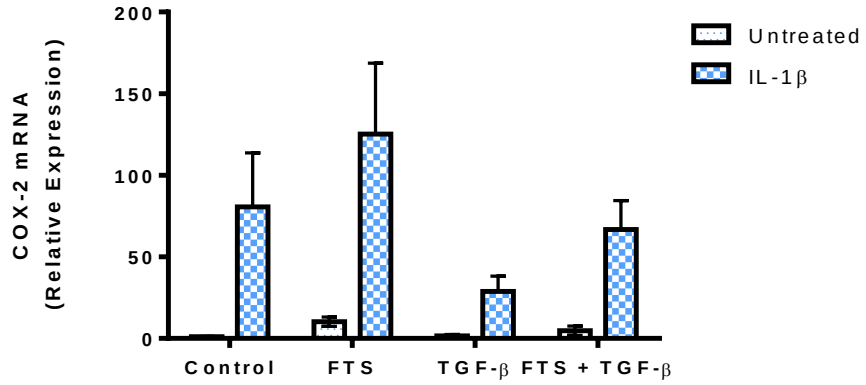


Figure 5-8 Effect of FTS on COX-2 mRNA Expression in TGF-β1-treated F-NL

Confluent F-NL were treated with TGF-β1 (2 ng/ml) alone, FTS (5 μM) alone or TGF-β1 (2 ng/ml) and FTS (5 μM) for three days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 4h) prior to the collection of total RNA for analysis by qPCR. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM.

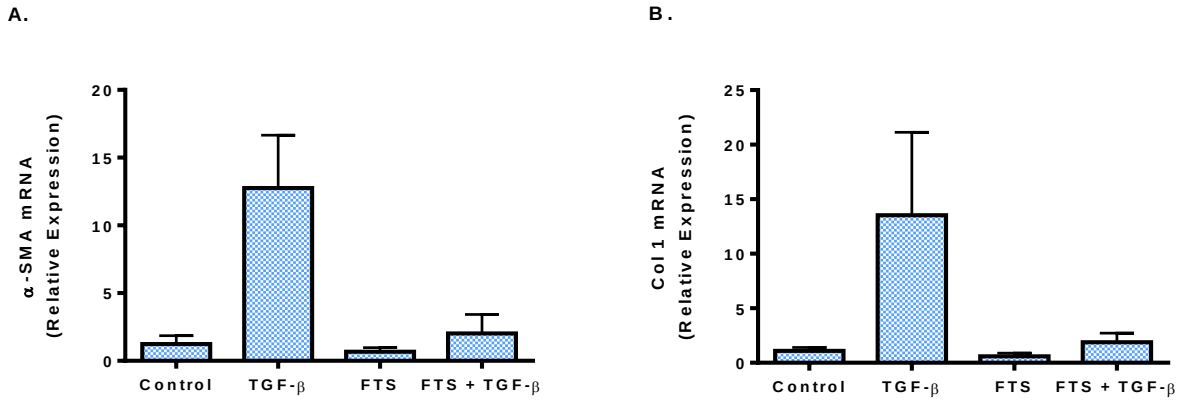


Figure 5-9 Effect of FTS on α-SMA and Col 1 mRNA Expression in TGF-β1 treated F-NL

Confluent F-NL were treated with TGF-β1 (2 ng/ml) alone, FTS (5 μM) alone or TGF-β1 (2 ng/ml) and FTS (5 μM) for 24 hours and serum starved for 24 hours prior to the collection of total RNA for analysis by qPCR of (A) α-SMA and (B) Col 1. This figure shows the amalgamation of data from separate experiments performed in duplicate of three different cell lines and is expressed as mean fold change over untreated control ± SEM.

5.4.3 FTS does not directly effect cAMP production in F-NL or F-IPF

Our data has shown that PGE_2 reverses fibroblast to myofibroblast differentiation and this is mimicked by FTS treatment. Results in the previous chapter suggest that PGE_2 increased IL-1β–induced COX-2 expression via increased cAMP. Cross talk between the cAMP pathway and Ras signalling pathways has been studied previously. It has been demonstrated that cAMP has differential effects on Ras signalling and can either activate or inhibit the Ras/Raf/ERK signalling pathway by several different mechanisms in a cell type specific manner (Stork and Schmitt, 2002). However, it is unknown whether the inhibition of Ras affects cAMP production. We therefore wanted to confirm if FTS, like PGE_2, increases cAMP production in F-NL or F-IPF. FTS treatment for 30 minutes did not induce cAMP production in F-NL nor F-IPF (Figure 5-10). Therefore, increased COX-2 expression by FTS treatment in F-NL and F-IPF is not due to cAMP signalling. We next wanted to determine if inhibiting Ras had any effect on cAMP production after stimulation with PGE_2, Form or FSK. Similarly, FTS pre-treatment had no effect on cAMP production following PGE_2, Form or FSK treatment in F-NL or F-IPF (Figure 5-11). Therefore, Ras inhibition does not directly effect cAMP production and inhibition of Ras is likely to have an indirect effect on cAMP production resulting in induced COX-2 expression, PGE_2 production and reversal of fibroblast to myofibroblast differentiation.

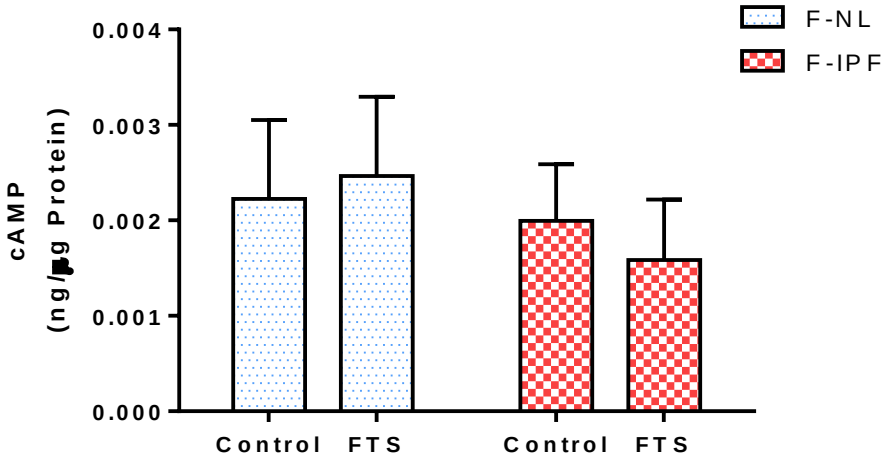


Figure 5-10 Effect of FTS on cAMP Production in F-NL and F-IPF

Confluent F-NL and F-IPF were serum starved for 24 hours and treated for 30 minutes with FTS (5 µM) and IBMX (1 mM). To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from three separate experiments performed in duplicate of three different cell lines and is expressed as mean ±SEM.

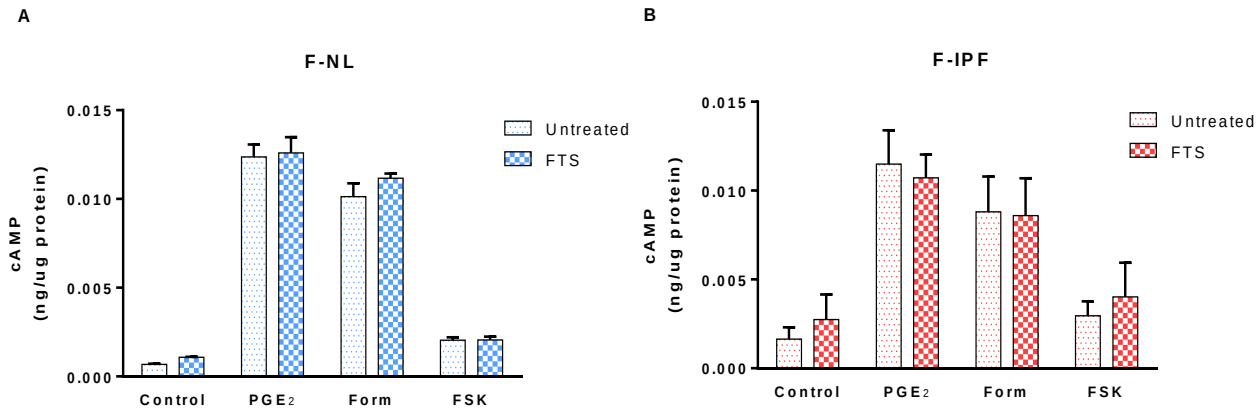


Figure 5-11 Effect of FTS Treatment on PGE_2, Form and FSK-induced cAMP Production in F-NL and F-IPF

Confluent (A) F-NL and (B) F-IPF were serum starved for 24 hours and treated with FTS (5 µM) and IBMX (1 mM) for 30 minutes prior to the addition of PGE_2 (1 µM), Form (10 µM) and FSK (10 µM) for a further 30 minutes. To terminate the reactions trichloroacetic acid (TCA) was added to the cell culture medium and cAMP content in TCA extracts was determined by radioimmunoassay. This figure shows the amalgamation of data from separate experiments performed in three different cell lines and is expressed as mean ±SEM.

5.4.4 Ras, Raf-1 and B-Raf activity is similar in F-NL and F-IPF but ERK-1/2 expression is reduced in F-IPF

Our data so far suggests that Ras activity may be important in regulating COX-2 expression and fibroblast to myofibroblast differentiation as Ras inhibition, by FTS, both prevents and reverses fibroblast to myofibroblast differentiation. We therefore hypothesised that Ras activity could be increased in F-IPF compared with F-NL and that hyperactive Ras could be responsible for COX-2 repression and the persistence of a pro-fibrotic phenotype in F-IPF. Consequently, we analysed Ras activity in F-NL and F-IPF. Ras activity and total Ras expression was comparable in F-NL and F-IPF (Figure 5-12A and Figure 5-12E). This suggests that Ras activity itself may not be responsible for regulating COX-2 expression so we investigated the activity and expression of downstream effectors of Ras. As previous studies have demonstrated that inhibition of ERK-1/2 prevented bleomycin-induced fibrosis in a mouse model (Galuppo et al., 2011) we focussed on the ERK-1/2 signalling pathway and its potential role in regulating COX-2 expression and fibroblast to myofibroblast differentiation. We first determined basal expression and activity of Raf-1, B-Raf and ERK-1/2. Raf-1, B-Raf and ERK-1/2 activity was determined using antibodies specific to the phosphorylation sites that are crucial for their activation. Similar to Ras activity, Phospho-Raf-1 (Figure 5-12B and Figure 5-12F), Phospho-B-Raf (Figure 5-12C and Figure 5-12G) and Phospho-ERK-1/2 (Figure 5-12D and Figure 5-12H) were comparable in F-NL and F-IPF. There was a trend for a decrease in Phopsho-ERK-1/2 in F-IPF compared with F-NL however this was likely due to one outlier cell line. In all, this data suggests that Ras activity and downstream effectors of Ras, Phopsho-Raf-1, Phospho-B-Raf and phospho-ERK-1/2 are similar in F-NL and F-NL. Despite no difference in Ras activity or expression of downstream effectors, inhibition of Ras activity by FTS promotes an anti-fibrotic phenotype and therefore Ras signalling may be necessary for the maintenance of a pro-fibrotic phenotype seen in F-IPF.

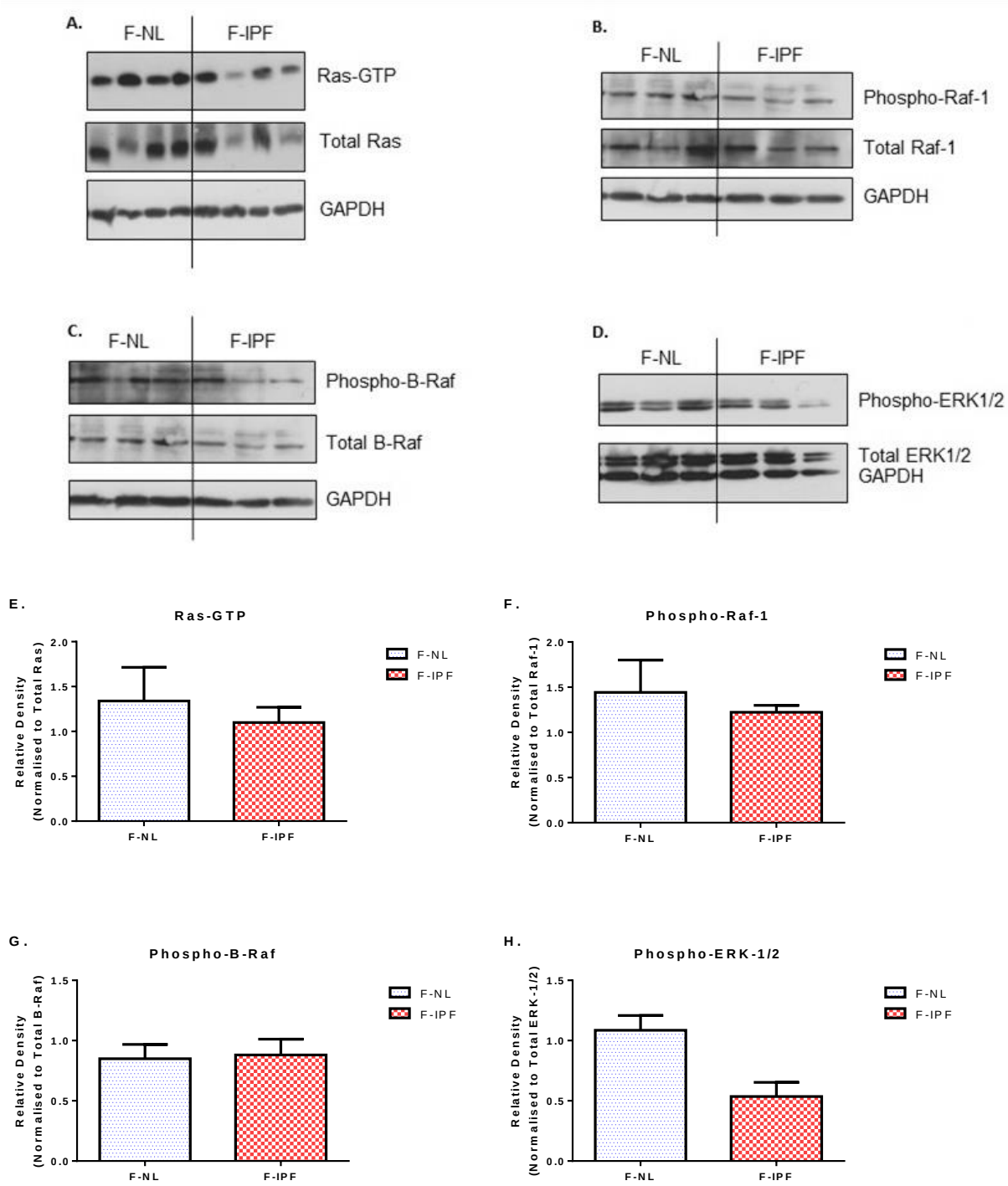


Figure 5-12 Ras, Raf-1, B-Raf and ERK-1/2 Protein Expression in F-NL and F-IPF

A) Confluent F-NL and F-IPF were serum starved for 24 hours prior to the collection of total cell lysate. 500 µg of total cell lysate was used in the GTP pull down assay and then analysed by Western blot. This figure shows data from four different cell lines. B, C & D). Confluent F-NL and F-IPF were serum starved for 24 hours prior to the collection of total cell lysate for

analysis by Western blot. This figure shows data from three different cell lines. Relative density was calculated by normalising the density of the (E) Ras-GTP bands, (F) Phospho-Raf-1 bands, (G) Phospho-B-Raf bands and (H) Phospho-ERK-1/2 bands against that of total Ras, total B-Raf, total Raf-1 and total ERK-1/2, respectively. This figure shows data from three different cell lines and is expressed as mean ± SEM.

5.4.5 TGF-β1 and PGE_2 treatment rapidly activates the Ras/Raf/ERK signalling pathway

In order to further investigate the role of Ras in regulating COX-2 expression and fibroblast to myofibroblast differentiation we next analysed the effect of TGF-β1 and PGE2 on Ras activity in F-NL and F-IPF, respectively. It is well known that many external stimuli, including TGF-β1, activate Ras to mediate distinct biological outcomes (Olson and Marais, 2000). Previous studies have demonstrated that Ras/MEK/ERK signalling is required for the induction of CTGF expression, a downstream mediator of TGF-β1, in mouse NIH 3T3 fibroblasts, a commercially available cell line originating from mouse embryos, and in human dermal fibroblasts (Stratton et al., 2002; Stratton et al., 2001). Stratton and colleagues also demonstrated that iIloprost, a prostacyclin analogue, inhibited TGF-β1-induced CTGF expression and concluded that iloprost, via increased cAMP and PKA activation, negatively regulated the Ras/MEK/ERK signalling pathway (Stratton et al., 2002). With regards to our data presented so far, PGE_2 and FTS have similar anti-fibrotic effects and thus PGE_2 could potentially reduce Ras activity to increase COX-2 expression. We therefore wanted to determine the effect of TGF-β1 and PGE_2 on Ras activity in F-NL and F-IPF. It has previously been demonstrated, in mouse intestinal epithelial cells, that TGF-β1 activated Ras within 3 – 6 minutes (Mulder and Morris, 1992). We therefore conducted time course experiments, 0, 5, and 15 minutes, to determine the direct effect of TGF-β1 and PGE_2 on Ras activity. Active Ras was detectable basally in both F-NL and F-IPF. TGF-β1 treatment reduced Ras activity after 15 minutes but total Ras expression did not change throughout the time course (Figure 5-13). Interestingly, PGE_2 increased Ras activation after 5 minutes which started to decrease at 15 minutes, but total Ras did not change (Figure 5-14). This demonstrated that TGF-β1 and PGE_2 both have different effects on Ras activity. The rapid nature of these effects strongly suggests that increases or decreases in Ras activation is a direct and not a secondary effect of TGF-β1 or PGE_2. As ERK-1 and ERK-2 are the final MAP kinases involved in the Ras/Raf/ERK signalling cascade we next sought to determine if changes in Ras activity, by TGF-β1 and PGE_2, effected downstream signalling

and thus measured ERK-1/2 phosphorylation. We found that ERK-1/2 was basally phosphorylated in F-NL and F-IPF and both TGF-β1 and PGE_2 significantly increased ERK-1/2 phosphorylation at 5 minutes which was sustained at 15 minutes and total ERK-1/2 expression remained unchanged (Figure 5-15 and Figure 5-16). This demonstrates that both TGF-β1 and PGE_2 activate the ERK signalling pathway suggesting that Ras activity and subsequent ERK-1/2 phosphorylation may be involved in TGF-β1 and PGE_2 signalling.

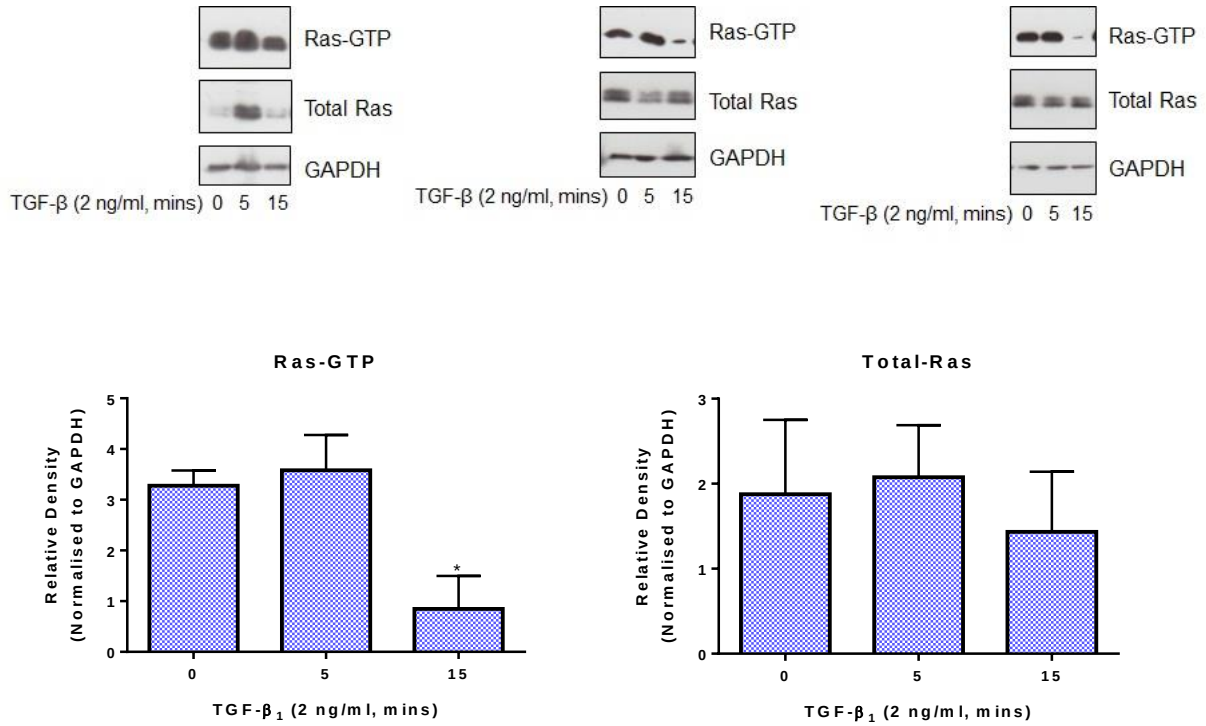


Figure 5-13 Effect of TGF-β1 on Ras Activity in F-NL

*F-NL were serum starved for 24 hours and treated with TGF-β1 (2 ng/ml) for 0, 5 and 15 minutes prior to the collection of total cell lysate. 500 µg of total cell lysate was used in the GTP pull down assay and then analysed by Western blot. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Ras-GTP and Total Ras bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with untreated control.*

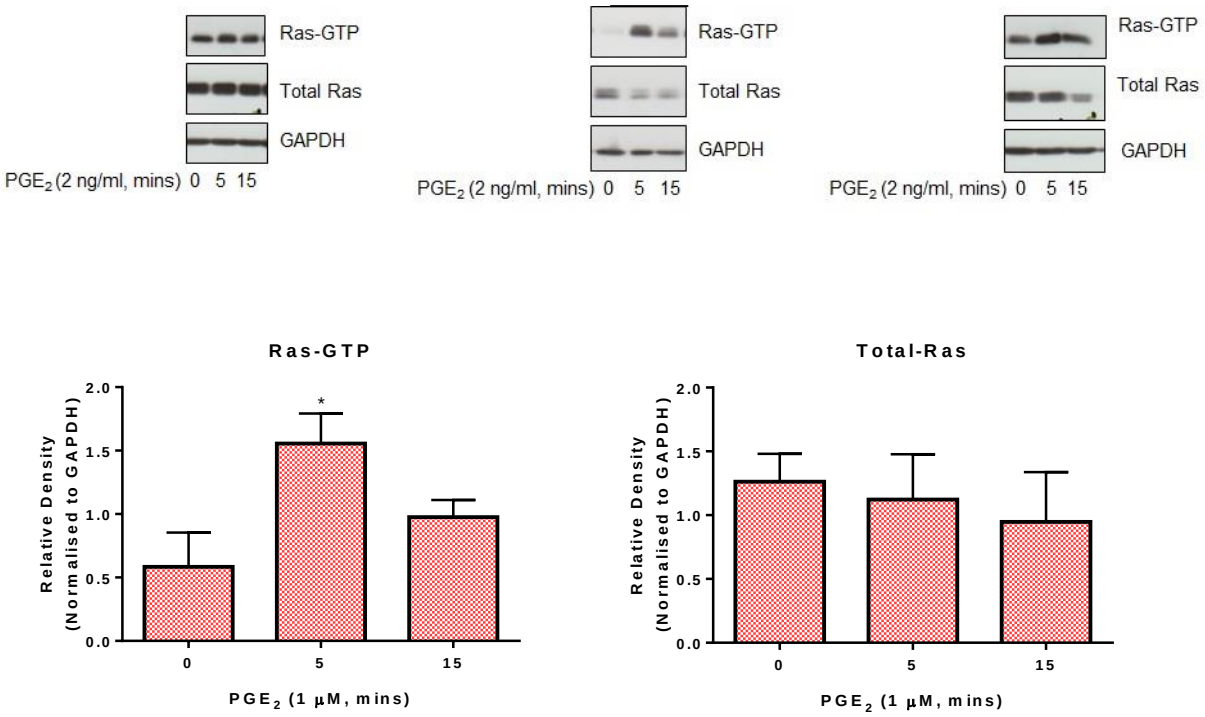


Figure 5-14 Effect of PGE_2 on Ras Activity in F-IPF

*F-IPF were serum starved for 24 hours and treated with PGE_2 (1 µM) for 0, 5 and 15 minutes prior to the collection of total cell lysate. 500 µg of total cell lysate was used in the GTP pull down assay and then analysed by Western blot. This figure is shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Ras-GTP and Total Ras bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared with untreated control.*

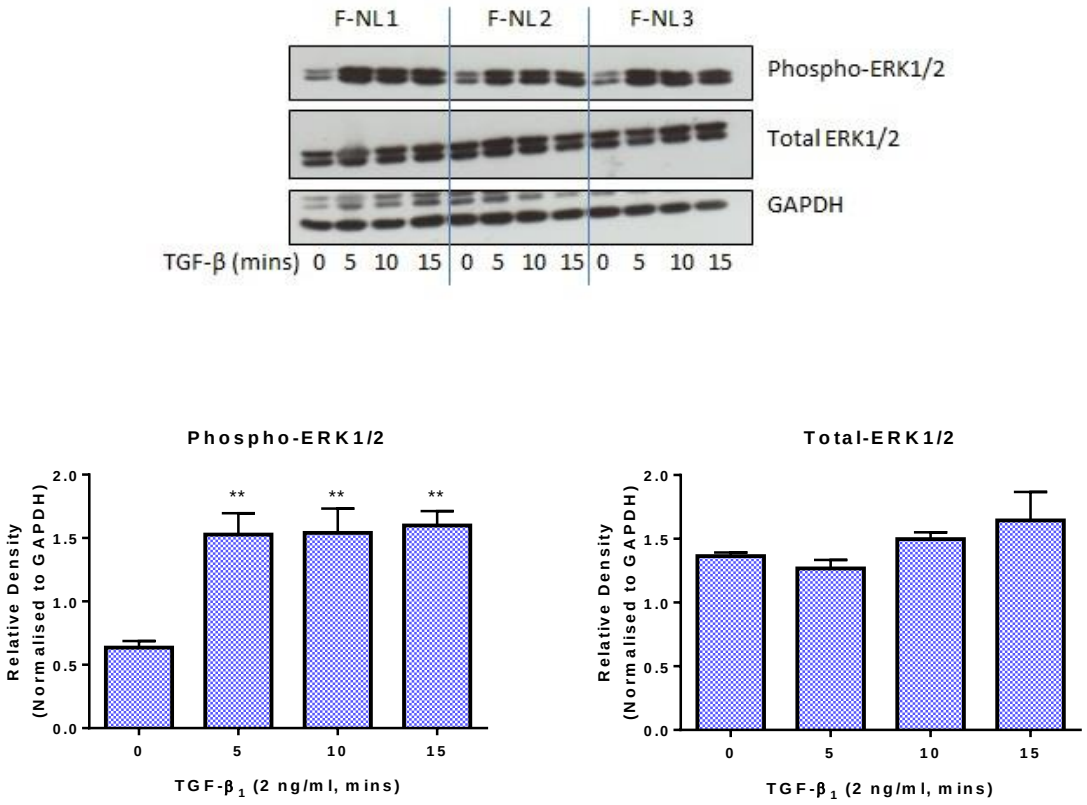


Figure 5-15 Effect of TGF-β1 on Phosphorylated ERK1/2 in F-NL

*A) F-NL were serum starved for 24 hours and treated with TGF-β1 (2 ng/ml) for 0, 5, 10 and 15 minutes and B) F-IPF were serum starved for 24 hours and treated with PGE_2 (1 μM) for 0, 5, 10 and 15 minutes prior to the collection of total cell lysate for analysis by Western blot. This figure shows one experiment performed in three different cell lines. Relative density was calculated by normalising the density of the Phospho-ERK-1/2 and Total ERK-1/2 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. **p<0.01 compared with untreated control.*

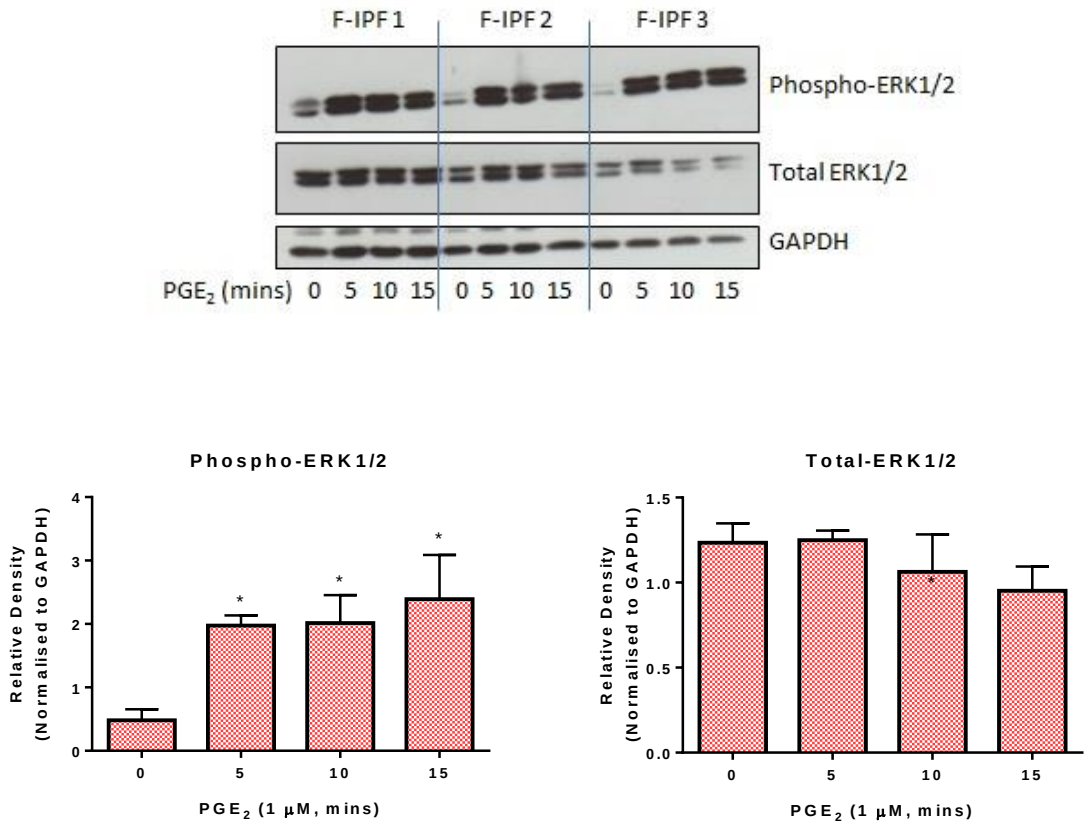


Figure 5-16 Effect of PGE_2 on Phosphorylated ERK1/2 in F-IPF

*(A) F-NL were serum starved for 24 hours and treated with TGF-β1 (2 ng/ml) for 0, 5, 10 and 15 minutes and (B) F-IPF were serum starved for 24 hours and treated with PGE_2 (1 µM) for 0, 5, 10 and 15 minutes prior to the collection of total cell lysate for analysis by Western blot. This figure shows one experiment performed in three different cell lines. Relative density was calculated by normalising the density of the Phospho-ERK-1/2 and Total ERK-1/2 bands against that of GAPDH. *p<0.05 compared with untreated control*.

5.4.6 Chronic TGF-β1 and PGE_2 Treatment has no effect on Ras activity but chronic PGE_2 treatment reduces ERK-1/2 activity

Our data showing that TGF-β1 treatment increased Ras activity and ERK-1/2 phosphorylation in F-NL is supported by several other studies in which TGF-β1 has been shown to signal via Ras in different cell lines (Hartsough et al., 1996; Mulder and Morris, 1992; Stratton et al., 2002). However, the fact that PGE_2 also increased Ras activity and phosphorylated ERK-1/2 was unexpected. Both PGE_2 treatment, which initially activated Ras, and FTS treatment, which antagonised Ras, increased COX-2 expression in F-IPF and prevented TGF-β1-induced COX-2 repression in F-NL. Therefore, long term treatment with PGE_2 may have different effects to short term treatment with regard to Ras activity and ERK-1/2 phosphorylation. The Ras/Raf/ERK signalling pathway is highly complex and ERK-1/2 exerts several positive and negative feedback loops in order to tightly control cellular outcome (Shin et al., 2009). Due to these complex feedback mechanisms it is plausible that the effects with short-term treatment differ significantly from long-term treatment. In addition, we are primarily interested in long-term treatment as the effect on COX-2 expression, by TGF-β1 and PGE_2, are maximal at 3 days. We therefore determined the effect of TGF-β1 and PGE_2 on Ras activity and phosphorylated ERK-1/2 after 3 days of treatment in F-NL and F-IPF, respectively. Ras activity remained the same after 3 day treatment with TGF-β1 and PGE_2 in F-NL and F-IPF, respectively (Figure 5-17). As Ras functions as a molecular switch its activation is usually transient and therefore we did not expect to detect changes after long-term treatment. As such, we investigated the effect of long-term treatment with TGF-β1 and PGE_2 on phosphorylated ERK-1/2. Three day treatment with TGF-β1 had no effect on ERK-1/2 phosphorylation in F-NL. However, three day treatment with PGE_2 resulted in a significant decrease in phosphorylation of ERK-1/2 in F-IPF (Figure 5-18). Therefore, ERK-1/2 activity may play a role in maintaining a pro-fibrotic phenotype in F-IPF. Activation of ERK-1/2 is usually rapid and transient however, in fibrotic dermal fibroblasts ERK-1/2 expression is increased at 15 minutes and remains elevated at 1, 3, 6 and 12 hours but starts to decrease

at 24 hours (Samuel et al., 2010). Therefore, TGF-β1 treatment may have caused pro-longed increases in ERK-1/2 in F-NL but only up to 24 hours. Based on these data a time course of TGF-β1 up to three days would confirm the kinetics of ERK-1/2 phosphorylation in F-NL. Overall, this data suggests that although Ras activation may be involved in TGF-β1 and PGE_2 signalling initially, basal Ras and ERK-1/2 activity may be necessary for maintaining the already differentiated pro-fibrotic phenotype. Therefore, PGE_2 may exert its anti-fibrotic effect partially through regulation of ERK-1/2 activity.

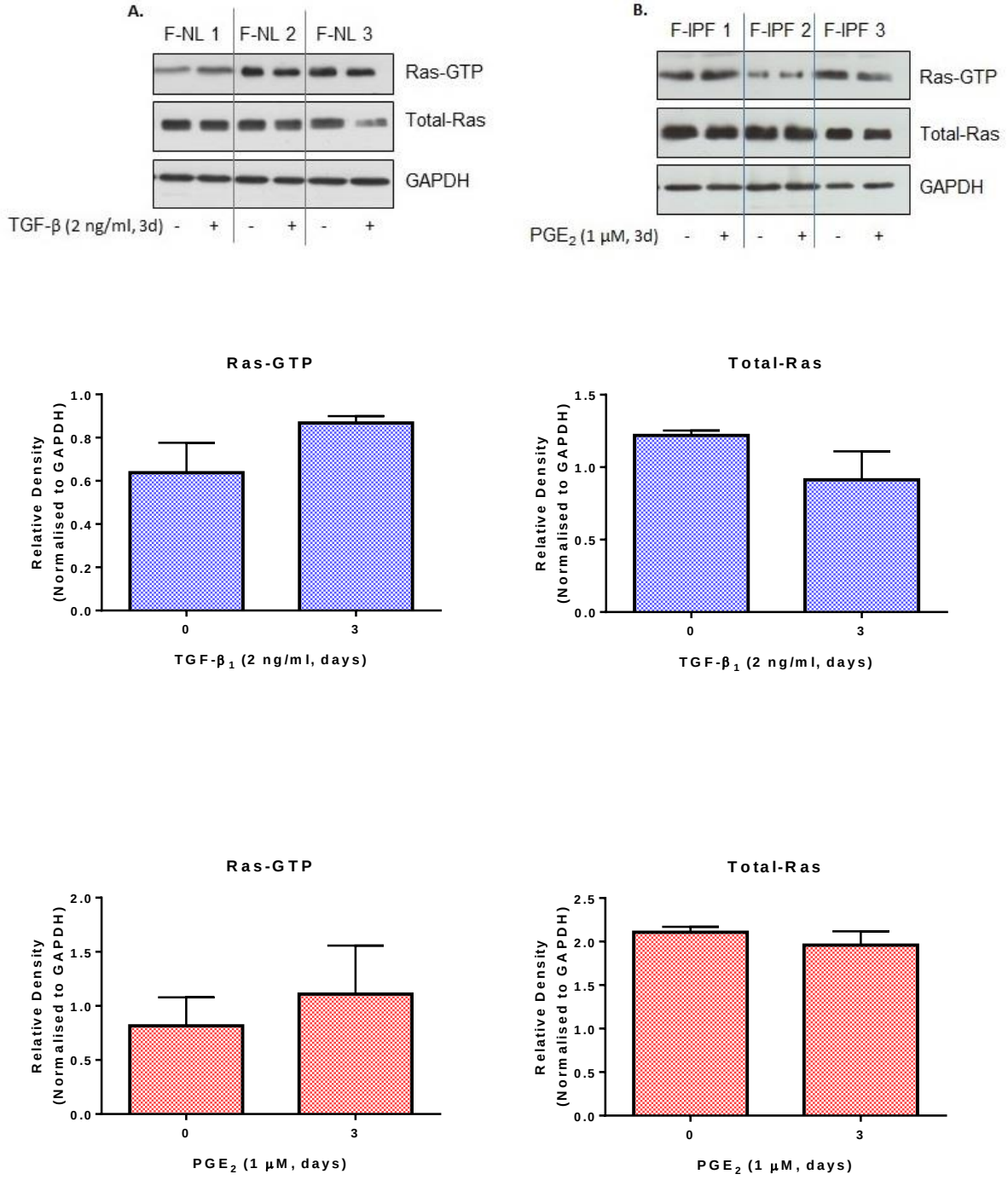
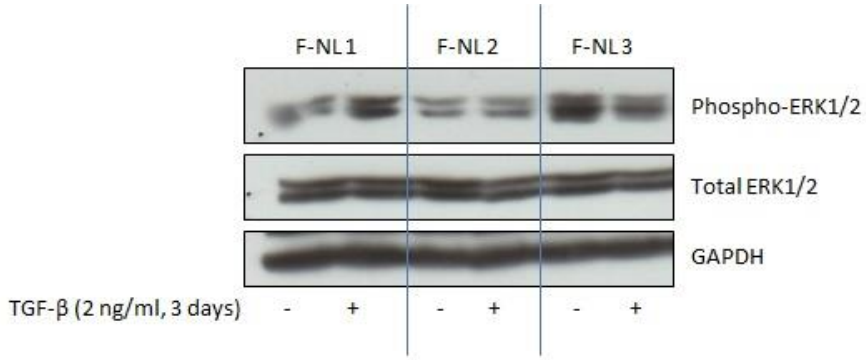


Figure 5-17 Effect of Chronic TGF-β1 and PGE_2 Treatment on Ras Activity in F-NL and F-IPF

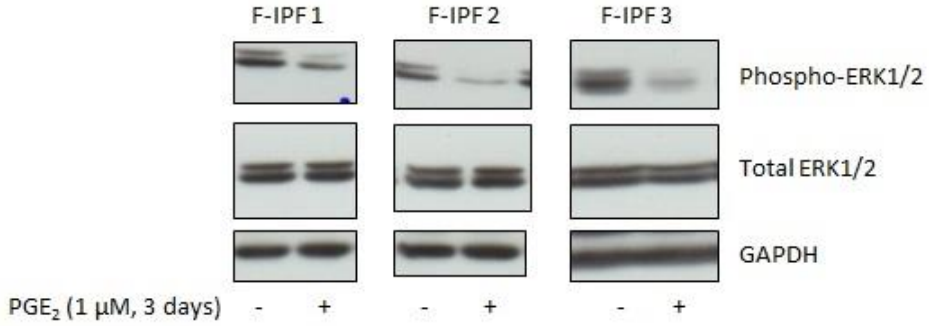
A) F-NL were treated with TGF-β1 (2 ng/ml) for 3 days and (B) F-IPF were treated with PGE_2 (1 µM) for 3 days and serum starved for 24 hours prior to the collection of total cell lysate. 500 µg of total cell lysate was used in the GTP pull down assay and then analysed by Western blot. This figures includes data from one experiment performed in three different cell lines.

Relative density was calculated by normalising the density of the Ras-GTP and Total Ras bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines.

A)


F-NL1
F-NL2
F-NL3
Phospho-ERK1/2
Total ERK1/2
GAPDH
TGF-β (2 ng/ml, 3 days)
- + - + - +

B)


F-IPF 1
F-IPF 2
F-IPF 3
Phospho-ERK1/2
Total ERK1/2
GAPDH
PGE2 (1 µM, 3 days)
- + - + - +

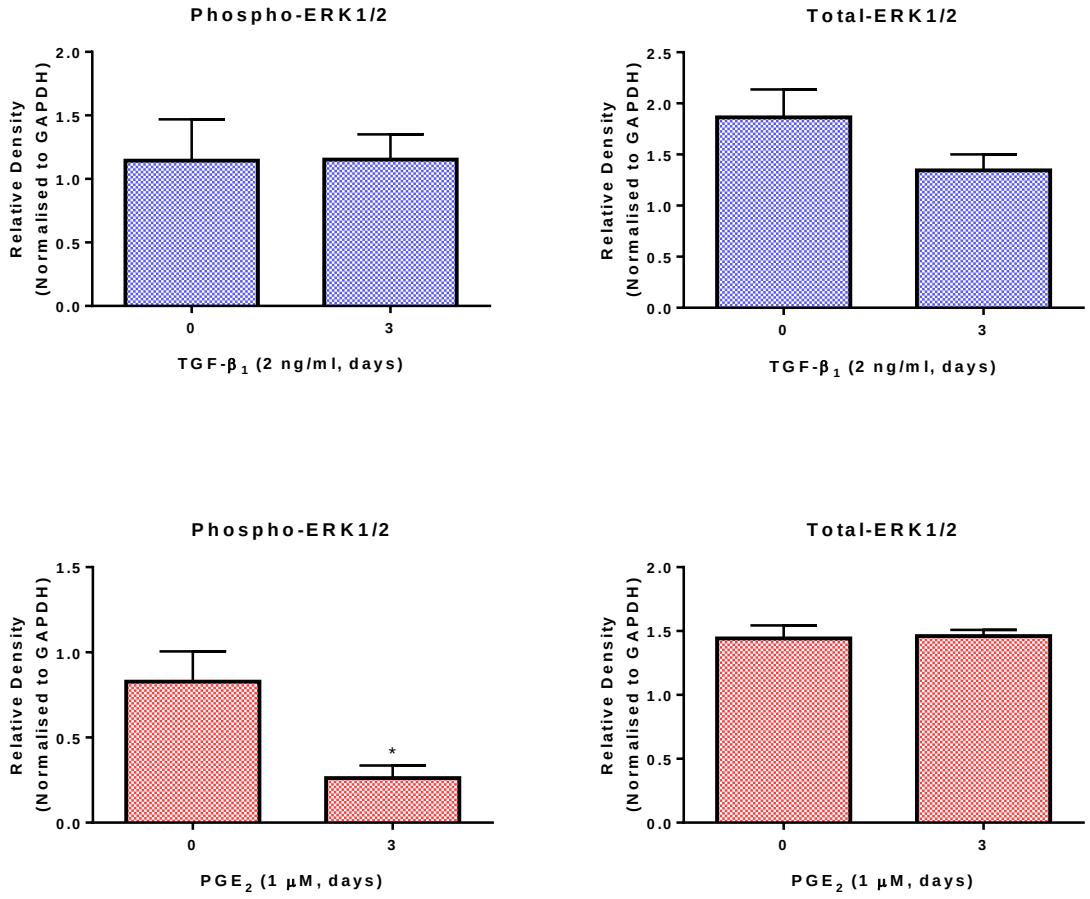


Figure 5-18 Effect of Chronic TGF-β1 and PGE_2 Treatment on ERK Phosphorylation in F-NL and F-IPF

A) F-NL were treated with TGF-β1 (2 ng/ml) for 3 days and serum starved 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows one experiment performed in three different cell lines. Relative density was calculated by normalising the density of the Phospho-ERK-1/2 and Total ERK-1/2 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. B) *F-IPF were treated with PGE_2 (1 µM) for 3 days and serum starved 24 hours prior to the collection of total cell lysate for Western blot analysis. This figure shows three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the Phospho-ERK-1/2 and Total ERK-1/2 bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05 compared to control.*

5.5 Discussion

Previous studies, in various animal models of fibrosis, have demonstrated that the inhibition of Ras ameliorated fibrosis and thus Ras signalling could play an important role in regulating fibroblast to myofibroblast differentiation (Bechtel et al., 2011; Nevo et al., 2011). In this chapter, we investigated whether Ras signalling was involved in regulating COX-2 expression and fibroblast to myofibroblast differentiation. The effect of FTS on COX-2 expression and fibroblast to myofibroblast differentiation has not been investigated prior to this study. We have shown that Ras inhibition, using FTS, increased IL-1β-induced COX-2 expression in F-IPF, prevented TGF-β1-induced COX-2 repression in F-NL and promoted an anti-fibrotic phenotype. Therefore, Ras may play a role in fibroblast to myofibroblast differentiation.

Although FTS has been widely used and proven to be a unique and potent Ras inhibitor in various cell lines (Halaschek-Wiener et al., 2000; Marom et al., 1995) we cannot rule out the possibility that FTS may interfere with the action of other prenylated proteins, such as proteins of the Rac/Rho family of GTPases, that are associated with the control of actin cytoskeleton, cell growth and cell motility (Hall, 2012). Thus, future studies using alternative methods to inhibit Ras, such as short interfering RNA against Ras, would be necessary to confirm the effects of FTS on fibroblast to myofibroblast regulation.

Due to the fact that Ras inhibition is able to increase IL-1β-induced COX-2, reduce Col 1 expression and prevent TGF-β1-induced repression of COX-2 we hypothesised that Ras activity could be increased in F-IPF compared with F-NL. Hyperactive Ras has been reported in fibroblasts isolated from mice with experimentally induced renal fibrosis (Bechtel et al., 2011) and liver fibrosis (Liu et al., 1994). Our results demonstrated that Ras activity is similar in both F-NL and F-IPF. This difference could be explained by the fact that the signalling properties of Ras are dependent upon the cellular context in which Ras operates. For example,

when Ras was over expressed in normal human foreskin diploid fibroblasts and thyroid epithelial cells, proliferation was inhibited in fibroblasts but stimulated in epithelial cells (Skinner et al., 2004). Despite no difference in basal Ras activity in F-NL and F-IPF, several studies have demonstrated that treatment with FTS, or inhibiting Ras, had a protective effect on animal models of fibrosis from various organs (Bechtel et al., 2011; Nevo et al., 2011; Reif et al., 2004; Stratton et al., 2002) and thus supports our finding that reducing Ras activity promotes an anti-fibrotic phenotype.

Our results demonstrate that TGF-β1 and PGE_2 have opposite effects on Ras activity. During the 15 minute time course TGF-β1 decreased Ras activity whereas PGE_2 increased Ras activity. Despite the differing effects on Ras activity, TGF-β1 and PGE_2 both caused rapid increases in ERK-1/2, a downstream mediator of Ras. The increase in ERK-1/2 happened after five minutes which is before the changes in Ras activity following TGF-β1 and PGE_2, treatment (fifteen minutes and ten minutes, respectively). This suggest that the ERK-1/2 signalling cascade may be involved in regulating fibroblast to myofibroblast differentiation but the role of Ras activity within this pathway remains unclear. Our laboratory has previously demonstrated that short term TGF-β1 treatment, i.e. 4 and 24 hours, increases COX-2 expression in F-NL (Coward et al., 2009). Therefore, Ras activation and ERK-1/2 signalling may be important for the initial induction of COX-2 expression. Future experiments should include the use of an ERK-1/2 inhibitor and measuring COX-2 expression after treatment with PGE_2 to determine if ERK-1/2 is necessary for IL-1β-induced COX-2 expression. Our data showing reduced ERK-1/2 in F-IPF is supported by numerous studies in various cell lines, such as WI-38, a foetal fibroblast cell line, and A549, a human pulmonary epithelial carcinoma cell line, in which short term ERK-1/2 inhibition resulted in reduced COX-2 expression (Chen et al., 2004; Shih et al., 2009). Our findings are also consistent with Liu and colleagues who demonstrated that short term treatment with cAMP elevating agents stimulate ERK-1/2 phosphorylation in adult rat cardiac fibroblasts (Liu et al., 2006a).

However, the long term effects of TGF-β1 and PGE_2 treatment on Ras activity and ERK-1/2 expression may differ significantly from short term effects as both the duration and intensity of Ras signalling are important for biological outcome. In support of this, short term treatment with TGF-β1 induces COX-2 expression (Coward et al., 2009) whereas our study demonstrates that long term treatment actually represses COX-2 expression. Although short term treatment with TGF-β1 decreased Ras activity and PGE_2 treatment resulted in activation of Ras, long term treatment with TGF-β1 and PGE_2 had no effect on Ras activity. The lack of effect on Ras activity following long term treatment is likely due to the fact that Ras activity is extremely transient. It has previous been demonstrated that despite continuous stimulation of receptor tyrosine kinase activity, active Ras returned to inactive GDP bound Ras within five minutes (Shin et al., 2009). Therefore, analysis of a downstream pathway of Ras, such as the ERK-1/2 signalling pathway, may provide further insight into the Ras signalling pathway involved after long term treatment. Three day treatment with TGF-β1 had no effect on ERK-1/2 phosphorylation, however, three day PGE_2 treatment resulted in significantly reduced ERK-1/2 phosphorylation. Therefore, the anti-fibrotic effects of PGE_2, such as increased IL-1β-induced COX-2 expression and reduced α-SMA and Col 1, could be partially mediated by ERK-1/2 inhibition. Currently, we are unsure as to why a reduction in ERK-1/2 phosphorylation promotes an anti-fibrotic phenotype and further investigation is required.

The signalling pathways involved in both short term and long term TGF-β1 and PGE_2 signalling also remains to be determined. The Ras/Raf/ERK pathway features a cascade of sequential phosphorylation and activation from Raf to ERK-1/2. This type of pathway architecture can lead to significant amplification of the original upstream signal, in addition, multiple and well characterised negative feedback loops can cause desensitisation or dampening of the signal (Chapnick et al., 2011) which could explain the difference in ERK-1/2 phosphorylation

following short and long term treatment. Furthermore, there are multiple integration points between the Ras signalling pathway and other pathways and thus the intricate interaction of these pathways produce complex and diverse biological outcomes (Chapnick et al., 2011). The signalling pathway(s) of PGE_2 that reduces ERK-1/2 phosphorylation is currently unknown. The different effect of cAMP on ERK-1/2 activation in other cells has been related to the expression levels of either Raf-1 or B-Raf and adaptor proteins that target PKA to Raf proteins (Dumaz and Marais, 2005). The relative expression and function of Raf isoforms and the various proteins that regulate them is not fully defined in pulmonary fibroblasts. The effect of PGE_2 on Raf signalling and adaptor proteins remains to be determined. Furthermore, although long term PGE_2 treatment reduced ERK-1/2 phosphorylation we have not yet studied other Ras signalling pathways. Previous studies have demonstrated that PGE_2 can inhibit other downstream effectors of Ras, for example, PGE_2 treatment resulted in inhibition of PI3K signalling in normal human foetal lung fibroblasts, (White, 2008) and thus we do not know the relative importance of ERK-1/2 in the regulation of COX-2 expression and fibroblast to myofibroblast differentiation.

In summary, our results demonstrate that inhibiting Ras activity is able to both prevent and reverse fibroblast to myofibroblast differentiation. Our results suggest that during TGF-β1-induced fibroblast to myofibroblast differentiation inhibition of Ras prevents TGF-β1-induced pro-fibrotic effects. Furthermore, ERK-1/2 expression is reduced following PGE_2 treatment. Therefore, Ras activity and possibly ERK-1/2 regulates fibroblast to myofibroblast differentiation. The effects of FTS may mimic PGE_2 due to its ability to inhibit ERK-1/2 activation. Thus, the anti-fibrotic effects of FTS support the fact that inhibitors of Ras, as an alternative to PGE_2 treatment, may be able to prevent and reverse fibroblast to myofibroblast differentiation.

6 EFFECT OF EPIGENETIC INHIBITORS ON COX-2 EXPRESSION AND FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION

6.1 Introduction

Epigenetics describes heritable changes in gene expression that are not caused by alterations in the DNA nucleotide sequence itself. Changes in DNA methylation and histone modifications can selectively activate or inactivate genes that control cell growth, proliferation and apoptosis and determine when and where a gene is expressed (Adcock et al., 2007). Histone tails are susceptible to covalent modifications, such as acetylation and methylation, which regulate gene expression. Histone acetylation is controlled by the balance of histone acetyltransferases (HATs) and histone deacetylases (HDACs) and histone methylation is controlled by histone methyltransferases (HMTase). Gene promoter regions with increased histone acetylation are usually associated with gene expression, in contrast, gene promoter regions associated with deacetylation of histones are usually transcriptionally repressed (Allfrey et al., 1964). Histone methylation is a more complicated process and depending on the site of methylation can result in gene activation or repression. DNA methylation, regulated by DNA methyltransferases (DNMTs), is another epigenetic modification whereby hypermethylation is usually associated with gene repression (Bian et al., 2013).

Previous studies have demonstrated that epigenetic modifications are likely to be involved in the regulation of genes involved in the pathogenesis of IPF. Previous data from our laboratory has demonstrated that COX-2 gene transcription in F-IPF was defective compared with F-NL due to deficient histone H3 and H4 acetylation. The deficient histone acetylation was a result of decreased HAT recruitment and increased recruitment of co-repressors to the COX-2 promoter (Coward et al., 2009). In addition, defective histone acetylation is also responsible for the repression of the anti-fibrotic mediator, IP-10 (IFN-γ-inducible protein of 10 kDa), a

strong inhibitor of angiogenesis (Coward et al., 2010b). Similarly, Thy-1 (CD90) is an important regulator of cell-cell and cell-matrix interactions that is expressed on normal lung fibroblasts, but its expression is absent on myofibroblasts isolated from patients with IPF. Down regulation of Thy-1 in rat lung fibroblasts is regulated by both promoter DNA hypermethylation (Sanders et al., 2008) and histone modifications (Sanders et al., 2011). Therefore, chromatin structural changes, including alterations in the histone acetylation/deacetylation balance, and changes in DNA methylation may contribute to the pathogenesis of IPF.

Increasing evidence suggests that epigenetic mechanisms are also critically involved in fibroblast to myofibroblast differentiation. For instance, HDAC inhibition has been shown to reduce TGF-β1-induced fibroblast to myofibroblast differentiation in normal human lung fibroblasts (Guo et al., 2009) and increased DNA methylation inhibited fibroblast to myofibroblast differentiation in human hepatic stellate cells (Mann et al., 2007). However, the role of epigenetic regulation on fibroblast to myofibroblast differentiation in IPF and the potential therapeutic value of epigenetic inhibitors have not yet been investigated.

6.2 Aims

This chapter aims to determine the epigenetic modifications involved in COX-2 repression and fibroblast to myofibroblast differentiation in TGF-β1-treated F-NL and F-IPF. We therefore investigated the role of histone acetylation, histone methylation and DNA methylation using specific epigenetic inhibitors, LBH589, a pan histone deacetylase inhibitor, BIX01294, an inhibitor for G9a, a histone methyltransferase specific for the repressive histone 3 lysine 9 (H3K9) methylation, and RG108, a DNA methyltransferase inhibitor. We sought to determine the effect of the above-mentioned epigenetic inhibitors on preventing TGF-β1-induced fibroblast to myofibroblast differentiation and reversing myofibroblast differentiation in F-IPF.

6.3 Experimental Protocol

F-IPF were cultured in 6 well culture plates and treated with epigenetic inhibitors LBH589 (10 nM), BIX01294 (100 nM) and RG108 (5 µM) for 3 days until confluent. The medium was changed every 48 hours. After 3 days of treatment F-IPF were growth arrested in serum free medium for 24 hours and left unstimulated or stimulated with 2 ng/ml IL-1β for a further 24 hours.

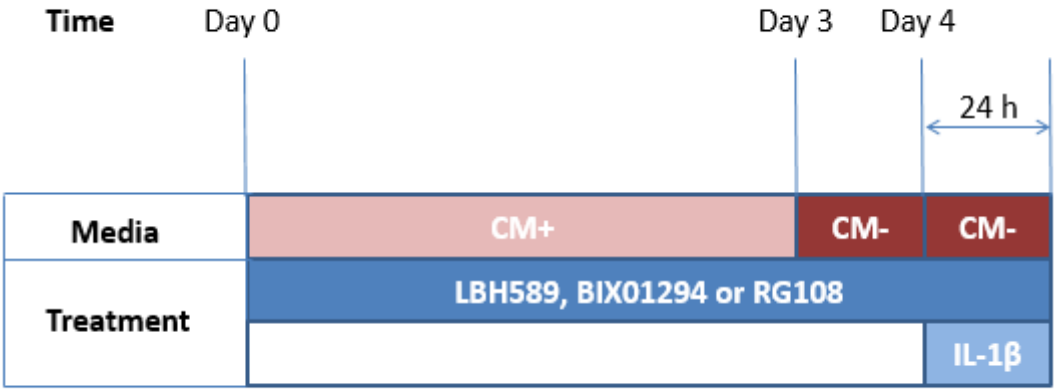


Figure 6-1 Experimental Protocol to assess the effect of epigenetic inhibitors in F-IPF

Schematic diagram of the timelines used for experimental protocol showing treatment, IL-1β stimulation and whether serum (CM^+) or serum free (CM^-) media was used.

F-NL were pre-treated with LBH589 (10 nM), BIX01294 (100 nM) and RG108 (5 µM) for 30 minutes prior to the addition of TGF-β1 (2 ng/ml) for 3 days. The medium was changed every 48 hours. After 3 days of treatment F-NL were growth arrested in serum free medium for 24 hours and left unstimulated or stimulated with 2 ng/ml IL-1β for a further 24 hours. After treatment cells were subject to protein extraction for Western blot analysis, as described in Chapter 2.

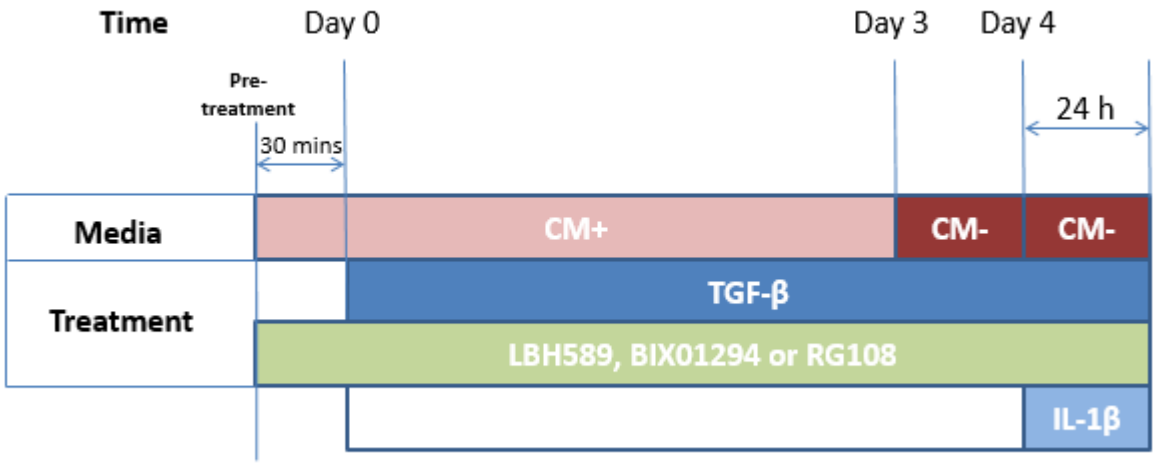


Figure 6-2 Experimental protocol of F-NL treated with epigenetic inhibitors

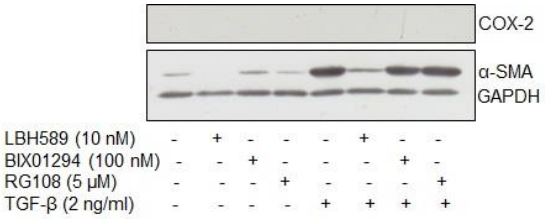
Schematic diagram of the timelines used for experimental protocol showing pre-treatment, treatment, IL-1ß stimulation and whether serum (CM^+) or serum free (CM^-) media was used.

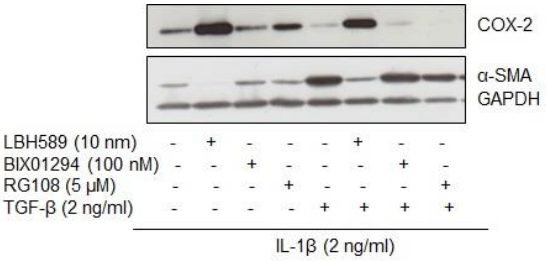
6.4 Results

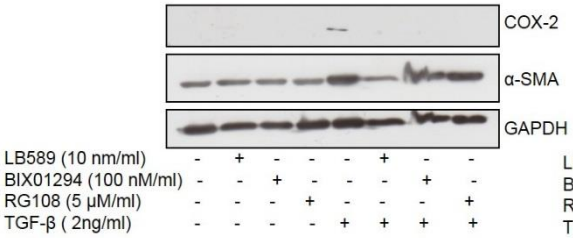
6.4.1 Histone acetylation is responsible for promoting a pro-fibrotic phenotype in TGF-β1-induced fibroblast to myofibroblast differentiation

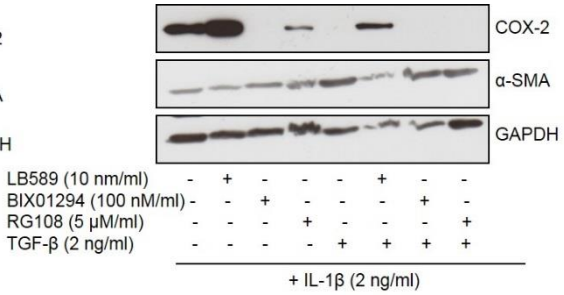
Our previous data has demonstrated that TGF-β1-induced fibroblast to myofibroblast differentiation maintains a pro-fibrotic phenotype even after the removal of TGF-β1 suggesting a stable epigenetic change. We therefore wanted to assess the effect of histone acetylation, histone 3 lysine 9 (H3K9) methylation (a repressive epigenetic mark) and DNA methylation on COX-2 expression and fibroblast to myofibroblast differentiation in TGF-β1-treated F-NL. We first determined the effect of LBH589, BIX01294 and RG108 on COX-2, α-SMA and Col 1 protein expression. LBH589 increased IL-1β-induced COX-2 expression compared with IL-1β control in F-NL. In addition, LBH589 prevented the TGF-β repression of IL-1β-induced COX-2 (Figure 6-3). In contrast, BIX01294 and RG108 had no effect on COX-2 or IL-1β-induced COX-2 expression either alone or in combination with TGF-β1. LBH589 treatment with IL-1β reduced TGF-β1-induced α-SMA expression (Figure 6-3). RG108 and BIX01294 had no effect on α-SMA expression either alone or in combination with TGF-β or IL-1β (Figure 6-3). LBH589 prevented TGF-β-induced Col 1 expression where as RG108 and BIX01294 had no effect (Figure 6-3Figure 6-4). However, this experiment was only performed in one cell line and thus further experiments are required to confirm this observation.

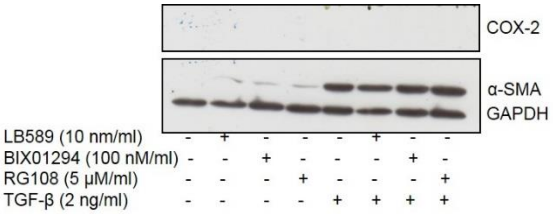
These data demonstrate a role for HDACs in TGF-β1-induced fibroblast to myofibroblast differentiation and suggests that TGF-β1 induces histone deacetylation at the COX-2 promoter to repress its expression and promote fibroblast to myofibroblast differentiation by increasing α-SMA and Col 1 expression. In contrast, H3K9 methylation and DNA methylation may not be initially involved in TGF-β1-induced repression of COX-2.

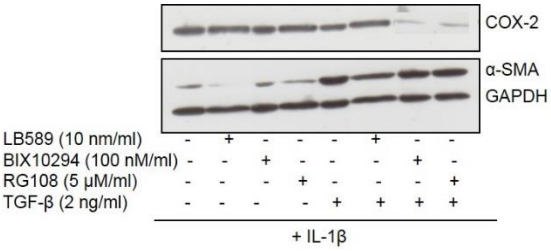

COX-2
α-SMA
GAPDH
LBH589 (10 nM) - + - - - + - -
BIX01294 (100 nM) - - + - - - + -
RG108 (5 μM) - - - + - - - +
TGF-β (2 ng/ml) - - - - + + + +


COX-2
α-SMA
GAPDH
LBH589 (10 nm) - + - - - + - -
BIX01294 (100 nM) - - + - - - + -
RG108 (5 μM) - - - + - - - +
TGF-β (2 ng/ml) - - - - + + + +
IL-1β (2 ng/ml)


COX-2
α-SMA
GAPDH
LB589 (10 nm/ml) - + - - - + - -
BIX01294 (100 nM/ml) - - + - - - + -
RG108 (5 μM/ml) - - - + - - - +
TGF-β (2ng/ml) - - - - + + + +


COX-2
α-SMA
GAPDH
LB589 (10 nm/ml) - + - - - + - -
BIX01294 (100 nM/ml) - - + - - - + -
RG108 (5 μM/ml) - - - + - - - +
TGF-β (2 ng/ml) - - - - + + + +
+ IL-1β (2 ng/ml)


COX-2
α-SMA
GAPDH
LB589 (10 nm/ml) - + - - - + - -
BIX01294 (100 nM/ml) - - + - - - + -
RG108 (5 μM/ml) - - - + - - - +
TGF-β (2 ng/ml) - - - - + + + +


COX-2
α-SMA
GAPDH
LB589 (10 nm/ml) - + - - - + - -
BIX10294 (100 nM/ml) - - + - - - + -
RG108 (5 μM/ml) - - - + - - - +
TGF-β (2 ng/ml) - - - - + + + +
+ IL-1β

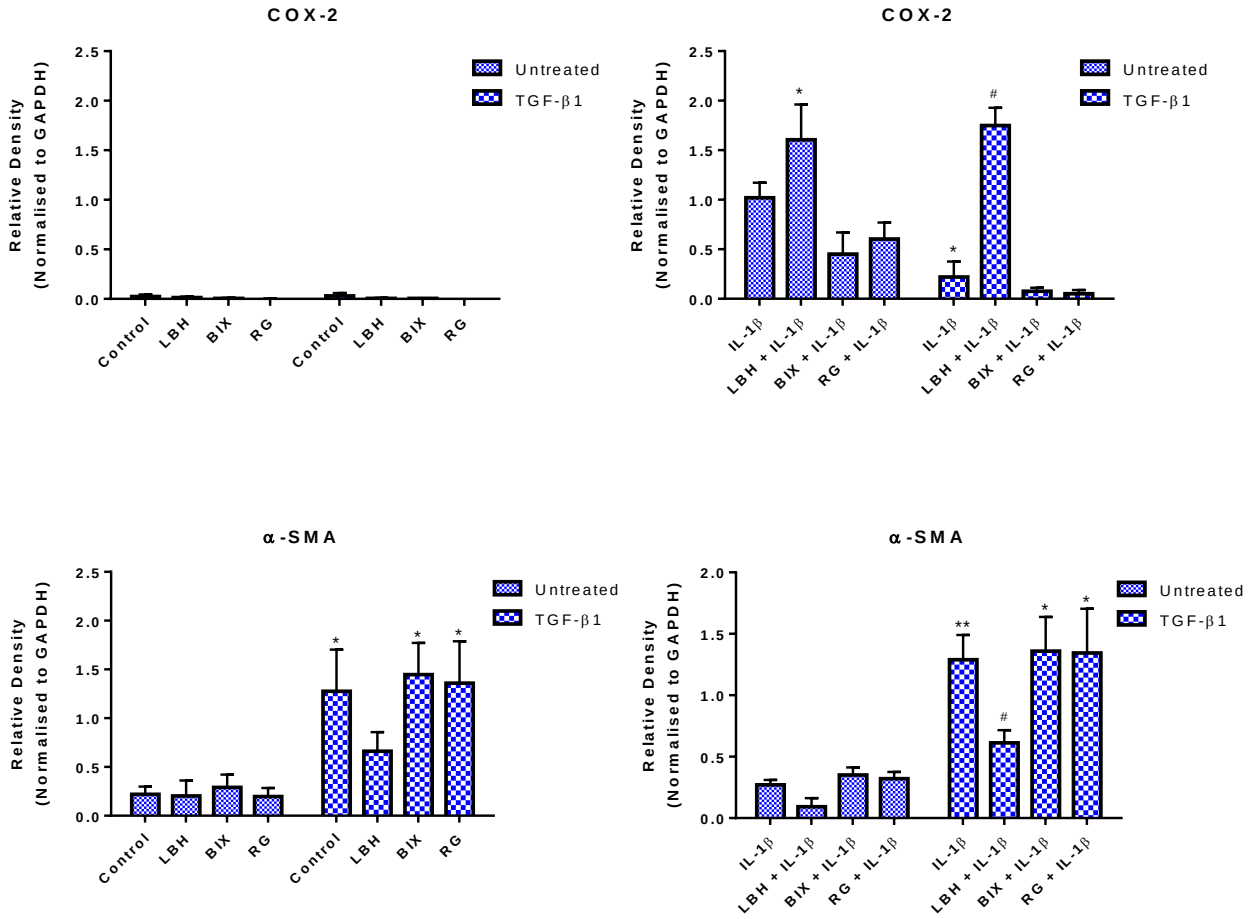


Figure 6-3 The Effect of LBH589, BIX01294 and RG108 on COX-2 and α-SMA Protein Expression in TGF-β1-treated F-NL

*F-NL were pre-treated with LBH589 (10 nM), BIX01294 (100 nM) or RG108 (5 μM) alone for 30 minutes before the addition of TGF-β1 (2 ng/ml) for 3 days, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 h) prior to the collection of total cell lysate for analysis by Western blot of COX-2 and α-SMA. This figure shows data from three separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines. *p<0.05, **p<0.01 compared with untreated control, #p<0.05, compared with IL-1β-treated control.*

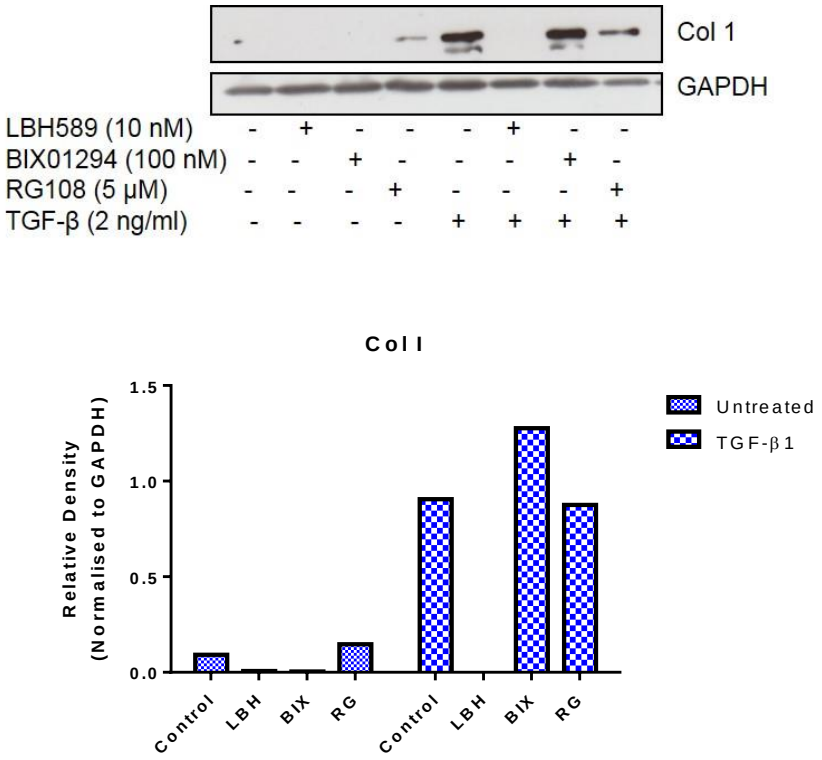


Figure 6-4 The Effect of LBH589, BIX01294 and RG108 on Col 1 Protein Expression in TGF-β1-treated F-NL

F-NL were pre-treated with LBH589 (10 nM), BIX01294 (100 nM) or RG108 (5 μM) alone for 30 minutes before the addition of TGF-β1 (2 ng/ml) for 3 days and serum starved for 24 hours prior to the collection of total cell lysate for analysis by Western blot of Col 1. This figure shows data from one experiment. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH.

6.4.2 Histone acetylation, H3K9 methylation and DNA methylation are involved in maintaining a pro-fibrotic phenotype in F-IPF

We next wanted to confirm whether histone acetylation, H3K9 methylation and DNA methylation are required for the maintenance of a pro-fibrotic phenotype seen in F-IPF. We therefore determined if the epigenetic inhibitors would be able to reverse fibroblast to myofibroblast differentiation in F-IPF. LBH589, BIX01294 and RG108 were all able to significantly increase IL-1β-induced COX-2 expression in F-IPF (Figure 6-5). Interestingly, none of them reduced α-SMA (Figure 6-5) or Col 1 (Figure 6-6) either with or without IL-1β treatment. These data suggest that histone acetylation, H3K9 methylation and DNA methylation all contribute to the repression of IL-1β-induced COX-2 seen in F-IPF. Collectively, the results in this chapter indicate that histone acetylation may be the initial epigenetic modification to repress COX-2 expression in TGF-β1-treated F-NL and that histone acetylation, H3K9 methylation and DNA methylation are all involved in maintaining COX-2 repression in F-IPF.

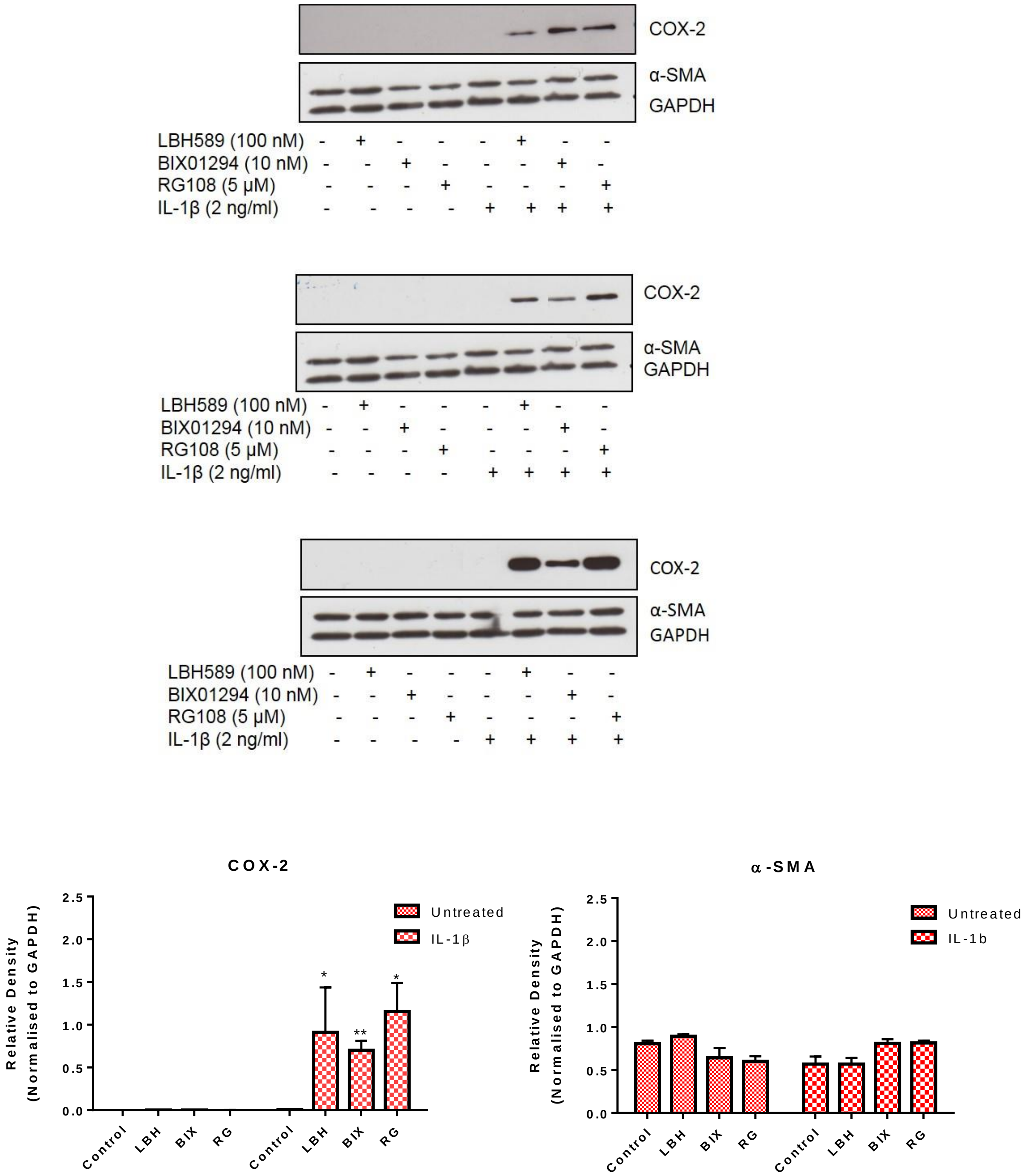


Figure 6-5 The Effect of BIX01294, LBH589 and RG108 on COX-2 and α-SMA Protein Expression in F-IPF

F-IPF were treated with LBH589 (10 nM), BIX01294 (100 nM) or RG108 (5 μM) alone, serum starved for 24 hours and left unstimulated or stimulated with IL-1β (2 ng/ml, 24 hr) prior to the collection of total cell lysate for analysis by Western blot. This figure shows data from three

*separate experiments performed in different cell lines. Relative density was calculated by normalising the density of the COX-2 and α-SMA bands against that of GAPDH. Each point represents the mean ± SEM of three different cell lines *p<0.05, **p<0.01 compared with IL-1β control.*

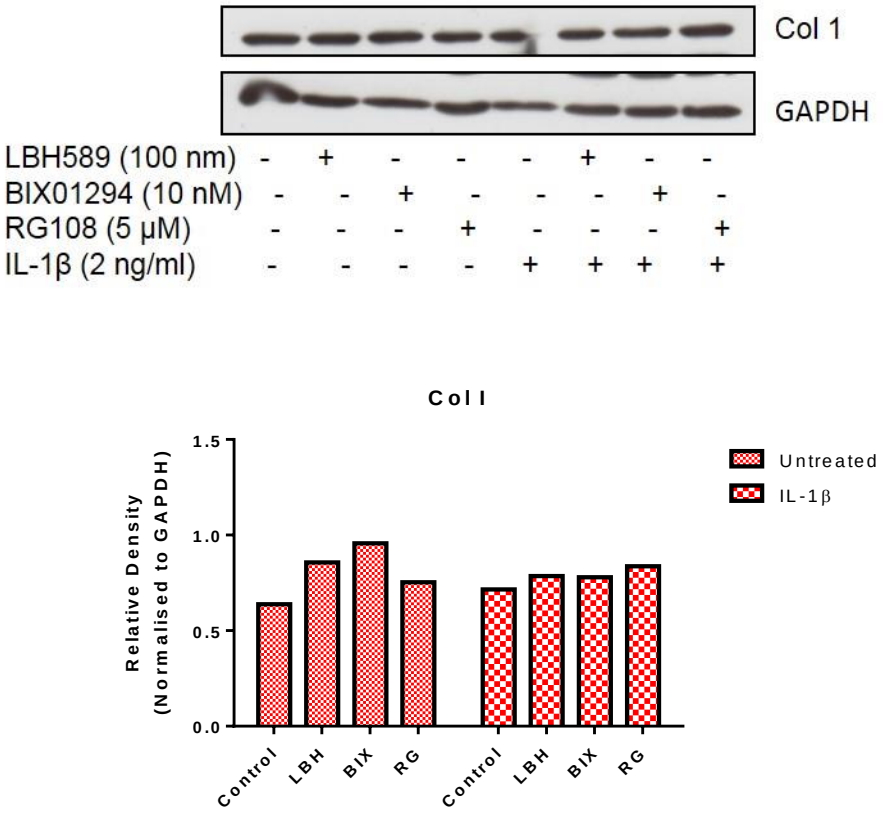


Figure 6-6 The Effect of BIX01294, LBH589 and RG108 on Col 1 Protein Expression in F-IPF

F-IPF were treated with LBH589 (10 nM), BIX01294 (100 nM) or RG108 (5 μM) alone, serum starved for 24 hours prior to the collection of total cell lysate for analysis by Western blot. This figure shows data from one experiment. Relative density was calculated by normalising the density of the Col 1 bands against that of GAPDH.

6.5 Discussion

This chapter investigates the anti-fibrotic effects of epigenetic inhibitors in both TGF-β1-treated F-NL and F-IPF. For the first time we have demonstrated the effects of epigenetic inhibitors on COX-2, α-SMA and Col 1 expression in F-NL and F-IPF. The data indicates that the initial epigenetic modification to repress COX-2 in TGF-β1-treated F-NL may be histone deacetylation. However, further epigenetic modifications in addition to histone acetylation, including H3K9 methylation and DNA methylation may be necessary to maintain the repression of COX-2, as seen in F-IPF. This is not surprising as the reversible nature of histone acetylation and methylation plays a critical role in regulating gene transcription whereas DNA methylation is a stable epigenetic mark linked to the maintenance of chromatin in a silent state. Evidence suggests that there is a link between DNA methylation and histone acetylation (Dobosy and Selker, 2001). HDAC inhibitors have been shown not only to change the acetylation of histones but also increase DNA demethylation (Arzenani et al., 2011). Therefore, communication between histone deacetylation and DNA methylation is likely to be a dynamic process in the repression of COX-2 during fibroblast to myofibroblast differentiation.

Interestingly, HDAC inhibition alone in TGF-β1-treated F-NL was able to reduce TGF-β1-induced expression of α-SMA and Col 1 despite histone acetylation being associated with gene activation. The reduction of α-SMA and Col 1 could be due to increased IL-1β COX-2 and subsequent PGE_2 production which then reduces α-SMA and Col 1 expression as demonstrated in chapter 2. Alternatively, it has been demonstrated that trichostatin A (TSA), a HDAC inhibitor, inhibits TGF-β1-induced α-SMA expression in human dermal fibroblasts via increased expression of Smad-7, an inhibitory Smad, to prevent TGF-β1 signalling (Rombouts et al., 2002). Therefore, LBH589 could have a similar mechanism of action that prevents TGF-β1-induced α-SMA and Col 1 expression in TGF-β1-treated F-NL. The mechanism by which LBH589 reduces TGF-β-induced α-SMA and Col 1 expression requires further investigation.

Our data is in agreement with previous studies that have shown inhibition of HDACs prevents TGF-β1-induced fibroblast to myofibroblast differentiation in rat dermal fibroblasts and rat hepatic fibroblasts (Mann et al., 2007; Rombouts et al., 2002). Although these studies only looked at myofibroblast markers such as α-SMA and Col 1 expression, the anti-fibrotic effect of inhibiting HDACs could be due to the increased expression of COX-2 and subsequent increases in PGE_2 production. Therefore, it is plausible that HDAC inhibitors could inhibit TGF-β1-induced expression of α-SMA and Col 1 indirectly through COX-2 induction *in vivo* due to potential gene activation of COX-2 inducers. Further experiments using COX-2 inhibitors, such as celecoxib and NS-398, would need to be completed to test this hypothesis.

Our data are further supported by the fact that COX-2 repression in F-IPF, is due to hypoacetylation of histone H3 and H4 at the COX-2 promoter (Coward et al., 2009). Unpublished data from our laboratory has also demonstrated that prolonged treatment of TGF-β1 in F-NL results in a marked reduction of IL-1β-induced histone H3 and H4 acetylation at the COX-2 promoter (unpublished data). Overall, these data suggest a close association of epigenetic remodelling, primarily histone acetylation, with reduced COX-2 expression and fibroblast to myofibroblast differentiation seen in TGF-β1-treated F-NL.

The epigenetic changes seen in TGF-β1-treated F-NL are also similar to those in F-IPF. Results from our laboratory have shown reduced histone acetylation, increased histone methylation and increased DNA methylation at the COX-2 promoter in F-IPF compared with F-NL (Coward et al., 2014). Collectively, these results suggest that histone acetylation, histone methylation and DNA methylation may play a role in the maintenance of COX-2 gene repression in F-IPF.

Although all of the epigenetic inhibitors increased COX-2 expression in F-IPF none of them reduced α-SMA or Col 1 expression. The epigenetic inhibitors that we used all promote gene expression and are therefore unlikely to directly inhibit α-SMA and Col 1 protein expression. We have shown in Chapter 3 that it takes 3 days for exogenous PGE_2 to inhibit α-SMA and Col 1 in F-IPF. Therefore, although the inhibitors restore COX-2 expression in these cells it may take longer than 24 hour IL-1β stimulation to see the inhibitory effect of endogenous PGE_2 on α-SMA and Col 1. Further experiments are necessary to confirm this.

Based on both our observations and previous studies epigenetic regulation is likely to play a key role in fibroblast to myofibroblast differentiation. However, the mediators and signalling pathways that direct these changes remain to be determined. Our data suggests that TGF-β1 promotes histone deacetylation in order to repress COX-2 expression and promote a pro-fibrotic phenotype. Since epigenetic inhibitors used in this study mimic the effect of PGE_2, with regard to COX-2 expression, it is possible that PGE_2 could also regulate epigenetic changes in order to promote an anti-fibrotic phenotype. Huang and colleagues have previously demonstrated that PGE_2 is able to increase gene-specific and global DNA methylation via increased DNMT3a expression in IMR-90 fibroblasts, a foetal fibroblast cell line (Huang et al., 2012). Therefore, this supports the fact that changes in the biosynthesis of PGE_2 may contribute to alterations in DNA methylation patterns. Whether or not PGE_2 has any effect on histone acetylation and histone methylation will require further investigation.

In summary, these data provide evidence that epigenetic remodelling is likely to play a key role in the altered expression of COX-2 and fibroblast to myofibroblast differentiation. Our results suggest that histone acetylation is initially involved in promoting a fibrotic phenotype in

TGF-β1-induced fibroblast to myofibroblast differentiation. However, histone acetylation, H3K9 methylation and DNA methylation may all be necessary in order to maintain the pro-fibrotic phenotype seen in F-IPF. Epigenetic therapeutics, such DNA methylation inhibitors and HDAC inhibitors, have emerged as promising molecules in drug development for cancer therapy (Razak et al., 2011) and therefore there is potential for this class of drug to be beneficial in the treatment of IPF.

CONCLUSIONS

7 CONCLUSIONS AND FUTURE STUDIES

7.1 Conclusions

Differentiation of fibroblasts into myofibroblasts and the persistence of the pro-fibrotic myofibroblast phenotype play a key role in the pathogenesis of IPF. Myofibroblasts are the principle effector cells that mediate tissue fibrosis via their capacities for migration, proliferation and collagen deposition (Lorena et al., 2002). Myofibroblast differentiation is stimulated by cytokines, the most potent of which is TGF-β1. However, much less is understood about the signals that inhibit fibroblast to myofibroblast differentiation (Selman et al., 2001). One of the best studied anti-fibrotic mediators is PGE_2 which has been shown to inhibit fibroblast proliferation (Huang et al., 2008b), migration (Kohyama et al., 2001) and fibroblast to myofibroblast differentiation (Kolodsick et al., 2003). Studies from our laboratory and others have shown that fibroblasts isolated from patients with IPF manifest impaired production of PGE_2 that is attributable to the epigenetic repression of COX-2 (Coward et al., 2009; Wilborn et al., 1995). However, there are no studies that have comprehensively examined if the loss of endogenous PGE_2 contributes to the pro-fibrotic phenotype of myofibroblasts and if exogenous PGE_2, or other cAMP elevating agents, can compensate for the loss of endogenous PGE_2.

The overall aim of this study was to explore if COX-2 and the subsequent loss of PGE_2 production is gradually lost during myofibroblast differentiation and determine if exogenous PGE_2, and cAMP elevating agents, can compensate for the loss of endogenous PGE_2 to prevent and reverse fibroblast to myofibroblast differentiation. The study also sought to determine the downstream signalling mechanism of PGE_2, in particular its cAMP and PKA/Epac dependence, in order to identify novel and specific therapeutic targets. Finally, we sought to identify if epigenetic events are involved in altered COX-2 expression in myofibroblast differentiation.

The present study demonstrates that the loss of PGE_2 is associated with fibroblast to myofibroblast differentiation and contributes to the pro-fibrotic phenotype observed in myofibroblasts such as increased α-SMA and Col 1 expression. In addition, we have shown that exogenous addition of PGE_2 compensates for the loss of endogenous PGE_2. Exogenous PGE_2 treatment increases IL-1β-induced COX-2 expression and subsequent PGE_2 production, which prevents the pro-fibrotic effects of TGF-β1 and reverses fibroblast to myofibroblast differentiation. Hence, augmenting COX-2 or PGE_2 levels in the lung may be of therapeutic value to IPF patients. We confirmed that the effects of PGE_2 on COX-2 expression were mediated mainly via the EP2 receptor and increased cAMP.

Although exogenous PGE_2 compensates for the loss of endogenous PGE_2, the use of PGE_2 as a clinical treatment for IPF has several limitations including its short half-life and possible side effects in other organs and tissues such as pain, fever, oedema and inflammation (Narumiya et al., 1999; Vancheri et al., 2004). Given the diverse effects of PGE_2 the use of selective EP2 agonists or cAMP elevating agents, such as β_2-agonists, which are already used in the treatment of Asthma are a more attractive therapeutic strategy. We therefore examined the effect of EP2 selective agonists and cAMP elevating agents on COX-2 expression and fibroblast to myofibroblast differentiation. Our results demonstrate that EP2 agonists and cAMP elevating agents inhibit the pro-fibrotic effects of TGF-β1 and reverse fibroblast to myofibroblast differentiation due to their ability to increase COX-2 expression and PGE_2 production. Our results confirm that increasing intracellular cAMP is able to mimic exogenous PGE_2 treatment and compensate for the loss of endogenous PGE_2 in myofibroblasts. The cAMP pathway thus appears to be an important regulator of COX-2 expression and PGE_2 production. Therefore, decreasing cAMP metabolism, via PDE inhibition, may also increase COX-2 expression. Yet, our results with roflumilast, a PDE4 inhibitor, showed no effect on

cAMP levels or COX-2 expression. Although the cAMP pathway is key mediator of fibroblast to myofibroblast differentiation our results suggest that PDE4 is not extensively involved in regulating cAMP production in pulmonary fibroblasts and as such other PDE isoforms or interventions that enhance cellular cAMP should be investigated.

PGE_2 is well known to inhibit several fibroblast specific functions such as fibroblast proliferation, migration and contraction and many of these functions are regulated by cAMP. However, the role of cAMP effectors, PKA and Epac, in modulating such functions remains relatively unknown. We have demonstrated that the activation of PKA, but not Epac, increases IL-1β-induced COX-2 expression. Neither PKA nor Epac activation had an effect on α-SMA expression. PGE_2 treatment decreased ERK-1/2 expression and it could be this pathway that controls α-SMA expression rather than PKA or Epac. The activation of this pathway provides another layer by which PGE_2 can exert its anti-fibrotic effects.

It is well documented that TGF-β1 promotes fibroblast to myofibroblast differentiation and as such strategies aimed at blocking TGF-β1 expression or signalling have gained much attention as a therapeutic target. TGF-β1 signals via Smad proteins in addition to Ras signalling cascades such as mitogen-activated protein kinase cascade, including ERK-1/2. Our results demonstrate the ability of PGE_2 and cAMP elevating agents to prevent TGF-β1-induced repression of COX-2 and subsequent impaired production of PGE_2. Nonetheless, the signalling mechanisms through which PGE_2 or cAMP act to inhibit the pro-fibrotic effects of TGF-β1 remain unknown. These data show that inhibition of Ras activity results in increased COX-2 expression and prevents the pro-fibrotic effects of TGF-β1. This novel data indicates that Ras activity may be important in TGF-β1-induced fibroblast to myofibroblast differentiation and maintaining the pro-fibrotic phenotype as seen in myofibroblasts. As such, TGF-β1-induced repression of COX-2 may be via TGF-β1 activation of Ras and downstream Ras

signalling. Whether or not PGE_2 induces COX-2 expression via inhibiting the activity of Ras has never been studied previously. Increased Ras activity and ERK-1/2 signalling are witnessed following short term PGE_2 treatment. However, following chronic treatment with PGE_2, ERK-1/2 phosphorylation was reduced, suggesting that reduced activity of ERK-1/2 results in increased expression of COX-2. The fact that PGE_2 can down regulate ERK-1/2 activity may provide a more selective way of blocking the pro-fibrotic effects of TGF-β1 and thus offers an alternative therapeutic strategy to treat IPF.

Finally, data from our laboratory has confirmed that COX-2 repression is regulated by epigenetic modifications (Coward et al., 2009). Our results suggest that TGF-β1-induced COX-2 repression is initially due to increased histone deacetylation at the COX-2 promoter, however, histone methylation and DNA methylation are not involved. In contrast, the maintenance of COX-2 repression, as seen in myofibroblasts, is likely due to histone deacetylation, histone methylation and DNA methylation. Our results confirm that the use of selective epigenetic inhibitors is able to prevent the pro-fibrotic effects of TGF-β1 and reverse the repression of COX-2 seen in myofibroblasts. Inhibition of histone deacetylases also prevented TGF-β1-induced expression of α-SMA and Col 1. All of the epigenetic inhibitors increased IL-1β-induced COX-2 expression in F-IPF but had no effect on α-SMA or Col 1 in our experimental setting.

To date there are very few effective treatments for IPF and thus the discovery of a novel therapeutic target is of paramount importance. The data shown here provides rationale for strategies that increase cAMP levels, inhibit Ras activity or reverse epigenetic modifications as a means to increase COX-2 expression and compensate for the loss of endogenous PGE_2, a key anti-fibrotic mediator. This therapy is likely to achieve two major anti-fibrotic effects: (i) to reverse the pro-fibrotic phenotype of myofibroblasts already present *in situ* and (ii) to

prevent the differentiation of newly recruited fibroblasts into myofibroblasts and thus is an attractive target for therapeutic drug development in IPF.

To summarise, the overall aim of this study was to investigate the molecular mechanisms of myofibroblast differentiation and cAMP mediated inhibition of this process in IPF. The results confirm our study hypothesis: COX-2 gene expression and subsequent PGE_2 production is gradually lost during myofibroblast differentiation and exogenous PGE_2 and other cAMP stimulating agents can compensate for the lack of endogenous PGE_2 and prevent and reverse myofibroblast differentiation. Furthermore, we demonstrate that Ras activity and epigenetic modifications also regulate COX-2 expression and are key molecular mechanisms involved in fibroblast to myofibroblast differentiation.

7.2 Future Directions

This thesis has highlighted different approaches to induce COX-2 expression and compensate for the endogenous loss of PGE_2 seen in myofibroblasts. Our results demonstrate that cAMP elevating agents, reducing Ras activity and epigenetic inhibitors can each induce COX-2 expression, prevent the pro-fibrotic effects of TGF-β1 and reverse fibroblast to myofibroblast differentiation.

Although this study confirms that PGE_2 acts mainly via the EP2 receptor and increased cAMP, the downstream pathways of PKA and Epac need further investigation. Our results suggest that PKA signalling, but not Epac, is involved in PGE_2-induced COX-2 expression however the lack of a selective Epac agonist and limited knowledge of signalling pathways downstream of Epac made further investigation difficult. To confirm that Epac is not involved in this signalling

pathway it would be beneficial to use short interfering RNA against Epac to assess PGE_2-induced COX-2 expression after Epac activity has been inhibited.

Our data clearly demonstrates that reducing Ras activity, using the Ras inhibitor FTS, enhances COX-2 production and is able to both prevent the pro-fibrotic effects of TGF-β1 and reverse fibroblast to myofibroblast differentiation. Although FTS has been widely used and proven to be a unique and potent Ras inhibitor in various cell lines (Halaschek-Wiener et al., 2000; Marom et al., 1995) we cannot rule out the possibility that FTS may interfere with the action of other prenylated proteins, such as proteins of the Rac/Rho family of GTPases, that are associated with the control of actin cytoskeleton, cell growth and cell motility (Hall, 2012). Thus, future studies using alternative methods to inhibit Ras, such as short interfering RNA against Ras or a transfection of a dominant negative Ras vector, would be necessary to confirm the effects of FTS on TGF-β1-induced fibroblast to myofibroblast differentiation and the reversal of myofibroblast differentiation. In addition, there are three isoforms of Ras, N-Ras, K-Ras and H-Ras, each Ras isoform is differentially expressed and perform distinct cellular roles *in vivo* (Olson and Marais, 2000). Several studies have demonstrated the involvement of H-Ras in modulating fibroblast functions (Fuentes-Calvo et al., 2012; Grande et al., 2010; Smaldone et al., 2011). Therefore, we plan to investigate the role of each isoform to determine which isoform is responsible for regulating COX-2 expression in pulmonary fibroblasts. Initial experiments will involve determining the basal expression of N-Ras, K-Ras and H-Ras in F-NL and F-IPF. Samples will be subject to the Ras-GTP Pull down assay and analysed by Western blot using isoform specific Ras antibodies. Once expression of the Ras isoforms has been determined we will then transfect cells with isoform specific dominant-negative Ras constructs to specifically determine the effect of inhibition of each isoform on COX-2, α-SMA and Col 1 on TGF-β1-treated F-NL and F-IPF. Together, the results should confirm which Ras isoform is responsible for regulating fibroblast to myofibroblast differentiation.

Our data suggests that ERK-1/2 may also be involved in regulating COX-2 expression, however further investigation is needed to confirm this. The effect of specific MEK antagonists, including U1026 and PD59089, on COX-2, α-SMA and Col 1 in TGF-β1-treated F-NL and F-IPF will be investigated. This will allow us to determine if ERK-1/2 is necessary for TGF-β1-induced repression of COX-2 and if inhibition of ERK-1/2 can reverse myofibroblast differentiation. Ras has several downstream signalling pathways in addition to the Ras/MEK/ERK signalling cascade. Future studies are planned to investigate the effect of inhibiting other downstream signalling pathways of Ras. Previous studies have demonstrated that inhibition of PI3K prevented fibroblast to myofibroblast differentiation in human lung fibroblasts (Conte et al., 2011) and as such we plan to assess the effect of PI3K inhibition on COX-2 expression and fibroblast to myofibroblast differentiation in TGF-β1-treated F-NL and F-IPF. Finally, we would like to determine the effect of TGF-β1 and PGE_2 on other downstream effectors of Ras including; PI3K, PKCδ and JNK.

Finally, as our current data has identified that fibroblast to myofibroblast differentiation is regulated via epigenetic modifications we will identify the key epigenetic events and modifying enzymes involved in altered COX-2, α-SMA and Col 1 expression in TGF-β1-treated F-NL compared with F-IPF. The repressive and active histone methylation marks and histone H3 and H4 acetylation at the COX-2, α-SMA and Col 1 promoters will be determined by chromatin immunoprecipitation (ChIP) assay. Bilsulfite and PCR sequencing will be performed to detect DNA methylation changes at the COX-2, α-SMA and Col 1 promoter. Once we have identified the key epigenetic events that are responsible for altered COX-2, α-SMA and Col 1 expression during TGF-β1-induced fibroblast to myofibroblast differentiation and F-IPF we will then examine the effect of PGE_2 and cAMP elevating agents.

APPENDIX

8 APPENDIX

8.1 Materials

Hyperfilm ECL™	Amersham BioSciences
PDVF Membrane	BioRad

8.2 Reagents

Adenosine 3’5’-cyclic Monophosphate, 8-(4-Chlorophenylthio)-2’-O-Methyl	Calbiochem
AH 6809	Cayman Chemicals
Ammonium persulphate	Sigma-Aldrich
Amphotericin B	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Deoxynucleosides (dNTPs)	Promega
Dimethyl sulphoixde (DMSO)	Sigma-Aldrich
Dithiothreitol	Sigma-Aldrich
Dulbecco’s modified Eagle’s medium (DMEM)	Sigma-Aldrich
ECL™ detection reagents	Amersham BioSciences
Emulsifier Safe Scintillation Cocktail	Perkin Elmer
Ethylene Glycol Tetraacetic Acid	Sigma Aldrich
Ethanol	BDH
Farnesyl Thiosalicyclic Acid	Cayman Chemicals
FluroSave TM Reagent	Merckmillipore
Foetal calf serum	PAA Laboratories
Formoterol	Sigma Aldrich
Forskolin	Sigma-Aldrich
Full Range Rainbow marker	Amersham Biosciences

Goat serum	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Interleukin 1-Beta	Peprotech
Leupeptin	Sigma-Aldrich
L-Glutamine	Sigma Aldrich
Methanol	BDH
M-MLV Reverse Transcriptase	Promega
Non-fat dry milk	Santa-Cruz
N,N,N,N-Tetramethylethylenediamine (Temed)	Sigma-Aldrich
NP40	Sigma Aldrich
Nuclease Free Water	Life Sciences
Oligo dT Primer	Roche
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich
Penicillin/streptomycin.	Sigma-Aldrich
Phemylmethanesulphonyylfluoride (PMSF)	Sigma-Aldrich
Prostaglandin E2	Sigma-Aldrich
Protein inhibitor cocktail	Sigma-Aldrich
RNasin RNase Inhibitor	Promega
RT Buffer	Promega
Salmeterol Xinafoate	Sigma-Aldrich

Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Transforming Growth Factor Beta	R&D Systems
Trizma® base	Sigma-Aldrich
Trypsin/EDTA	Sigma-Aldrich
Tween-20	Sigma-Aldrich
3-Isobutyl-1-methlyxanthine	Sigma-Aldrich
6-Bnz-cAMP	Sigma-Aldrich

8.3 Antibodies

Alpha Smooth Muscle Actin	Sigma-Aldrich
Alpha Smooth Muscle Actin-Phycoerythrin	R&D Systems
Collagen I	Abcam
COX-2	Caymen Chemicals
EP2 (H-75)	Santa Cruz
EP4 (C-4)	Santa Cruz
FITC Conjugated Rat Anti-Mouse IgM.	BD Biosciences
GAPDH	Santa Cruz
PE Conjugated Goat Anti-Mouse IgG	Abcam

8.4 Kits

BCA Protein Assay	Thermo Scientific
Cyclic Nucleotide Phosphodiesterase Assay KIT	Enzo Life Sciences
NucleoSpin RNA II RNA Extraction Kit	Macherey Nagal
Ras Activation ELISA Assay Kit	Millipore

8.5 Western Blot Buffers

8.5.1 RIPA Buffer

Reagent	Amount
50mM Tris-HCl pH7.4	10ml
1% NP40	2ml
0.25% Sodium Deoxycholate	5ml
150mM NaCl	6ml
1mM EDTA	400µl
1mM PMSF*	20µl
1mM NaF*	20µl
0.1mM DTT*	20µl
Leupeptin*	20µl
Protease Inhibitor Cocktail*	20µl

* Added on day of use

8.5.2 Buffer 1

Reagent	Amount
Tris Base	18.5g
10% SDS	4ml
dH20	50ml

Adjust pH to 8.8 then add dH20 to 100ml

8.5.3 Buffer 2

Reagent	Amount
Tris Base	6g
10% SDS	4ml
dH20	60ml

Adjust to pH 6.8 then add dH20 to 100ml

8.5.4 Resolving Gel

Reagent	Amount
30% Bis/acrylamide	6.66ml
Buffer 1	5.2ml
dH20	7.92ml
10% Ammonium Persulphate	200µl
Tetramethylenediamine (Temed)	20µl

8.5.5 Stacking Gel

Reagent	Amount
30% Bis/acrylamide	1.3ml
Buffer 2	2.5ml
dH20	6.1ml
10% Ammonium Persulphate	50µl
Tetramethylenediamine (Temed)	10µl

8.5.6 Lamellae Buffer

Reagent	Amount
0.5M Tris-HCl pH 6.8	5ml
100% Glycerol	4ml
10% SDS	4ml
Bromophenol Blue	40mg
dH20	7ml
B-mercaptoethanol	60ul/ml

8.5.7 10X Running Buffer

Reagent	Amount
Tris-base	24.4g
Glycine	144g
SDS	10g
dH20	1000ml

Diluted 1:10 with dH20 for 1X Running Buffer

8.5.8 10X Transfer Buffer

Reagent	Amount
Tris-Base	24.2g
Glycine	144g
dH20	1000ml

8.5.9 1X Transfer Buffer

Reagent	Amount
10X Transfer Buffer	100ml
Methanol	200ml
dH20	700ml

8.5.10 10X Tris Buffered Saline with Tween (TBST)

Reagent	Amount
Tris-HCL (pH 6.8)	24.2g
NaCl	87.6g
dH20	1000ml
Tween 20	10ml

Adjust pH to 7.4-7.6

Diluted 1:10 with dH20 for 1X TBST

8.6 Immunocytochemistry Buffers

8.6.1 Blocking Buffer

Reagent	Amount
Bovine Serum Albumin	0.1g
Goat Serum	1ml
PBS	99ml

8.7 PCR Primers and RT-PCR cycling conditions

Gene	Primer Sequences	Annealing Temperature
COX-2	F:GGaACACAACAGAGTATGCG R:AAGGGGATGCCAGTGATAGA	60^{o}C
α-SMA	F:ACCCTGGCATTGCCGACCGA R:GAAGGCCCGGCTTCATCGTAT	60^{o}C
Collagen 1	F:ATGCCTGGTGAACGTGGT R:AGGAGAGCCATCAGCACCT	60^{o}C
B2M	F:GAGTATGCCTGCCGTGTG R:AATCCAAATGCGGCATCT	60^{o}C

REFERENCES

9 REFERENCES

2000, American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS): Am J Respir Crit Care Med, v. 161, p. 646-64.

Abe, R., S. C. Donnelly, T. Peng, R. Bucala, and C. N. Metz, 2001, Peripheral blood fibrocytes: Differentiation pathway and migration to wound sites: Journal of Immunology, v. 166, p. 7556-7562.

Adamson, I. Y. R., L. Young, and D. H. Bowden, 1988, RELATIONSHIP OF ALVEOLAR EPITHELIAL INJURY AND REPAIR TO THE INDUCTION OF PULMONARY FIBROSIS: American Journal of Pathology, v. 130, p. 377-383.

Adcock, I. M., L. Tsaprouni, P. Bhavsar, and K. Ito, 2007, Epigenetic regulation of airway inflammation: Current Opinion in Immunology, v. 19, p. 694-700.

Aharonson, Z., M. Gana-Weisz, T. Varsano, R. Haklai, D. Marciano, and Y. Kloog, 1998, Stringent structural requirements for anti-Ras activity of S-prenyl analogues: Biochimica Et Biophysica Acta-Molecular Basis of Disease, v. 1406, p. 40-50.

Allfrey, V. G., R. Faulkner, and A. E. Mirsky, 1964, ACETYLATION + METHYLATION OF HISTONES + THEIR POSSIBLE ROLE IN REGULATION OF RNA SYNTHESIS: Proceedings of the National Academy of Sciences of the United States of America, v. 51, p. 786-&.

Andersson-Sjoland, A., C. G. de Alba, K. Nihlberg, C. Becerril, R. Ramirez, A. Pardo, G. Westergren-Thorsson, and M. Selman, 2008, Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis: International Journal of Biochemistry & Cell Biology, v. 40, p. 2129-2140.

Annes, J. P., J. S. Munger, and D. B. Rifkin, 2003, Making sense of latent TGF beta activation: Journal of Cell Science, v. 116, p. 217-224.

Antoniou, K. M., G. A. Margaritopoulos, G. Soufla, E. Symvoulakis, E. Vassalou, R. Lymbouridou, K. D. Samara, D. Kappou, D. A. Spandidos, and N. M. Siafakas, 2010, Expression analysis of Akt and MAPK signaling pathways in lung tissue of patients with idiopathic pulmonary fibrosis (IPF): Journal of Receptors and Signal Transduction, v. 30, p. 262-269.

Araya, J., S. Cambier, J. A. Markovics, P. Wolters, D. Jablons, A. Hill, W. Finkbeiner, K. Jones, V. C. Broaddus, D. Sheppard, A. Barzcak, Y. Xiao, D. J. Erle, and S. L. Nishimura, 2007, Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients: J Clin Invest, v. 117, p. 3551-62.

Arras, M., J. Louahed, J. F. Heilier, M. Delos, F. Brombacher, J. C. Renauld, D. Lison, and F. Huaux, 2005, IL-9 protects against bleomycin-induced lung injury - Involvement of prostaglandins: American Journal of Pathology, v. 166, p. 107-115.

Arzenani, M. K., A. E. Zade, Y. Ming, S. J. H. Vijverberg, Z. Zhang, Z. Khan, S. Sadique, L. Kallenbach, L. Hu, V. Vukojevic, and T. J. Ekstrom, 2011, Genomic DNA Hypomethylation by Histone Deacetylase Inhibition Implicates DNMT1 Nuclear Dynamics: Molecular and Cellular Biology, v. 31, p. 4119-4128.

Ask, K., G. E. M. Martin, M. Kolb, and J. Gauldie, 2006, Targeting genes for treatment in idiopathic pulmonary fibrosis: challenges and opportunities, promises and pitfalls: Proc Am Thorac Soc, v. 3, p. 389-93.

Assoian, R. K., A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn, 1983, TRANSFORMING GROWTH FACTOR-BETA IN HUMAN-PLATELETS - IDENTIFICATION OF A MAJOR STORAGE SITE, PURIFICATION, AND CHARACTERIZATION: Journal of Biological Chemistry, v. 258, p. 7155-7160.

Awad, M. R., A. El-Gamel, P. Hasleton, D. M. Turner, P. J. Sinnott, and I. V. Hutchinson, 1998, Genotypic variation in the transforming growth factor-beta 1 gene - Association with transforming growth factor-pi production, fibrotic lung disease, and graft fibrosis after lung transplantation: Transplantation, v. 66, p. 1014-1020.

Bakin, A. V., A. K. Tomlinson, N. A. Bhowmick, H. L. Moses, and C. L. Arteaga, 2000, Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration: Journal of Biological Chemistry, v. 275, p. 36803-36810.

Bannister, A. J., and T. Kouzarides, 2005, Reversing histone methylation: Nature, v. 436, p. 1103-1106.

Baouz, S., J. Giron-Michel, B. Azzarone, M. Giuliani, F. Cagnoni, S. Olsson, R. Testi, G. Gabbiani, and G. W. Canonica, 2005, Lung myofibroblasts as targets of salmeterol and fluticasone propionate: inhibition of alpha-SMA and NF-kappa B: International Immunology, v. 17, p. 1473-1481.

Baran, C. P., J. M. Opalek, S. McMaken, C. A. Newland, J. M. O'Brien, Jr., M. G. Hunter, B. D. Bringardner, M. M. Monick, D. R. Brigstock, P. C. Stromberg, G. W. Hunninghake, and C. B. Marsh, 2007, Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 176, p. 78-89.

Barlo, N. P., C. H. van Moorsel, N. M. Korthagen, M. Heron, G. T. Rijkers, H. J. Ruven, J. M. van den Bosch, and J. C. Grutters, 2011, Genetic variability in the IL1RN gene and the balance between interleukin (IL)-1 receptor agonist and IL-1β in idiopathic pulmonary fibrosis: Clin Exp Immunol, v. 166, p. 346-51.

Basset, F., V. J. Ferrans, P. Soler, T. Takemura, Y. Fukuda, and R. G. Crystal, 1986, INTRALUMINAL FIBROSIS IN INTERSTITIAL LUNG DISORDERS: American Journal of Pathology, v. 122, p. 443-461.

Battegay, E. J., E. W. Raines, T. Colbert, and R. Ross, 1995, TNF-alpha stimulation of fibroblast proliferation. Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression: J Immunol, v. 154, p. 6040-7.

Baum, B. J., J. Moss, S. D. Breul, R. A. Berg, and R. G. Crystal, 1980, EFFECT OF CYCLIC-AMP ON THE INTRACELLULAR DEGRADATION OF NEWLY SYNTHESIZED COLLAGEN: Journal of Biological Chemistry, v. 255, p. 2843-2847.

Bauman, K. A., S. H. Wettlaufer, K. Okunishi, K. M. Vannella, J. S. Stoolman, S. K. Huang, A. J. Courey, E. S. White, C. M. Hogaboam, R. H. Simon, G. B. Toews, T. H. Sisson, B. B. Moore, and M. Peters-Golden, 2010, The antifibrotic effects of plasminogen

activation occur via prostaglandin E-2 synthesis in humans and mice: Journal of Clinical Investigation, v. 120, p. 1950-1960.

Baumgartner, K. B., J. M. Samet, C. A. Stidley, T. V. Colby, J. A. Waldron, D. B. Coultas, G. S. Davis, J. G. N. Garcia, G. W. Hunninghake, M. C. Kallay, T. E. King, M. J. Krowka, S. I. Rennard, J. H. Ryu, C. B. Sherman, L. J. Smith, G. Toews, and R. H. Winterbauer, 1997, Cigarette smoking: A risk factor for idiopathic pulmonary fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 155, p. 242-248.

Bechtel, W., S. McGoohan, E. Zeisberg, G. Muller, H. Kalbacher, D. J. Salant, C. Muller, R. Kalluri, and M. Zeisberg, 2011, Methylation determines fibroblast activation and fibrogenesis in the kidney: Internist, v. 52, p. 736-736.

Belinsky, S. A., W. A. Palmisano, F. D. Gilliland, L. A. Crooks, K. K. Divine, S. A. Winters, M. J. Grimes, H. J. Harms, C. S. Tellez, T. M. Smith, P. P. Moots, J. F. Lechner, C. A. Stidley, and R. E. Crowell, 2002, Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers: Cancer Research, v. 62, p. 2370-2377.

Berger, S. L., 2007, The complex language of chromatin regulation during transcription: Nature, v. 447, p. 407-412.

Bian, E.-B., C. Huang, H. Wang, B.-M. Wu, L. Zhang, X.-w. Lv, and J. Li, 2013, DNA methylation: New therapeutic implications for hepatic fibrosis: Cellular Signalling, v. 25, p. 355-358.

Bissell, D. M., S. S. Wang, W. R. Jarnagin, and F. J. Roll, 1995, CELL-SPECIFIC EXPRESSION OF TRANSFORMING GROWTH-FACTOR-BETA IN RAT-LIVER - EVIDENCE FOR AUTOCRINE REGULATION OF HEPATOCYTE PROLIFERATION: Journal of Clinical Investigation, v. 96, p. 447-455.

Blom, I. E., A. J. van Dijk, R. A. de Weger, M. G. J. Tilanus, and R. Goldschmeding, 2001, Identification of human ccn2*(connective tissue growth factor) promoter polymorphisms: Journal of Clinical Pathology-Molecular Pathology, v. 54, p. 192-196.

Boehm, U., T. Klamp, M. Groot, and J. C. Howard, 1997, Cellular responses to interferon-gamma: Annual Review of Immunology, v. 15, p. 749-795.

Bonner, J. C., 2004, Regulation of PDGF and its receptors in fibrotic diseases: Cytokine & Growth Factor Reviews, v. 15, p. 255-273.

Bonniaud, P., M. Kolb, T. Galt, J. Robertson, C. Robbins, M. Stampfli, C. Lavery, P. J. Margetts, A. B. Roberts, and J. Gauldie, 2004, Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis: Journal of Immunology, v. 173, p. 2099-2108.

Bonniaud, P., P. J. Margetts, M. Kolb, J. A. Schroeder, A. M. Kapoun, D. Damm, A. Murphy, S. Chakravarty, S. Dugar, L. Higgins, A. A. Protter, and J. Gauldie, 2005, Progressive transforming growth factor beta 1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor: American Journal of Respiratory and Critical Care Medicine, v. 171, p. 889-898.

Borok, Z., A. Gillissen, R. Buhl, R. F. Hoyt, R. C. Hubbard, T. Ozaki, S. I. Rennard, and R. G. Crystal, 1991, AUGMENTATION OF FUNCTIONAL PROSTAGLANDIN-E LEVELS ON THE RESPIRATORY EPITHELIAL SURFACE BY AEROSOL ADMINISTRATION

OF PROSTAGLANDIN-E: American Review of Respiratory Disease, v. 144, p. 1080-1084.

Bottinger, E. P., and M. Bitzer, 2002, TGF-beta signaling in renal disease: Journal of the American Society of Nephrology, v. 13, p. 2600-2610.

Bottinger, E. P., J. J. Letterio, and A. B. Roberts, 1997, Biology of TGF-beta in knockout and transgenic mouse models: Kidney International, v. 51, p. 1355-1360.

Bradbury, D. A., R. Newton, Y. M. Zhu, H. El-Haroun, L. Corbett, and A. J. Knox, 2003, Cyclooxygenase-2 induction by bradykinin in human pulmonary artery smooth muscle cells is mediated by the cyclic AMP response element through a novel autocrine loop involving endogenous prostaglandin E-2, E-prostanoid 2 (EP2), and EP4 receptors: Journal of Biological Chemistry, v. 278, p. 49954-49964.

Breyer, R. M., C. K. Bagdassarian, S. A. Myers, and M. D. Breyer, 2001, Prostanoid receptors: Subtypes and signaling: Annual Review of Pharmacology and Toxicology, v. 41, p. 661-690.

Brunton, L. L., J. S. Hayes, and S. E. Mayer, 1981, FUNCTIONAL COMPARTMENTATION OF CYCLIC-AMP AND PROTEIN-KINASE IN HEART: Advances in Cyclic Nucleotide Research, v. 14, p. 391-397.

Bucala, R., L. A. Spiegel, J. Chesney, M. Hogan, and A. Cerami, 1994, CIRCULATING FIBROCYTES DEFINE A NEW LEUKOCYTE SUBPOPULATION THAT MEDIATES TISSUE-REPAIR: Molecular Medicine, v. 1.

Burke, S. J., J. J. Collier, and D. K. Scott, 2009, cAMP Prevents Glucose-Mediated Modifications of Histone H3 and Recruitment of the RNA Polymerase II Holoenzyme to the L-PK Gene Promoter: Journal of Molecular Biology, v. 392, p. 578-588.

Campbell, S. L., R. Khosravi-Far, K. L. Rossman, G. J. Clark, and C. J. Der, 1998, Increasing complexity of Ras signaling: Oncogene, v. 17, p. 1395-1413.

Carey, K. D., R. T. Watson, J. E. Pessin, and P. J. S. Stork, 2003, The requirement of specific membrane domains for Raf-1 phosphorylation and activation: Journal of Biological Chemistry, v. 278, p. 3185-3196.

Chambers, R. C., 2003, Role of coagulation cascade proteases in lung repair and fibrosis: European Respiratory Journal, v. 22, p. 33S-35S.

Chang, Y., X. Zhang, J. R. Horton, A. K. Upadhyay, A. Spannhoff, J. Liu, J. P. Snyder, M. T. Bedford, and X. Cheng, 2009, Structural basis for G9a-like protein lysine methyltransferase inhibition by BIX-01294: Nature Structural & Molecular Biology, v. 16, p. 312-317.

Chapnick, D. A., L. Warner, J. Bernet, T. Rao, and X. Liu, 2011, Partners in crime: the TGF beta and MAPK pathways in cancer progression: Cell and Bioscience, v. 1.

Charbeneau, R. P., and M. Peters-Golden, 2005, Eicosanoids: mediators and therapeutic targets in fibrotic lung disease: Clinical Science, v. 108, p. 479-491.

Cheifetz, S., J. A. Weatherbee, M. L. S. Tsang, J. K. Anderson, J. E. Mole, R. Lucas, and J. Massague, 1987, THE TRANSFORMING GROWTH-FACTOR-BETA SYSTEM, A

COMPLEX PATTERN OF CROSS-REACTIVE LIGANDS AND RECEPTORS: Cell, v. 48, p. 409-415.

Chen, B. C., C. C. Yu, H. C. Lei, M. S. Chang, M. J. Hsu, C. L. Huang, M. C. Chen, J. R. Sheu, T. F. Chen, T. L. Chen, H. Inoue, and C. H. Lin, 2004, Bradykinin B2 receptor mediates NF-kappa B activation and cyclooxygenase-2 expression via the Ras/Raf-1/ERK pathway in human airway epithelial cells: Journal of Immunology, v. 173, p. 5219-5228.

Chen, T. S., R. W. Cho, P. J. S. Stork, and M. J. Weber, 1999, Elevation of cyclic adenosine 3 ',5 '-monophosphate potentiates activation of mitogen-activated protein kinase by growth factors in LNCaP prostate cancer cells: Cancer Research, v. 59, p. 213-218.

Chen, Y., T. Ali, V. Todorovic, J. M. O'Leary, A. K. Downing, and D. B. Rifkin, 2005, Amino acid requirements for formation of the TGF-beta-latent TGF-beta binding protein complexes: Journal of Molecular Biology, v. 345, p. 175-186.

Cheng, X. D., Z. Y. Ji, T. Tsalkova, and F. Mei, 2008, Epac and PKA: a tale of two intracellular cAMP receptors: Acta Biochimica Et Biophysica Sinica, v. 40, p. 651-662.

Chesney, J., C. Metz, A. B. Stavitsky, M. Bacher, and R. Bucala, 1998, Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes: Journal of Immunology, v. 160, p. 419-425.

Cheung, P., C. D. Allis, and P. Sassone-Corsi, 2000, Signaling to chromatin through histone modifications: Cell, v. 103, p. 263-271.

Choung, J., L. Taylor, K. Thomas, X. Zhou, H. Kagan, X. Yang, and P. Polgar, 1998, Role of EP2 receptors and cAMP in prostaglandin E-2 regulated expression of type I collagen alpha 1, lysyl oxidase, and cyclooxygenase-1 genes in human embryo lung fibroblasts: Journal of Cellular Biochemistry, v. 71, p. 254-263.

Christensen, A. E., F. Selheim, J. de Rooij, S. Dremier, F. Schwede, K. K. Dao, A. Martinez, C. Maenhaut, J. L. Bos, H. G. Genieser, and S. O. Doskeland, 2003, cAMP analog mapping of Epac1 and cAMP kinase - Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension: Journal of Biological Chemistry, v. 278, p. 35394-35402.

Coker, R. K., G. J. Laurent, S. Shahzeidi, P. A. Lympany, R. M. duBois, P. K. Jeffery, and R. J. McAnulty, 1997, Transforming growth factors-beta(1), -beta(2), and -beta(3) stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis: American Journal of Pathology, v. 150, p. 981-991.

Constantinescu, A., A. S. Gordon, and I. Diamond, 2002, CAMP-dependent protein kinase types I and II differentially regulate cAMP response element-mediated gene expression - Implications for neuronal responses to ethanol: Journal of Biological Chemistry, v. 277, p. 18810-18816.

Conte, E., M. Fruciano, E. Fagone, E. Gili, F. Caraci, M. Iemmolo, N. Crimi, and C. Vancheri, 2011, Inhibition of PI3K Prevents the Proliferation and Differentiation of Human Lung Fibroblasts into Myofibroblasts: The Role of Class I P110 Isoforms: Plos One, v. 6.

Conti, M., W. Richter, C. Mehats, G. Livera, J. Y. Park, and C. Jin, 2003, Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling: Journal of Biological Chemistry, v. 278, p. 5493-5496.

Cooper, D. M. F., 2003, Regulation and organization of adenylyl cyclases and cAMP: Biochemical Journal, v. 375, p. 517-529.

Cottin, V., 2013, Interstitial lung disease: European respiratory review : an official journal of the European Respiratory Society, v. 22, p. 26-32.

Coward, W. R., C. A. Feghali-Bostwick, G. Jenkins, A. J. Knox, and L. Pang, 2014, A central role for G9a and EZH2 in the epigenetic silencing of cyclooxygenase-2 in idiopathic pulmonary fibrosis: FASEB J.

Coward, W. R., G. Saini, and G. Jenkins, 2010a, The pathogenesis of idiopathic pulmonary fibrosis: Ther Adv Respir Dis, v. 4, p. 367-88.

Coward, W. R., K. Watts, C. A. Feghali-Bostwick, G. Jenkins, and L. Pang, 2010b, Repression of IP-10 by Interactions between Histone Deacetylation and Hypermethylation in Idiopathic Pulmonary Fibrosis: Molecular and Cellular Biology, v. 30, p. 2874-2886.

Coward, W. R., K. Watts, C. A. Feghali-Bostwick, A. Knox, and L. H. Pang, 2009, Defective Histone Acetylation Is Responsible for the Diminished Expression of Cyclooxygenase 2 in Idiopathic Pulmonary Fibrosis: Molecular and Cellular Biology, v. 29, p. 4325-4339.

Crystal, R. G., J. D. Fulmer, W. C. Roberts, M. L. Moss, B. R. Line, and H. Y. Reynolds, 1976, IDIOPATHIC PULMONARY FIBROSIS - CLINICAL, HISTOLOGIC, RADIOGRAPHIC, PHYSIOLOGIC, SCINTIGRAPHIC, CYTOLOGIC, AND BIOCHEMICAL ASPECTS: Annals of Internal Medicine, v. 85, p. 769-788.

Dackor, R. T., J. Cheng, J. W. Voltz, J. W. Card, C. D. Ferguson, R. C. Garrett, J. A. Bradbury, L. M. DeGraff, F. B. Lih, K. B. Tomer, G. P. Flake, G. S. Travlos, R. W. Ramsey, M. L. Edin, D. L. Morgan, and D. C. Zeldin, 2011, Prostaglandin E(2) protects murine lungs from bleomycin-induced pulmonary fibrosis and lung dysfunction: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 301, p. L645-L655.

Dao, K. K., K. Teigen, R. Kopperud, E. Hodneland, F. Schwede, A. E. Christensen, A. Martinez, and S. O. Doskeland, 2006, Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition: Journal of Biological Chemistry, v. 281, p. 21500-21511.

Datta, A., C. J. Scotton, and R. C. Chambers, 2011, Novel therapeutic approaches for pulmonary fibrosis: British Journal of Pharmacology, v. 163.

de Caestecker, M. P., W. T. Parks, C. J. Frank, P. Castagnino, D. P. Bottaro, A. B. Roberts, and R. J. Lechleider, 1998, Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases: Genes & Development, v. 12, p. 1587-1592.

de Rooij, J., F. J. T. Zwartkruis, M. H. G. Verheijen, R. H. Cool, S. M. B. Nijman, A. Wittinghofer, and J. L. Bos, 1998, Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP: Nature, v. 396, p. 474-477.

De Ruijter, A. J. M., A. H. Van Gennip, H. N. Caron, S. Kemp, and A. B. P. Van Kuilenburg, 2003, Histone deacetylases (HDACs): characterization of the classical HDAC family: Biochemical Journal, v. 370, p. 737-749.

De Ruiter, N. D., B. M. T. Burgering, and J. L. Bos, 2001, Regulation of the forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451: Molecular and Cellular Biology, v. 21, p. 8225-8235.

Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel, 1985, HUMAN TRANSFORMING GROWTH FACTOR-BETA COMPLEMENTARY-DNA SEQUENCE AND EXPRESSION IN NORMAL AND TRANSFORMED-CELLS: Nature, v. 316, p. 701-705.

Derynck, R., and Y. E. Zhang, 2003, Smad-dependent and Smad-independent pathways in TGF-beta family signalling: Nature, v. 425, p. 577-584.

Desmouliere, A., A. Geinoz, F. Gabbiani, and G. Gabbiani, 1993, TRANSFORMING GROWTH-FACTOR-BETA-1 INDUCES ALPHA-SMOOTH MUSCLE ACTIN EXPRESSION IN GRANULATION-TISSUE MYOFIBROBLASTS AND IN QUIESCENT AND GROWING CULTURED FIBROBLASTS: Journal of Cell Biology, v. 122, p. 103-111.

Diaz, A., E. Munoz, R. Johnston, J. H. Korn, and S. A. Jimenez, 1993, REGULATION OF HUMAN LUNG FIBROBLAST-ALPHA-1(I) PROCOLLAGEN GENE-EXPRESSION BY TUMOR-NECROSIS-FACTOR-ALPHA, INTERLEUKIN-1-BETA, AND PROSTAGLANDIN-E(2): Journal of Biological Chemistry, v. 268, p. 10364-10371.

Dinarello, C. A., 1996, Biologic basis for interleukin-1 in disease: Blood, v. 87, p. 2095-147.

Dobosy, J. R., and E. U. Selker, 2001, Emerging connections between DNA methylation and histone acetylation: Cellular and Molecular Life Sciences, v. 58, p. 721-727.

Downward, J., 1996, Control of ras activation: Cancer Surveys, v. 27, p. 87-100.

Downward, J., 2003, Targeting ras signalling pathways in cancer therapy: Nature Reviews Cancer, v. 3, p. 11-22.

du Bois, R. M., 2010, Strategies for treating idiopathic pulmonary fibrosis: Nature Reviews Drug Discovery, v. 9, p. 129-140.

Dumaz, N., and R. Marais, 2005, Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways - Based on the Anniversary Prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the special FEBS meeting in Brussels: Febs Journal, v. 272, p. 3491-3504.

Dunkern, T. R., D. Feurstein, G. A. Rossi, F. Sabatini, and A. Hatzelmann, 2007, Inhibition of TGF-beta induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase: European Journal of Pharmacology, v. 572, p. 12-22.

Dwivedi, R. S., J. G. Herman, T. A. McCaffrey, and D. S. C. Raj, 2011, Beyond genetics: epigenetic code in chronic kidney disease: Kidney International, v. 79, p. 23-32.

Dworniczak, S., D. Ziora, M. Kapral, U. Mazurek, G. Niepsuj, R. Rauer, T. Wilczok, and J. Kozielski, 2004, Human cytomegalovirus DNA level in patients with idiopathic pulmonary fibrosis: J Physiol Pharmacol, v. 55 Suppl 3, p. 67-75.

Ebihara, Y., M. Masuya, A. C. LaRue, P. A. Fleming, R. P. Visconti, H. Minamiguchi, C. J. Drake, and M. Ogawa, 2006, Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes: Experimental Hematology, v. 34, p. 219-229.

Elias, J. A., M. D. Rossman, R. B. Zurier, and R. P. Daniele, 1985, HUMAN ALVEOLAR MACROPHAGE INHIBITION OF LUNG FIBROBLAST GROWTH - A PROSTAGLANDIN-DEPENDENT PROCESS: American Review of Respiratory Disease, v. 131, p. 94-99.

Engel, M. E., M. A. McDonnell, B. K. Law, and H. L. Moses, 1999, Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription: Journal of Biological Chemistry, v. 274, p. 37413-37420.

Epler, G. R., C. B. Carrington, and E. A. Gaensler, 1978, CRACKLES (RALES) IN INTERSTITIAL PULMONARY-DISEASES: Chest, v. 73, p. 333-339.

Evans, R. A., Y. C. Tian, R. Steadman, and A. O. Phillips, 2003, TGF-beta 1-mediated fibroblast-myofibroblast terminal differentiation - the role of Smad proteins: Experimental Cell Research, v. 282, p. 90-100.

Eyden, B., 2008, The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine: Journal of Cellular and Molecular Medicine, v. 12, p. 22-37.

Failla, M., T. Genovese, E. Mazzon, M. Fruciano, E. Fagone, E. Gili, A. Barera, C. La Rosa, E. Conte, N. Crimi, S. Cuzzocrea, and C. Vancheri, 2009, 16,16-Dimethyl Prostaglandin E-2 Efficacy on Prevention and Protection from Bleomycin-Induced Lung Injury and Fibrosis: American Journal of Respiratory Cell and Molecular Biology, v. 41, p. 50-58.

Ferguson, R. E., H. P. Carroll, A. Harris, E. R. Maher, P. J. Selby, and R. E. Banks, 2005, Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies: Proteomics, v. 5, p. 566-571.

Fine, A., C. F. Poliks, L. P. Donahue, B. D. Smith, and R. H. Goldstein, 1989, THE DIFFERENTIAL EFFECT OF PROSTAGLANDIN-E2 ON TRANSFORMING GROWTH FACTOR-BETA AND INSULIN-INDUCED COLLAGEN FORMATION IN LUNG FIBROBLASTS: Journal of Biological Chemistry, v. 264, p. 16988-16991.

Fischer, A., F. J. Pfalzgraf, C. A. Feghali-Bostwick, T. M. Wright, D. Curran-Everett, S. G. West, and K. K. Brown, 2006, Anti-Th/To-positivity in a cohort of patients with idiopathic pulmonary fibrosis: Journal of Rheumatology, v. 33, p. 1600-1605.

Fischle, W., B. S. Tseng, H. L. Dormann, B. M. Ueberheide, B. A. Garcia, J. Shabanowitz, D. F. Hunt, H. Funabiki, and C. D. Allis, 2005, Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation: Nature, v. 438, p. 1116-1122.

Fletcher, T. M., and J. C. Hansen, 1995, CORE HISTONE TAIL DOMAINS MEDIATE OLIGONUCLEOSOME FOLDING AND NUCLEOSOMAL DNA ORGANIZATION THROUGH DISTINCT MOLECULAR MECHANISMS: Journal of Biological Chemistry, v. 270, p. 25359-25362.

Fonseca, C., D. Abraham, and E. A. Renzoni, 2011, Endothelin in Pulmonary Fibrosis: American Journal of Respiratory Cell and Molecular Biology, v. 44, p. 1-10.

Francis, S. H., M. A. Blount, and J. D. Corbin, 2011, Mammalian Cyclic Nucleotide Phosphodiesterases: Molecular Mechanisms and Physiological Functions: Physiological Reviews, v. 91, p. 651-690.

Fuentes-Calvo, I., A. M. Blazquez-Medela, N. Eleno, E. Santos, J. M. Lopez-Novoa, and C. Martinez-Salgado, 2012, H-Ras isoform modulates extracellular matrix synthesis, proliferation, and migration in fibroblasts: American Journal of Physiology-Cell Physiology, v. 302, p. C686-C697.

Furuyama, A., T. Hosokawa, and K. Mochitate, 2008, Interleukin-1beta and tumor necrosis factor-alpha have opposite effects on fibroblasts and epithelial cells during basement membrane formation: Matrix Biol, v. 27, p. 429-40.

Gabbiani, G., 2003, The myofibroblast in wound healing and fibrocontractive diseases: Journal of Pathology, v. 200, p. 500-503.

Gabbiani, G., G. B. Ryan, and G. Majno, 1971, PRESENCE OF MODIFIED FIBROBLASTS IN GRANULATION TISSUE AND THEIR POSSIBLE ROLE IN WOUND CONTRACTION: Experientia, v. 27, p. 549-&.

Galie, N., M. Humbert, J. L. Vachiery, C. D. Vizza, M. Kneussl, A. Manes, O. Sitbon, A. Torbicki, M. Delcroix, R. Naeije, M. Hoeper, A. Chaouat, S. Morand, B. Besse, G. Simonneau, H. Arterial Pulm, and Berap, 2002, Effects of beraprost sodium, an oral prostacyclin analogue, in patients with pulmonary arterial hypertension: With pulmonary arterial hypertension: A randomized, double-blind, placebo-controlled trial: Journal of the American College of Cardiology, v. 39, p. 1496-1502.

Galuppo, M., E. Esposito, E. Mazzon, R. Di Paola, I. Paterniti, D. Impellizzeri, and S. Cuzzocrea, 2011, MEK inhibition suppresses the development of lung fibrosis in the bleomycin model: Naunyn-Schmiedebergs Archives of Pharmacology, v. 384, p. 21-37.

Garrison, G., S. K. Huang, K. Okunishi, J. P. Scott, L. R. K. Penke, A. M. Scruggs, and M. Peters-Golden, 2013, Reversal of Myofibroblast Differentiation by Prostaglandin E-2: American Journal of Respiratory Cell and Molecular Biology, v. 48, p. 550-558.

Gauvreau, G. M., R. M. Watson, and P. M. O'Byrne, 1999, Protective effects of inhaled PGE(2) on allergen-induced airway responses and airway inflammation: American Journal of Respiratory and Critical Care Medicine, v. 159, p. 31-36.

Gharaee-Kermani, M., M. R. Gyetko, B. Hu, and S. H. Phan, 2007, New insights into the pathogenesis and treatment of idiopathic pulmonary fibrosis: A potential role for stem cells in the lung parenchyma and implications for therapy: Pharmaceutical Research, v. 24, p. 819-841.

Ghosh, A. K., Y. Mori, E. Dowling, and J. Varga, 2007, Trichostatin A blocks TGF-beta-induced collagen gene expression in skin fibroblasts: Involvement of Sp1: Biochemical and Biophysical Research Communications, v. 354, p. 420-426.

Gilman, A. G., 1970, A PROTEIN BINDING ASSAY FOR ADENOSINE 3'-5'-CYCLIC MONOPHOSPHATE: Proceedings of the National Academy of Sciences of the United States of America, v. 67, p. 305-&.

Glenisson, W., V. Castronovo, and D. Waltregny, 2007, Histone deacetylase 4 is required for TGF beta 1-induced myofibroblastic differentiation: Biochimica Et Biophysica Acta-Molecular Cell Research, v. 1773, p. 1572-1582.

Goldstein, R. H., and P. Polgar, 1982, THE EFFECT AND INTERACTION OF BRADYKININ AND PROSTAGLANDINS ON PROTEIN AND COLLAGEN PRODUCTION BY LUNG FIBROBLASTS: Journal of Biological Chemistry, v. 257, p. 8630-8633.

Goodwin, A., and G. Jenkins, 2009, Role of integrin-mediated TGF beta activation in the pathogenesis of pulmonary fibrosis: Biochemical Society Transactions, v. 37, p. 849-854.

Gorgoni, B., M. Caivano, C. Arizmendi, and V. Poli, 2001, The transcription factor C/EBP beta is essential for inducible expression of the cox-2 gene in macrophages but not in fibroblasts: Journal of Biological Chemistry, v. 276, p. 40769-40777.

Goulet, S., M. P. Bihl, F. Gambazzi, M. Tamm, and M. Roth, 2007, Opposite effect of corticosteroids and long-acting beta 2-agonits on serum- and TGF-beta(1)-induced extracellular matrix deposition by primary human lung fibroblasts: Journal of Cellular Physiology, v. 210, p. 167-176.

Grande, M. T., I. Fuentes-Calvo, M. Arevalo, F. Heredia, E. Santos, C. Martinez-Salgado, D. Rodriguez-Puyol, M. A. Nieto, and J. M. Lopez-Novoa, 2010, Deletion of H-Ras decreases renal fibrosis and myofibroblast activation following ureteral obstruction in mice: Kidney International, v. 77, p. 509-518.

Gribbin, J., R. B. Hubbard, I. Le Jeune, C. J. P. Smith, J. West, and L. J. Tata, 2006, Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK: Thorax, v. 61, p. 980-985.

Griffiths, M. J. D., D. Bonnet, and S. M. Janes, 2005, Stem cells of the alveolar epithelium: Lancet, v. 366, p. 249-260.

Gross, T. J., and G. W. Hunninghake, 2001, Medical progress: Idiopathic pulmonary fibrosis: New England Journal of Medicine, v. 345, p. 517-525.

Grunert, S., M. Jechlinger, and H. Beug, 2003, Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis: Nature Reviews Molecular Cell Biology, v. 4, p. 657-665.

Guo, W., B. Shan, R. C. Klingsberg, X. Qin, and J. A. Lasky, 2009, Abrogation of TGF-beta 1-induced fibroblast-myofibroblast differentiation by histone deacetylase inhibition: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 297, p. L864-L870.

Hagimoto, N., K. Kuwano, I. Inoshima, M. Yoshimi, N. Nakamura, M. Fujita, T. Maeyama, and N. Hara, 2002, TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells: Journal of Immunology, v. 168, p. 6470-6478.

Haklai, R., M. G. Weisz, G. Elad, A. Paz, D. Marciano, Y. Egozi, G. Ben-Baruch, and Y. Kloog, 1998, Dislodgment and accelerated degradation of Ras: Biochemistry, v. 37, p. 1306-1314.

Halaschek-Wiener, J., V. Wacheck, H. Schlagbauer-Wadl, K. Wolff, Y. Kloog, and B. Jansen, 2000, A novel Ras antagonist regulates both oncogenic Ras and the tumor suppressor p53 in colon cancer cells: Molecular Medicine, v. 6, p. 693-704.

Hall, A., 2012, Rho family GTPases: Biochemical Society Transactions, v. 40, p. 1378-1382.

Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall, 1989, ALL RAS PROTEINS ARE POLYISOPRENYLATED BUT ONLY SOME ARE PALMITOYLATED: Cell, v. 57, p. 1167-1177.

Hardie, W. D., J. S. Hagood, V. Dave, A.-K. T. Perl, J. A. Whitsett, T. R. Korfhagen, and S. Glasser, 2010, Signaling pathways in the epithelial origins of pulmonary fibrosis: Cell Cycle, v. 9, p. 2769-2776.

Hartl, D., M. Griese, T. Nicolai, G. Zissel, C. Prell, D. Reinhardt, D. J. Schendel, and S. Krauss-Etschmann, 2005, A role for MCP-1/CCR2 in interstitial lung disease in children: Respiratory Research, v. 6.

Hartsough, M. T., R. S. Frey, P. A. Zipfel, A. Buard, S. J. Cook, F. McCormick, and K. M. Mulder, 1996, Altered transforming growth factor beta signaling in epithelial cells when Ras activation is blocked: Journal of Biological Chemistry, v. 271, p. 22368-22375.

Hayashida, T., M. deCaestecker, and H. W. Schnaper, 2003, Cross-talk between ERK MAP kinase and Smad-signaling pathways enhances TGF-beta dependent responses in human mesangial cells: Faseb Journal, v. 17, p. 1576-+.

Hayes, J. S., and L. L. Brunton, 1982, FUNCTIONAL COMPARTMENTS IN CYCLIC-NUCLEOTIDE ACTION: Journal of Cyclic Nucleotide Research, v. 8, p. 1-16.

Heise, C. E., B. F. O'Dowd, D. J. Figueroa, N. Sawyer, T. Nguyen, D. S. Im, R. Stocco, J. N. Bellefeuille, M. Abramovitz, R. Cheng, D. L. Williams, Z. Z. Zeng, Q. Y. Liu, L. Ma, M. K. Clements, N. Coulombe, Y. Liu, C. P. Austin, S. R. George, G. P. O'Neill, K. M. Metters, K. R. Lynch, and J. F. Evans, 2000, Characterization of the human cysteinyl leukotriene 2 receptor: Journal of Biological Chemistry, v. 275, p. 30531-30536.

Heldin, C. H., K. Miyazono, and P. tenDijke, 1997, TGF-beta signalling from cell membrane to nucleus through SMAD proteins: Nature, v. 390, p. 465-471.

Hetzel, M., M. Bachem, D. Anders, G. Trischler, and M. Faehling, 2005, Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts: Lung, v. 183, p. 225-237.

Hilberg, F., G. J. Roth, M. Krssak, S. Kautschitsch, W. Sommergruber, U. Tontsch-Grunt, P. Garin-Chesa, G. Bader, A. Zoephel, J. Quant, A. Heckel, and W. J. Rettig, 2008, BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy: Cancer Res, v. 68, p. 4774-82.

Hinz, B., 2010, The myofibroblast: Paradigm for a mechanically active cell: Journal of Biomechanics, v. 43, p. 146-155.

Hinz, B., S. H. Phan, V. J. Thannickal, A. Galli, M. L. Bochaton-Piallat, and G. Gabbiani, 2007a, The myofibroblast - One function, multiple origins: American Journal of Pathology, v. 170, p. 1807-1816.

Hinz, B., S. H. Phan, V. J. Thannickal, A. Galli, M. L. Bochaton-Piallat, and G. Gabbiani, 2007b, The myofibroblast: one function, multiple origins: Am J Pathol, v. 170, p. 1807-16.

Hinz, B., S. H. Phan, V. J. Thannickal, A. Galli, M. L. Bochaton-Piallat, and G. Gabbiani, 2007c, The myofibroblast: one function, multiple origins.: Am J Pathol, v. 170, p. 1807-16.

Hodges, R. J., R. G. Jenkins, C. P. D. Wheeler-Jones, D. M. Copeman, S. E. Bottoms, G. J. Bellingan, C. B. Nanthakumar, G. J. Laurent, S. L. Hart, M. L. Foster, and R. J. McAnulty, 2004, Severity of lung injury in cyclooxygenase-2-deficient mice is dependent on reduced prostaglandin E-2 production: American Journal of Pathology, v. 165, p. 1663-1676.

Holz, G. G., O. G. Chepurny, and F. Schwede, 2008, Epac-selective cAMP analogs: New tools with which to evaluate the signal transduction properties of cAMP-regulated guanine nucleotide exchange factors: Cellular Signalling, v. 20, p. 10-20.

Hong, K. M., J. A. Belperio, M. P. Keane, M. D. Burdick, and R. M. Strieter, 2007, Differentiation of human circulating fibrocytes as mediated by transforming growth factor-beta and peroxisome proliferator-activated receptor gamma: Journal of Biological Chemistry, v. 282, p. 22910-22920.

Horan, G. S., S. Wood, V. Ona, D. J. Li, M. E. Lukashev, P. H. Weinreb, K. J. Simon, K. Hahm, N. E. Allaire, N. J. Rinaldi, J. Goyal, C. A. Feghali-Bostwick, E. L. Matteson, C. O'Hara, R. Lafyatis, G. S. Davis, X. Huang, D. Sheppard, and S. M. Violette, 2008, Partial inhibition of integrin alpha(v)beta6 prevents pulmonary fibrosis without exacerbating inflammation: Am J Respir Crit Care Med, v. 177, p. 56-65.

Hu, A., G. Nino, J. S. Grunstein, S. Fatma, and M. M. Grunstein, 2008, Prolonged heterologous beta 2-adrenoceptor desensitization promotes proasthmatic airway smooth muscle function via PKA/ERK1/2-mediated phosphodiesterase-4 induction: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 294, p. L1055-L1067.

Hu, B., M. Gharaee-Kermani, Z. Wu, and S. H. Phan, 2010, Epigenetic Regulation of Myofibroblast Differentiation by DNA Methylation: American Journal of Pathology, v. 177, p. 21-28.

Huang, S., S. H. Wettlaufer, C. Hogaboam, D. M. Aronoff, and M. Peters-Golden, 2007, Prostaglandin E-2 inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 292, p. L405-L413.

Huang, S. K., and M. Peters-Golden, 2008, Eicosanoid lipid mediators in fibrotic lung diseases: Ready for prime time?: Chest, v. 133, p. 1442-1450.

Huang, S. K., A. M. Scruggs, J. Donaghy, R. C. McEachin, A. S. Fisher, B. C. Richardson, and M. Peters-Golden, 2012, Prostaglandin E-2 increases fibroblast gene-specific and global DNA methylation via increased DNA methyltransferase expression: Faseb Journal, v. 26, p. 3703-3714.

Huang, S. K., S. H. Wettlaufer, C. M. Hogaboam, K. R. Flaherty, F. J. Martinez, J. L. Myers, T. V. Colby, W. D. Travis, G. B. Toews, and M. Peters-Golden, 2008a, Variable prostaglandin E-2 resistance in fibroblasts from patients with usual interstitial pneumonia: American Journal of Respiratory and Critical Care Medicine, v. 177, p. 66-74.

Huang, S. K., S. H. Wettlauferl, L. Chung, and M. Peters-Golden, 2008b, Prostaglandin E-2 inhibits specific lung fibroblast functions via selective actions of PKA and Epac-1: American Journal of Respiratory Cell and Molecular Biology, v. 39, p. 482-489.

Huang, S. K., E. S. White, S. H. Wettlaufer, H. Grifka, C. M. Hogaboam, V. J. Thannickal, J. C. Horowitz, and M. Peters-Golden, 2009, Prostaglandin E-2 induces fibroblast

apoptosis by modulating multiple survival pathways: Faseb Journal, v. 23, p. 4317-4326.

Huang, X., J. Wu, W. Zhu, R. Pytela, and D. Sheppard, 1998, Expression of the human integrin beta6 subunit in alveolar type II cells and bronchiolar epithelial cells reverses lung inflammation in beta6 knockout mice: Am J Respir Cell Mol Biol, v. 19, p. 636-42.

Hubbard, R., I. Johnston, D. B. Coultas, and J. Britton, 1996, Mortality rates from cryptogenic fibrosing alveolitis in seven countries: Thorax, v. 51, p. 711-716.

Häkkinen, L., L. Koivisto, H. Gardner, U. Saarialho-Kere, J. M. Carroll, M. Lakso, H. Rauvala, M. Laato, J. Heino, and H. Larjava, 2004, Increased expression of beta6-integrin in skin leads to spontaneous development of chronic wounds: Am J Pathol, v. 164, p. 229-42.

Insel, P. A., F. Murray, U. Yokoyama, S. Romano, H. Yun, L. Brown, A. Snead, D. Lu, and N. Aroonsakool, 2012, cAMP and Epac in the regulation of tissue fibrosis: British Journal of Pharmacology, v. 166, p. 447-456.

Itoh, S., M. Landstrom, A. Hermansson, F. Itoh, C. H. Heldin, N. E. Heldin, and P. ten Dijke, 1998, Transforming growth factor beta 1 induces nuclear export of inhibitory Smad7: Journal of Biological Chemistry, v. 273, p. 29195-29201.

Ivanova, V., O. B. Garbuzenko, K. R. Reuhl, D. C. Reimer, V. P. Pozharov, and T. Minko, 2013, Inhalation treatment of pulmonary fibrosis by liposomal prostaglandin E2: European Journal of Pharmaceutics and Biopharmaceutics, v. 84, p. 335-344.

Iwano, M., D. Plieth, T. M. Danoff, C. Xue, H. Okada, and E. G. Neilson, 2002, Evidence that fibroblasts derive from epithelium during tissue fibrosis: Journal of Clinical Investigation, v. 110, p. 341-350.

Janda, E., K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, and S. Grunert, 2002, Ras and TGF beta cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways: Journal of Cell Biology, v. 156, p. 299-313.

Jenkins, G., 2008, The role of proteases in transforming growth factor-beta activation: International Journal of Biochemistry & Cell Biology, v. 40, p. 1068-1078.

Jenkins, G., S. L. Hart, R. J. Hodges, Q. H. Meng, C. Kinnon, G. J. Laurent, and R. J. McAnulty, 2002, Cyclooxygenase-2 overexpression, using an integrin-targeted gene delivery system (the LID vector), inhibits fibroblast proliferation in vitro and leads to increased prostaglandin E-2 in the lung: Chest, v. 121, p. 102S-104S.

Jenkins, R. G., X. Su, G. Su, C. J. Scotton, E. Camerer, G. J. Laurent, G. E. Davis, R. C. Chambers, M. A. Matthay, and D. Sheppard, 2006, Ligation of protease-activated receptor 1 enhances alpha(v)beta(6) integrin-dependent TGF-beta activation and promotes acute lung injury: Journal of Clinical Investigation, v. 116, p. 1606-1614.

Johnson, L., D. Greenbaum, K. Cichowski, K. Mercer, E. Murphy, E. Schmitt, R. T. Bronson, H. Umanoff, W. Edelmann, R. Kucherlapati, and T. Jacks, 1997, K-ras is an essential gene in the mouse with partial functional overlap with N-ras: Genes & Development, v. 11, p. 2468-2481.

Kalluri, R., and E. G. Neilson, 2003, Epithelial-mesenchymal transition and its implications for fibrosis: Journal of Clinical Investigation, v. 112, p. 1776-1784.

Kaminski, N., 2003, Microarray analysis of idiopathic pulmonary fibrosis: American Journal of Respiratory Cell and Molecular Biology, v. 29, p. S32-S36.

Kapanci, Y., A. Desmouliere, J. C. Pache, M. Redard, and G. Gabbiani, 1995, CYTOSKELETAL PROTEIN MODULATION IN PULMONARY ALVEOLAR MYOFIBROBLASTS DURING IDIOPATHIC PULMONARY FIBROSIS - POSSIBLE ROLE OF TRANSFORMING GROWTH-FACTOR-BETA AND TUMOR-NECROSIS-FACTOR-ALPHA: American Journal of Respiratory and Critical Care Medicine, v. 152, p. 2163-2169.

Katzenstein, A. L. A., and J. L. Myers, 1998, Idiopathic pulmonary fibrosis - Clinical relevance of pathologic classification: American Journal of Respiratory and Critical Care Medicine, v. 157, p. 1301-1315.

KauffmanZeh, A., P. RodriguezViciana, E. Ulrich, C. Gilbert, P. Coffer, J. Downward, and G. Evan, 1997, Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB: Nature, v. 385, p. 544-548.

Keane, M. P., J. A. Belperio, D. A. Arenberg, M. D. Burdick, Z. J. Xu, Y. Y. Xue, and R. M. Strieter, 1999, IFN-gamma-inducible protein-10 attenuates bleomycin-induced pulmonary fibrosis via inhibition of angiogenesis: Journal of Immunology, v. 163, p. 5686-5692.

Keerthisingam, C. B., R. G. Jenkins, N. K. Harrison, N. A. Hernandez-Rodriguez, H. Booth, G. J. Laurent, S. L. Hart, M. L. Foster, and R. J. McAnulty, 2001, Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice: American Journal of Pathology, v. 158, p. 1411-1422.

Kelley, G. G., S. E. Reks, J. M. Ondrako, and A. V. Smrcka, 2001, Phospholipase C epsilon: a novel Ras effector: Embo Journal, v. 20, p. 743-754.

Keogh, B. A., and R. G. Crystal, 1982, ALVEOLITIS - THE KEY TO THE INTERSTITIAL LUNG DISORDERS: Thorax, v. 37, p. 1-10.

Khalil, N., R. N. Oconnor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay, and A. H. Greenberg, 1991, INCREASED PRODUCTION AND IMMUNOHISTOCHEMICAL LOCALIZATION OF TRANSFORMING GROWTH-FACTOR-BETA IN IDIOPATHIC PULMONARY FIBROSIS: American Journal of Respiratory Cell and Molecular Biology, v. 5, p. 155-162.

Khwaja, A., P. RodriguezViciana, S. Wennstrom, P. H. Warne, and J. Downward, 1997, Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway: Embo Journal, v. 16, p. 2783-2793.

Khym, J. X., 1975, ANALYTICAL SYSTEM FOR RAPID SEPARATION OF TISSUE NUCLEOTIDES AT LOW-PRESSURES ON CONVENTIONAL ANION-EXCHANGERS: Clinical Chemistry, v. 21, p. 1245-1252.

Kievit, P., J. D. Lauten, and R. A. Maurer, 2001, Analysis of the role of the mitogen-activated protein kinase in mediating cyclic-adenosine 3 ',5 '-monophosphate effects on prolactin promoter activity: Molecular Endocrinology, v. 15, p. 614-624.

Kim, J. M., and H. Y. Kim, 2010, Pathogenesis of rheumatoid arthritis: Journal of the Korean Medical Association, v. 53, p. 853-861.

Kim, K. K., M. C. Kugler, P. J. Wolters, L. Robillard, M. G. Galvez, A. N. Brumwell, D. Sheppard, and H. A. Chapman, 2006, Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix: Proceedings of the National Academy of Sciences of the United States of America, v. 103, p. 13180-13185.

Kim, S., J. J. Shim, P. R. Burgel, I. F. Ueki, D. P. Trang, D. C. W. Tam, and J. A. Nadel, 2002, IL-13-induced Clara cell secretory protein expression in airway epithelium: role of EGFR signaling pathway: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 283, p. L67-L75.

King, T. E., W. Z. Bradford, S. Castro-Bernardini, E. A. Fagan, I. Glaspole, M. K. Glassberg, E. Gorina, P. M. Hopkins, D. Kardatzke, L. Lancaster, D. J. Lederer, S. D. Nathan, C. A. Pereira, S. A. Sahn, R. Sussman, J. J. Swigris, P. W. Noble, and A. S. Group, 2014, A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis: N Engl J Med, v. 370, p. 2083-92.

Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda, 1989, A RAS-RELATED GENE WITH TRANSFORMATION SUPPRESSOR ACTIVITY: Cell, v. 56, p. 77-84.

Kohyama, T., R. F. Ertl, V. Valenti, J. Spurzem, M. Kawamoto, Y. Nakamura, T. Veys, L. Allegra, D. Romberger, and S. I. Rennard, 2001, Prostaglandin E-2 inhibits fibroblast chemotaxis: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 281, p. L1257-L1263.

Kolb, M., P. J. Margetts, D. C. Anthony, F. Pitossi, and J. Gauldie, 2001a, Transient expression of IL-1 beta induces acute lung injury and chronic repair leading to pulmonary fibrosis: Journal of Clinical Investigation, v. 107, p. 1529-1536.

Kolb, M., P. J. Margetts, D. C. Anthony, F. Pitossi, and J. Gauldie, 2001b, Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis: J Clin Invest, v. 107, p. 1529-36.

Kolodsick, J. E., M. Peters-Golden, J. Larios, G. B. Toews, V. J. Thannickal, and B. B. Moore, 2003, Prostaglandin E-2 inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation: American Journal of Respiratory Cell and Molecular Biology, v. 29, p. 537-544.

Korn, J. H., P. V. Halushka, and E. C. Leroy, 1980, MONONUCLEAR CELL MODULATION OF CONNECTIVE-TISSUE FUNCTION - SUPPRESSION OF FIBROBLAST GROWTH BY STIMULATION OF ENDOGENOUS PROSTAGLANDIN PRODUCTION: Journal of Clinical Investigation, v. 65, p. 543-554.

Kotani, I., A. Sato, H. Hayakawa, T. Urano, Y. Takada, and A. Takada, 1995, INCREASED PROCOAGULANT AND ANTIFIBRINOLYTIC ACTIVITIES IN THE LUNGS WITH IDIOPATHIC PULMONARY FIBROSIS: Thrombosis Research, v. 77, p. 493-504.

Kottmann, R. M., C. M. Hogan, R. P. Phipps, and P. J. Sime, 2009, Determinants of initiation and progression of idiopathic pulmonary fibrosis: Respirology, v. 14, p. 917-933.

Kretzschmar, M., J. Doody, I. Timokhina, and J. Massague, 1999, A mechanism of repression of TGF beta/Smad signaling by oncogenic Ras: Genes & Development, v. 13, p. 804-816.

Kuhn, C., and J. A. McDonald, 1991, THE ROLES OF THE MYOFIBROBLAST IN IDIOPATHIC PULMONARY FIBROSIS - ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL FEATURES OF SITES OF ACTIVE EXTRACELLULAR-MATRIX SYNTHESIS: American Journal of Pathology, v. 138, p. 1257-1265.

Kumar, R. K., 2005, Morphological methods for assessment of fibrosis: Fibrosis Research: Methods and Protocols, v. 117, p. 179-188.

Lachner, M., N. O'Carroll, S. Rea, K. Mechtler, and T. Jenuwein, 2001, Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins: Nature, v. 410, p. 116-120.

Landells, L. J., B. Naidoo, J. Robertson, and P. Clark, 2013, NICE guidance on pirfenidone for treating idiopathic pulmonary fibrosis: Lancet Respiratory Medicine, v. 1, p. 191-192.

Lawson, W. E., V. V. Polosukhin, O. Zoia, G. T. Stathopoulos, W. Han, D. Plieth, J. E. Loyd, E. G. Neilson, and T. S. Blackwell, 2005, Characterization of fibroblast-specific protein 1 in pulmonary fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 171, p. 899-907.

Leask, A., and D. J. Abraham, 2003, The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology: Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire, v. 81, p. 355-363.

Leask, A., and D. J. Abraham, 2004, TGF-beta signaling and the fibrotic response: Faseb Journal, v. 18, p. 816-827.

Lefkowitz, R. J., W. P. Hausdorff, and M. G. Caron, 1990, ROLE OF PHOSPHORYLATION IN DESENSITIZATION OF THE BETA-ADRENOCEPTOR: Trends in Pharmacological Sciences, v. 11, p. 190-194.

Levine, S. J., 1995, Bronchial epithelial cell-cytokine interactions in airway inflammation: J Investig Med, v. 43, p. 241-9.

Ley, B., H. R. Collard, and T. E. King, Jr., 2011, Clinical Course and Prediction of Survival in Idiopathic Pulmonary Fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 183, p. 431-440.

Ley, B., C. J. Ryerson, E. Vittinghoff, J. H. Ryu, S. Tomassetti, J. S. Lee, V. Poletti, M. Buccioli, B. M. Elicker, K. D. Jones, T. E. King, Jr., and H. R. Collard, 2012, A Multidimensional Index and Staging System for Idiopathic Pulmonary Fibrosis: Annals of Internal Medicine, v. 156, p. 684-U58.

Li, J. H., Y. Guo, F. A. Schroeder, R. M. Youngs, T. W. Schmidt, C. Ferris, C. Konradi, and S. Akbarian, 2004, Dopamine D-2-like antagonists induce chromatin remodeling in striatal neurons through cyclic AMP-protein kinase A and NMDA receptor signaling: Journal of Neurochemistry, v. 90, p. 1117-1131.

Li, M., M. S. Krishnaveni, C. Li, B. Zhou, Y. Xing, A. Banfalvi, A. Li, V. Lombardi, O. Akbari, Z. Borok, and P. Minoo, 2011, Epithelium-specific deletion of TGF-β receptor type II protects mice from bleomycin-induced pulmonary fibrosis: J Clin Invest, v. 121, p. 277-87.

Lindenschmidt, R. C., and H. Witschi, 1985, ATTENUATION OF PULMONARY FIBROSIS IN MICE BY AMINOPHYLLINE: Biochemical Pharmacology, v. 34, p. 4269-4273.

Liu, B., C. L. Dou, L. Prabhu, and E. S. Lai, 1999, FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor beta signals: Molecular and Cellular Biology, v. 19, p. 424-430.

Liu, C. H., M. D. A. Gaca, E. S. Swenson, V. F. Vellucci, M. Reiss, and R. G. Wells, 2003, Smads 2 and 3 are differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells - Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent: Journal of Biological Chemistry, v. 278, p. 11721-11728.

Liu, J. Y., D. M. Brass, G. W. Hoyle, and A. R. Brody, 1998, TNF-alpha receptor knockout mice are protected from the fibroproliferative effects of inhaled asbestos fibers: Am J Pathol, v. 153, p. 1839-47.

Liu, P., J. F. Flejou, G. Feldmann, and D. Bernuau, 1994, ACTIVATION OF RAS ONCOGENE IN LIVERS WITH CIRRHOSIS: Journal of Hepatology, v. 21, p. 1103-1108.

Liu, X. Q., F. Y. Li, S. Q. Sun, M. Thangavel, J. Kaminsky, L. Balazs, and R. S. Ostrom, 2010, Fibroblast-specific expression of AC6 enhances beta-adrenergic and prostacyclin signaling and blunts bleomycin-induced pulmonary fibrosis: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 298, p. L819-L829.

Liu, X. Q., R. S. Ostrom, and P. A. Insel, 2004, CAMP-elevating agents and adenylyl cyclase overexpression promote an antifibrotic phenotype in pulmonary fibroblasts: American Journal of Physiology-Cell Physiology, v. 286, p. C1089-C1099.

Liu, X. Q., S. Q. Sun, A. Hassid, and R. S. Ostrom, 2006a, CAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts: Molecular Pharmacology, v. 70, p. 1992-2003.

Liu, X. Q., S. Q. Sun, and R. S. Ostrom, 2005a, Fibrotic lung fibroblasts show blunted inhibition by cAMP due to deficient cAMP response element-binding protein phosphorylation: Journal of Pharmacology and Experimental Therapeutics, v. 315, p. 678-687.

Liu, X. Q., S. Q. Sun, and R. S. Ostrom, 2005b, Fibrotic pulmonary fibroblasts show blunted inhibition by cAMP-elevating agents due to decreased CREB phosphorylation: Faseb Journal, v. 19, p. A1296-A1296.

Liu, X. Q., S. Q. Sun, and R. S. Ostrom, 2006b, TGF beta signaling via ERK1/2 and smad is inhibited by cAMP-elevating agents in rat cardiac fibroblasts: Faseb Journal, v. 20, p. A1465-A1465.

Lorena, D., K. Uchio, A. M. A. Costa, and A. Desmouliere, 2002, Normal scarring: importance of myofibroblasts: Wound Repair and Regeneration, v. 10, p. 86-92.

Lu, Y., N. Azad, L. Wang, A. K. V. Iyer, V. Castranova, B.-H. Jiang, and Y. Rojanasakul, 2010, Phosphatidylinositol-3-Kinase/Akt Regulates Bleomycin-Induced Fibroblast Proliferation and Collagen Production: American Journal of Respiratory Cell and Molecular Biology, v. 42, p. 432-441.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond, 1997, Crystal structure of the nucleosome core particle at 2.8 angstrom resolution: Nature, v. 389, p. 251-260.

Lugnier, C., 2006, Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents: Pharmacology & Therapeutics, v. 109, p. 366-398.

Lynch, J. P., and W. J. McCune, 1997, Immunosuppressive and cytotoxic pharmacotherapy for pulmonary disorders: American Journal of Respiratory and Critical Care Medicine, v. 155, p. 395-420.

Lynch, K. R., G. P. O'Neill, Q. Y. Liu, D. S. Im, N. Sawyer, K. M. Metters, N. Coulombe, M. Abramovitz, D. J. Figueroa, Z. Z. Zeng, B. M. Connolly, C. Bai, C. P. Austin, A. Chateauneuf, R. Stocco, G. M. Greig, S. Kargman, S. B. Hooks, E. Hosfield, D. L. Williams, A. W. Ford-Hutchinson, C. T. Caskey, and J. F. Evans, 1999, Characterization of the human cysteinyl leukotriene CysLT(1) receptor: Nature, v. 399, p. 789-793.

Maeda, A., K. Hiyama, H. Yamakido, S. Ishioka, and M. Yamakido, 1996, Increased expression of platelet-derived growth factor A and insulin-like growth factor-I in BAL cells during the development of bleomycin-induced pulmonary fibrosis in mice: Chest, v. 109, p. 780-6.

Maher, T. M., I. C. Evans, S. E. Bottoms, P. F. Mercer, A. J. Thorley, A. G. Nicholson, G. J. Laurent, T. D. Tetley, R. C. Chambers, and R. J. McAnulty, 2010, Diminished Prostaglandin E-2 Contributes to the Apoptosis Paradox in Idiopathic Pulmonary Fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 182.

Malumbres, M., and M. Barbacid, 2003, Timeline - RAS oncogenes: the first 30 years: Nature Reviews Cancer, v. 3, p. 459-465.

Mann, J., F. Oakley, F. Akiboye, A. Elsharkawy, A. W. Thorne, and D. A. Mann, 2007, Regulation of myofibroblast transdifferentiation by DNA methylation and MeCP2: implications for wound healing and fibrogenesis: Cell Death and Differentiation, v. 14, p. 275-285.

Marais, R., Y. Light, H. F. Paterson, and C. J. Marshall, 1995, RAS RECRUITS RAF-1 TO THE PLASMA-MEMBRANE FOR ACTIVATION BY TYROSINE PHOSPHORYLATION: Embo Journal, v. 14, p. 3136-3145.

Margueron, R., P. Trojer, and D. Reinberg, 2005, The key to development: interpreting the histone code?: Current Opinion in Genetics & Development, v. 15, p. 163-176.

Marom, M., R. Haklai, G. Benbaruch, D. Marciano, Y. Egozi, and Y. Kloog, 1995, SELECTIVE-INHIBITION OF RAS-DEPENDENT CELL-GROWTH BY FARNESYLTHIOSALICYLIC ACID: Journal of Biological Chemistry, v. 270, p. 22263-22270.

Marshall, C. J., 1995, SPECIFICITY OF RECEPTOR TYROSINE KINASE SIGNALING - TRANSIENT VERSUS SUSTAINED EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION: Cell, v. 80, p. 179-185.

Martin, M. M., J. A. Buckenberger, J. Jiang, G. E. Malana, D. L. Knoell, D. S. Feldman, and T. S. Elton, 2007, TGF-beta 1 stimulates human AT(1) receptor expression in lung

fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 293, p. L790-L799.

Mayr, B., and M. Montminy, 2001, Transcriptional regulation by the phosphorylation-dependent factor CREB: Nature Reviews Molecular Cell Biology, v. 2, p. 599-609.

Mehrad, B., M. D. Burdick, D. A. Zisman, M. P. Keane, J. A. Belperio, and R. M. Strieter, 2007, Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease: Biochemical and Biophysical Research Communications, v. 353, p. 104-108.

Mei, F. C., J. B. Qiao, O. M. Tsygankova, J. L. Meinkoth, L. A. Quilliam, and X. D. Cheng, 2002, Differential signaling of cyclic AMP - Opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation: Journal of Biological Chemistry, v. 277, p. 11497-11504.

Meneghin, A., and C. M. Hogaboam, 2007, Infectious disease, the innate immune response, and fibrosis: Journal of Clinical Investigation, v. 117, p. 530-538.

Mercer, K. E., and C. A. Pritchard, 2003, Raf proteins and cancer: B-Raf is identified as a mutational target: Biochimica Et Biophysica Acta-Reviews on Cancer, v. 1653, p. 25-40.

Mia, M. M., M. Boersema, and R. A. Bank, 2014, Interleukin-1β attenuates myofibroblast formation and extracellular matrix production in dermal and lung fibroblasts exposed to transforming growth factor-β1: PLoS One, v. 9, p. e91559.

Mischak, H., T. Seitz, P. Janosch, M. Eulitz, H. Steen, M. Schellerer, A. Philipp, and W. Kolch, 1996, Negative regulation of Raf-1 by phosphorylation of serine 621: Molecular and Cellular Biology, v. 16, p. 5409-5418.

Miyazono, K., 2009, Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer: Proceedings of the Japan Academy Series a-Mathematical Sciences, v. 85, p. 314-323.

Moeller, A., S. E. Gilpin, K. Ask, G. Cox, D. Cook, J. Gauldie, P. J. Margetts, L. Farkas, J. Dobranowski, C. Boylan, P. M. O'Byrne, R. M. Strieter, and M. Kolb, 2009, Circulating Fibrocytes Are an Indicator of Poor Prognosis in Idiopathic Pulmonary Fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 179, p. 588-594.

Montminy, M., 1997, Transcriptional regulation by cyclic AMP: Annual Review of Biochemistry, v. 66, p. 807-822.

Moodley, Y. P., P. Caterina, A. K. Scaffidi, N. L. Misso, J. M. Papadimitriou, R. J. McAnulty, G. J. Laurent, P. J. Thompson, and D. A. Knight, 2004, Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis: J Pathol, v. 202, p. 486-95.

Moore, B. B., M. N. Ballinger, E. S. White, M. E. Green, A. B. Herrygers, C. A. Wilke, G. B. Toews, and M. Peters-Golden, 2005, Bleomycin-induced E prostanoid receptor changes alter fibroblast responses to prostaglandin E-2: Journal of Immunology, v. 174, p. 5644-5649.

Moore, B. B., M. Peters-Golden, P. J. Christensen, V. Lama, W. A. Kuziel, R. Paine, and G. B. Toews, 2003, Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE2: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 284, p. L342-L349.

Moustakas, A., and C. H. Heldin, 2005, Non-Smad TGF-beta signals: Journal of Cell Science, v. 118, p. 3573-3584.

Mu, Y., S. K. Gudey, and M. Landstrom, 2012, Non-Smad signaling pathways: Cell and Tissue Research, v. 347, p. 11-20.

Mulder, K. M., 2000, Role of Ras and Mapks in TGF beta signaling: Cytokine & Growth Factor Reviews, v. 11, p. 23-35.

Mulder, K. M., and S. L. Morris, 1992, ACTIVATION OF P21RAS BY TRANSFORMING GROWTH-FACTOR-BETA IN EPITHELIAL-CELLS: Journal of Biological Chemistry, v. 267, p. 5029-5031.

Munger, J. S., X. Z. Huang, H. Kawakatsu, M. J. D. Griffiths, S. L. Dalton, J. F. Wu, J. F. Pittet, N. Kaminski, C. Garat, M. A. Matthay, D. B. Rifkin, and D. Sheppard, 1999, The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis: Cell, v. 96, p. 319-328.

Murakami, S., N. Nagaya, T. Itoh, M. Kataoka, T. Iwase, T. Horio, Y. Miyahara, Y. Sakai, K. Kangawa, and H. Kimura, 2006, Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 290, p. L59-L65.

Nakao, A., M. Afrakhte, A. Moren, T. Nakayama, J. L. Christian, R. Heuchel, S. Itoh, N. Kawabata, N. E. Heldin, C. H. Heldin, and P. tenDijke, 1997, Identification of Smad7, a TGF beta-inducible antagonist of TGF-beta signalling: Nature, v. 389, p. 631-635.

Narumiya, S., Y. Sugimoto, and F. Ushikubi, 1999, Prostanoid receptors: Structures, properties, and functions: Physiological Reviews, v. 79, p. 1193-1226.

Nevo, Y., S. Aga-Mizrachi, E. Elmakayes, N. Yanay, K. Ettinger, M. Elbaz, Z. Brunschwig, O. Dadush, G. Elad-Sfadia, R. Haklai, Y. Kloog, J. Chapman, and S. Reif, 2011, The Ras Antagonist, Farnesylthiosalicylic Acid (FTS), Decreases Fibrosis and Improves Muscle Strength in dy(2J)/dy(2J) Mouse Model of Muscular Dystrophy: Plos One, v. 6.

Nie, M., A. J. Knox, and L. H. Pang, 2005, B-2-Adrenoceptor agonists, like glucocorticoids, repress eotaxin gene transcription by selective inhibition of histone H4 acetylation: Journal of Immunology, v. 175, p. 478-486.

Nishigaki, N., M. Negishi, and A. Ichikawa, 1996, Two G(S)-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist: Molecular Pharmacology, v. 50, p. 1031-1037.

Noda, M., M. Ko, A. Ogura, D. G. Liu, T. Amano, T. Takano, and Y. Ikawa, 1985, SARCOMA-VIRUSES CARRYING RAS ONCOGENES INDUCE DIFFERENTIATION-ASSOCIATED PROPERTIES IN A NEURONAL CELL-LINE: Nature, v. 318, p. 73-75.

Nonomura, A., G. Ohta, M. Hayashi, R. Izumi, K. Watanabe, N. Takayanagi, Y. Mizukami, and F. Matsubara, 1987, IMMUNOHISTOCHEMICAL DETECTION OF RAS ONCOGENE-

P21 PRODUCT IN LIVER-CIRRHOSIS AND HEPATOCELLULAR-CARCINOMA: American Journal of Gastroenterology, v. 82, p. 512-518.

Obayashi, Y., I. Yamadori, J. Fujita, T. Yoshinouchi, N. Ueda, and J. Takahara, 1997, The role of neutrophils in the pathogenesis of idiopathic pulmonary fibrosis: Chest, v. 112, p. 1338-1343.

Oft, M., J. Peli, C. Rudaz, H. Schwarz, H. Beug, and E. Reichmann, 1996, TGF-beta 1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells: Genes & Development, v. 10, p. 2462-2477.

Okano, M., D. W. Bell, D. A. Haber, and E. Li, 1999, DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development: Cell, v. 99, p. 247-257.

Olman, M. A., 2003, Epithelial cell modulation of airway fibrosis in asthma: American Journal of Respiratory Cell and Molecular Biology, v. 28, p. 125-128.

Olson, M. F., and R. Marais, 2000, Ras protein signalling: Seminars in Immunology, v. 12, p. 63-73.

Omerovic, J., A. J. Laude, and I. A. Prior, 2007, Ras proteins: paradigms for compartmentalised and isoform-specific signalling: Cellular and Molecular Life Sciences, v. 64, p. 2575-2589.

Otte, J. M., I. M. Rosenberg, and D. K. Podolsky, 2003, Intestinal myofibroblasts in innate immune responses of the intestine: Gastroenterology, v. 124, p. 1866-1878.

Pan, L. H., H. Ohtani, K. Yamauchi, and H. Nagura, 1996, Co-expression of TNF alpha and IL-1 beta in human acute pulmonary fibrotic diseases: an immunohistochemical analysis: Pathol Int, v. 46, p. 91-9.

Pang, L. H., E. Holland, and A. J. Knox, 1998a, Impaired cAMP production in human airway smooth muscle cells by bradykinin: role of cyclooxygenase products: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 275, p. L322-L329.

Pang, L. H., E. Holland, and A. J. Knox, 1998b, Role of cyclo-oxygenase-2 induction in interleukin-1 beta induced attenuation of cultured human airway smooth muscle cell cyclic AMP generation in response to isoprenaline: British Journal of Pharmacology, v. 125, p. 1320-1328.

Pantelidis, P., G. C. Fanning, A. U. Wells, K. I. Welsh, and R. M. Du Bois, 2001, Analysis of tumor necrosis factor-alpha, lymphotoxin-alpha, tumor necrosis factor receptor II, and interleukin-6 polymorphisms in patients with idiopathic pulmonary fibrosis: Am J Respir Crit Care Med, v. 163, p. 1432-6.

Pardo, A., and M. Selman, 2002, Idiopathic pulmonary fibrosis: new insights in its pathogenesis: International Journal of Biochemistry & Cell Biology, v. 34, p. 1534-1538.

Peters-Golden, M., M. Bailie, T. Marshall, C. Wilke, S. H. Phan, G. B. Toews, and B. B. Moore, 2002, Protection from pulmonary fibrosis in leukotriene-deficient mice: American Journal of Respiratory and Critical Care Medicine, v. 165, p. 229-235.

Peterson, C. L., and M. A. Laniel, 2004, Histones and histone modifications: Current Biology, v. 14, p. R546-R551.

Petkova, D. K., C. A. Clelland, J. E. Ronan, S. Lewis, and A. J. Knox, 2003, Reduced expression of cyclooxygenase (COX) in idiopathic pulmonary fibrosis and sarcoidosis: Histopathology, v. 43, p. 381-386.

Phan, S. H., 2002a, The myofibroblast in pulmonary fibrosis: Chest, v. 122, p. 286S-289S.

Phan, S. H., 2002b, The myofibroblast in pulmonary fibrosis.: Chest, v. 122, p. 286S-289S.

Phan, S. H., and S. L. Kunkel, 1992, Lung cytokine production in bleomycin-induced pulmonary fibrosis: Exp Lung Res, v. 18, p. 29-43.

Phillips, P. G., L. Long, M. R. Wilkins, and N. W. Morrell, 2005, cAMP phosphodiesterase inhibitors potentiate effects of prostacyclin analogs in hypoxic pulmonary vascular remodeling: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 288, p. L103-L115.

Phillips, R. J., M. D. Burdick, K. Hong, M. A. Lutz, L. A. Murray, Y. Y. Xue, J. A. Belperio, M. P. Keane, and R. M. Strieter, 2004, Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis: Journal of Clinical Investigation, v. 114, p. 438-446.

Phillips, R. J., M. D. Burdick, M. Lutz, J. A. Belperio, M. P. Keane, and R. M. Strieter, 2003, The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases: American Journal of Respiratory and Critical Care Medicine, v. 167, p. 1676-1686.

Piguet, P. F., C. Vesin, G. E. Grau, and R. C. Thompson, 1993, Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica: Cytokine, v. 5, p. 57-61.

Pilewski, J. M., L. X. Liu, A. C. Henry, A. V. Knauer, and C. A. Feghali-Bostwick, 2005, Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition: American Journal of Pathology, v. 166, p. 399-407.

Polgar, P., and L. Taylor, 1980, Alterations in prostaglandin synthesis during senescence of human lung fibroblasts: Mech Ageing Dev, v. 12, p. 305-10.

Ponticos, M., A. M. Holmes, S.-w. Xu, P. Leoni, K. Khan, V. S. Rajkumar, R. K. Hoyles, G. Bou-Gharios, C. M. Black, C. P. Denton, D. J. Abraham, A. Leask, and G. E. Lindahl, 2009, Pivotal Role of Connective Tissue Growth Factor in Lung Fibrosis MAPK-Dependent Transcriptional Activation of Type I Collagen: Arthritis and Rheumatism, v. 60, p. 2142-2155.

Pozharskaya, V., E. Torres-Gonzalez, M. Rojas, A. Gal, M. Amin, S. Dollard, J. Roman, A. A. Stecenko, and A. L. Mora, 2009, Twist: A Regulator of Epithelial-Mesenchymal Transition in Lung Fibrosis: Plos One, v. 4.

Prokhortchouk, E., and P. A. Defossez, 2008, The cell biology of DNA methylation in mammals: Biochimica Et Biophysica Acta-Molecular Cell Research, v. 1783, p. 2167-2173.

Pruitt, K., and C. J. Der, 2001, Ras and Rho regulation of the cell cycle and oncogenesis: Cancer Letters, v. 171, p. 1-10.

Puthawala, K., N. Hadjiangelis, S. C. Jacoby, E. Bayongan, Z. Zhao, Z. Yang, M. L. Devitt, G. S. Horan, P. H. Weinreb, M. E. Lukashev, S. M. Violette, K. S. Grant, C. Colarossi, S. C. Formenti, and J. S. Munger, 2008, Inhibition of integrin alpha(v)beta6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis: Am J Respir Crit Care Med, v. 177, p. 82-90.

Rabe, K. F., 2011, Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease: British Journal of Pharmacology, v. 163, p. 53-67.

Rabinovich, E. I., M. G. Kapetanaki, I. Steinfeld, K. F. Gibson, K. V. Pandit, G. Yu, Z. Yakhini, and N. Kaminski, 2012, Global Methylation Patterns in Idiopathic Pulmonary Fibrosis: Plos One, v. 7.

Racke, K., S. Haag, K. U. Juergens, and M. Warnken, 2008, Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts: Naunyn-Schmiedebergs Archives of Pharmacology, v. 377, p. 38.

Raghu, G., K. J. Anstrom, T. E. King, J. A. Lasky, F. J. Martinez, and I. P. F. C. R. Network, 2012, Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis: N Engl J Med, v. 366, p. 1968-77.

Raghu, G., H. R. Collard, J. J. Egan, F. J. Martinez, J. Behr, K. K. Brown, T. V. Colby, J. F. Cordier, K. R. Flaherty, J. A. Lasky, D. A. Lynch, J. H. Ryu, J. J. Swigris, A. U. Wells, J. Ancochea, D. Bouros, C. Carvalho, U. Costabel, M. Ebina, D. M. Hansell, T. Johkoh, D. S. Kim, T. E. King, Y. Kondoh, J. Myers, N. L. Muller, A. G. Nicholson, L. Richeldi, M. Selman, R. F. Dudden, B. S. Griss, S. L. Protzko, H. J. Schunemann, and A. E. J. A. Comm, 2011, An Official ATS/ERSARS/ALAT Statement: Idiopathic Pulmonary Fibrosis: Evidence-based Guidelines for Diagnosis and Management: American Journal of Respiratory and Critical Care Medicine, v. 183, p. 788-824.

Raghu, G., T. D. Freudenberger, S. Yang, J. R. Curtis, C. Spada, J. Hayes, J. K. Sillery, C. E. Pope, and C. A. Pellegrini, 2006, High prevalence of abnormal acid gastro-oesophageal reflux in idiopathic pulmonary fibrosis: European Respiratory Journal, v. 27, p. 136-142.

Raines, E. W., S. K. Dower, and R. Ross, 1989, Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA: Science, v. 243, p. 393-6.

Ramos, C., M. Montano, J. Garcia-Alvarez, V. Ruiz, B. D. Uhal, M. Selman, and A. Pardo, 2001, Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression: American Journal of Respiratory Cell and Molecular Biology, v. 24, p. 591-598.

Razak, A. R. A., S. J. Hotte, L. L. Siu, E. X. Chen, H. W. Hirte, J. Powers, W. Walsh, L. A. Stayner, A. Laughlin, V. Novotny-Diermayr, J. Zhu, and E. A. Eisenhauer, 2011, Phase I clinical, pharmacokinetic and pharmacodynamic study of SB939, an oral histone deacetylase (HDAC) inhibitor, in patients with advanced solid tumours: British Journal of Cancer, v. 104, p. 756-762.

Regan, J. W., 2003, EP2 and EP4 prostanoid receptor signaling: Life Sciences, v. 74, p. 143-153.

Reif, S., H. Aeed, Y. Shilo, R. Reich, Y. Kloog, Y. O. Kweon, and R. Bruck, 2004, Treatment of thioacetamide-induced liver cirrhosis by the Ras antagonist, farnesylthiosalicylic acid: Journal of Hepatology, v. 41, p. 235-241.

Richeldi, L., R. M. du Bois, G. Raghu, A. Azuma, K. K. Brown, U. Costabel, V. Cottin, K. R. Flaherty, D. M. Hansell, Y. Inoue, D. S. Kim, M. Kolb, A. G. Nicholson, P. W. Noble, M. Selman, H. Taniguchi, M. Brun, F. Le Maulf, M. Girard, S. Stowasser, R. Schlenker-Herceg, B. Disse, H. R. Collard, and I. T. Investigators, 2014, Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis: N Engl J Med, v. 370, p. 2071-82.

Roberts, A. B., 1999, TGF-beta signaling from receptors to the nucleus: Microbes and Infection, v. 1, p. 1265-1273.

Robertson, K. D., and A. P. Wolffe, 2000, DNA methylation in health and disease: Nature Reviews Genetics, v. 1, p. 11-19.

Rodriguezviciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward, 1994, PHOSPHATIDYLINOSITOL-3-OH KINASE AS A DIRECT TARGET OF RAS: Nature, v. 370, p. 527-532.

Rombouts, K., T. Niki, P. Greenwel, A. Vandermonde, A. Wielant, K. Hellemans, P. De Bleser, M. Yoshida, D. Schuppan, M. Rojkind, and A. Geerts, 2002, Trichostatin A, a histone deacetylase inhibitor, suppresses collagen synthesis and prevents TGF-beta(1)-induced fibrogenesis in skin fibroblasts: Experimental Cell Research, v. 278, p. 184-197.

Roth, S. Y., J. M. Denu, and C. D. Allis, 2001, Histone acetyltransferases: Annual Review of Biochemistry, v. 70, p. 81-120.

Roy, S. G., Y. Nozaki, and S. H. Phan, 2001, Regulation of alpha-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts: International Journal of Biochemistry & Cell Biology, v. 33, p. 723-734.

Saharinen, J., and J. Keski-Oja, 2000, Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-beta: Molecular Biology of the Cell, v. 11, p. 2691-2704.

Sakai, N., and A. M. Tager, 2013, Fibrosis of two: Epithelial cell-fibroblast interactions in pulmonary fibrosis: Biochim Biophys Acta.

Sam, R., L. Wanna, K. P. Gudehithlu, S. L. Garber, G. Dunea, J. A. L. Arruda, and A. K. Singh, 2006, Glomerular epithelial cells transform to myofibroblasts: early but not late removal of TGF-beta(1) reverses transformation: Translational Research, v. 148, p. 142-148.

Samuel, G. H., A. M. Bujor, S. S. Nakerakanti, F. N. Hant, and M. Trojanowska, 2010, Autocrine transforming growth factor beta signaling regulates extracellular signal-regulated kinase 1/2 phosphorylation via modulation of protein phosphatase 2A expression in scleroderma fibroblasts: Fibrogenesis & tissue repair, v. 3, p. 25-25.

Sanchez-Elsner, T., L. M. Botella, B. Velasco, A. Corbi, L. Attisano, and C. Bernabeu, 2001, Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression: Journal of Biological Chemistry, v. 276, p. 38527-38535.

Sanders, Y. Y., A. Pardo, M. Selman, G. J. Nuovo, T. O. Tollefsbol, G. P. Siegal, and J. S. Hagood, 2008, Thy-1 Promoter Hypermethylation A Novel Epigenetic Pathogenic Mechanism in Pulmonary Fibrosis: American Journal of Respiratory Cell and Molecular Biology, v. 39, p. 610-618.

Sanders, Y. Y., T. O. Tollefsbol, B. M. Varisco, and J. S. Hagood, 2011, Epigenetic Regulation of Thy-1 by Histone Deacetylase Inhibitor in Rat Lung Fibroblasts: American Journal of Respiratory Cell and Molecular Biology, v. 45, p. 16-23.

Sassone-Corsi, P., 2012, The Cyclic AMP Pathway: Cold Spring Harbor Perspectives in Biology, v. 4.

Schmitt, J. M., and P. J. S. Stork, 2000, beta(2)-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein Rap1 and the serine/threonine kinase B-Raf: Journal of Biological Chemistry, v. 275, p. 25342-25350.

Schmitt, J. M., and P. J. S. Stork, 2002, G alpha and G beta gamma require distinct Src-dependent pathways to activate Rap1 and ras: Journal of Biological Chemistry, v. 277, p. 43024-43032.

Schmitz, N., M. Kurrer, M. F. Bachmann, and M. Kopf, 2005, Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection: J Virol, v. 79, p. 6441-8.

Scotton, C. J., and R. C. Chambers, 2007, Molecular targets in pulmonary fibrosis - The myofibroblast in focus: Chest, v. 132, p. 1311-1321.

Scotton, C. J., M. A. Krupiczojc, M. Koenigshoff, P. F. Mercer, Y. C. G. Lee, N. Kaminski, J. Morser, J. M. Post, T. M. Maher, A. G. Nicholson, J. D. Moffatt, G. J. Laurent, C. K. Derian, O. Eickelberg, and R. C. Chambers, 2009, Increased local expression of coagulation factor X contributes to the fibrotic response in human and murine lung injury: Journal of Clinical Investigation, v. 119, p. 2550-2563.

Selige, J., A. Hatzelmann, and T. Dunkern, 2011, The Differential Impact of PDE4 Subtypes in Human Lung Fibroblasts on Cytokine-Induced Proliferation and Myofibroblast Conversion: Journal of Cellular Physiology, v. 226, p. 1970-1980.

Selman, M., G. Carrillo, A. Estrada, M. Mejia, C. Becerril, J. Cisneros, M. Gaxiola, R. Perez-Padilla, C. Navarro, T. Richards, J. Dauber, T. E. King, Jr., A. Pardo, and N. Kaminski, 2007, Accelerated Variant of Idiopathic Pulmonary Fibrosis: Clinical Behavior and Gene Expression Pattern: Plos One, v. 2.

Selman, M., T. E. King, and A. Pardo, 2001, Idiopathic pulmonary fibrosis: Prevailing and evolving hypotheses about its pathogenesis and implications for therapy: Annals of Internal Medicine, v. 134, p. 136-151.

Selman, M., and A. Pardo, 2006, Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers: Proc Am Thorac Soc, v. 3, p. 364-72.

Selman, M., A. Pardo, L. Richeldi, and S. Cerri, 2011, Emerging drugs for idiopathic pulmonary fibrosis: Expert Opinion on Emerging Drugs, v. 16, p. 341-362.

Selman, M. S., V. J. Thannickal, A. Pardo, D. A. Zisman, F. J. Martinez, and J. P. Lynch, 2004, Idiopathic pulmonary fibrosis - Pathogenesis and therapeutic approaches: Drugs, v. 64, p. 405-430.

Shahbazian, M. D., and M. Grunstein, 2007, Functions of site-specific histone acetylation and deacetylation.: Annu Rev Biochem, v. 76, p. 75-100.

Shen, X., P. P. Hu, N. T. Liberati, M. B. Datto, J. P. Frederick, and X. F. Wang, 1998, TGF-beta-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein: Molecular Biology of the Cell, v. 9, p. 3309-3319.

Shih, C.-H., M.-Y. Bien, L.-L. Chiang, C.-L. Su, C.-H. Lin, and B.-C. Chen, 2009, Thrombin induces cyclooxygenase-2 expression via the ERK and NF-kappa B pathways in human lung fibroblasts: European Journal of Pharmacology, v. 618, p. 70-75.

Shin, S.-Y., O. Rath, S.-M. Choo, F. Fee, B. McFerran, W. Kolch, and K.-H. Cho, 2009, Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway: Journal of Cell Science, v. 122, p. 425-435.

Sime, P. J., Z. Xing, F. L. Graham, K. G. Csaky, and J. Gauldie, 1997, Adenovector-mediated gene transfer of active transforming growth factor-beta 1 induces prolonged severe fibrosis in rat lung: Journal of Clinical Investigation, v. 100, p. 768-776.

Skinner, J., A. Bounacer, J. A. Bond, M. F. Haughton, C. DeMicco, and D. Wynford-Thomas, 2004, Opposing effects of mutant ras oncoprotein on human fibroblast and epithelial cell proliferation: implications for models of human tumorigenesis: Oncogene, v. 23, p. 5994-5999.

Smaldone, S., J. Olivieri, G. L. Gusella, G. Moroncini, A. Gabrielli, and F. Ramirez, 2011, Ha-Ras stabilization mediates pro-fibrotic signals in dermal fibroblasts: Fibrogenesis & tissue repair, v. 4, p. 8-8.

Sorrentino, A., N. Thakur, S. Grimsby, A. Marcusson, V. von Bulow, N. Schuster, S. Zhang, C.-H. Heldin, and M. Landstroem, 2008, The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner: Nature Cell Biology, v. 10, p. 1199-1207.

Sterner, D. E., and S. L. Berger, 2000, Acetylation of histones and transcription-related factors: Microbiology and Molecular Biology Reviews, v. 64, p. 435-+.

Stewart, J. P., J. J. Egan, A. J. Ross, B. G. Kelly, S. S. Lok, P. S. Hasleton, and A. A. Woodcock, 1999, The detection of Epstein-Barr virus DNA in lung tissue from patients with idiopathic pulmonary fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 159, p. 1336-1341.

Stork, P. J. S., 2003, Does Rap1 deserve a bad Rap?: Trends in Biochemical Sciences, v. 28, p. 267-275.

Stork, P. J. S., and J. M. Schmitt, 2002, Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation: Trends in Cell Biology, v. 12, p. 258-266.

Stratton, R., V. Rajkumar, M. Ponticos, B. Nichols, S. W. Xu, C. M. Black, D. J. Abraham, and A. Leask, 2002, Prostacyclin derivatives prevent the fibrotic response to TGF beta 2 by inhibiting the Ras/MEK/ERK pathway: Faseb Journal, v. 16, p. 1949-+.

Stratton, R., S. W. Xu, G. Martini, A. Holmes, A. Leask, T. Haberberger, G. R. Martin, C. M. Black, and D. Abraham, 2001, Iloprost suppresses connective tissue growth factor

production in fibroblasts and in the skin of scleroderma patients: Journal of Clinical Investigation, v. 108, p. 241-250.

Strieter, R. M., and B. Mehrad, 2009, New Mechanisms of Pulmonary Fibrosis: Chest, v. 136, p. 1364-1370.

Sullivan, D. E., M. B. Ferris, D. Pociask, and A. R. Brody, 2005, Tumor necrosis factor-alpha induces transforming growth factor-alpha expression in lung fibroblasts through the extracellular signal-regulated kinase pathway: American Journal of Respiratory Cell and Molecular Biology, v. 32, p. 342-349.

Suzawa, T., C. Miyaura, M. Inada, T. Maruyama, Y. Sugimoto, F. Ushikubi, A. Ichikawa, S. Narumiya, and T. Suda, 2000, The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: An analysis using specific agonists for the respective EPs: Endocrinology, v. 141, p. 1554-1559.

Swigris, J. J., and K. K. Brown, 2010, The Role of Endothelin-1 in the Pathogenesis of Idiopathic Pulmonary Fibrosis: Biodrugs, v. 24, p. 49-54.

Taipale, J., K. Koli, and J. Keskioja, 1992, RELEASE OF TRANSFORMING GROWTH FACTOR-BETA-1 FROM THE PERICELLULAR MATRIX OF CULTURED FIBROBLASTS AND FIBROSARCOMA CELLS BY PLASMIN AND THROMBIN: Journal of Biological Chemistry, v. 267, p. 25378-25384.

Takai, Y., T. Sasaki, and T. Matozaki, 2001, Small GTP-binding proteins: Physiological Reviews, v. 81, p. 153-208.

Tang, Y. W., J. E. Johnson, P. J. Browning, R. A. Cruz-Gervis, A. Davis, B. S. Graham, K. L. Brigham, J. A. Oates, J. E. Loyd, and A. A. Stecenko, 2003, Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis: Journal of Clinical Microbiology, v. 41, p. 2633-2640.

Tanjore, H., X. C. C. Xu, V. V. Polosukhin, A. L. Degryse, B. Li, W. Han, T. P. Sherrill, D. Plieth, E. G. Neilson, T. S. Blackwell, and W. E. Lawson, 2009, Contribution of Epithelial-derived Fibroblasts to Bleomycin-induced Lung Fibrosis: American Journal of Respiratory and Critical Care Medicine, v. 180, p. 657-665.

Tannheimer, S. L., C. D. Wright, and M. Salmon, 2012, Combination of roflumilast with a beta-2 adrenergic receptor agonist inhibits proinflammatory and profibrotic mediator release from human lung fibroblasts: Respiratory Research, v. 13.

Taskar, V. S., and D. B. Coultas, 2006, Is idiopathic pulmonary fibrosis an environmental disease?: Proc Am Thorac Soc, v. 3, p. 293-8.

Tasken, K., and E. M. Aandahl, 2004, Localized effects of cAMP mediated by distinct routes of protein kinase A: Physiological Reviews, v. 84, p. 137-167.

Tatler, A. L., J. Porte, A. Knox, G. Jenkins, and L. H. Pang, 2008, Tryptase activates TGF beta in human airway smooth muscle cells via direct proteolysis: Biochemical and Biophysical Research Communications, v. 370, p. 239-242.

Taylor, S. S., D. R. Knighton, J. Zheng, L. F. Teneyck, and J. M. Sowadski, 1992, CAMP-DEPENDENT PROTEIN-KINASE AND THE PROTEIN-KINASE FAMILY: Faraday Discussions, v. 93, p. 143-152.

Tessner, T. G., F. Muhale, T. E. Riehl, S. Anant, and W. F. Stenson, 2004, Prostaglandin E-2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation: Journal of Clinical Investigation, v. 114, p. 1676-1685.

Thannickal, V. J., and J. C. Horowitz, 2006, Evolving concepts of apoptosis in idiopathic pulmonary fibrosis: Proc Am Thorac Soc, v. 3, p. 350-6.

Thomas, P. E., M. Peters-Golden, E. S. White, V. J. Thannickal, and B. B. Moore, 2007, PGE(2) inhibition of TGF-beta 1-induced myofibroblast differentiation is Smad-independent but involves cell shape and adhesion-dependent signaling: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 293, p. L417-L428.

Togo, S., X. D. Liu, X. Q. Wang, H. Sugiura, K. Kamio, S. Kawasaki, T. Kobayashi, R. F. Ertl, Y. Ahn, O. Holz, H. Magnussen, K. Fredriksson, C. M. Skold, and S. I. Rennard, 2009, PDE4 inhibitors roflumilast and rolipram augment PGE(2) inhibition of TGF-beta 1-stimulated fibroblasts: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 296, p. L959-L969.

Travis, M. A., B. Reizis, A. C. Melton, E. Masteller, Q. Z. Tang, J. M. Proctor, Y. L. Wang, X. Bernstein, X. Z. Huang, L. F. Reichardt, J. A. Bluestone, and D. Sheppard, 2007, Loss of integrin alpha(v)beta(8) on dendritic cells causes autoimmunity and colitis in mice: Nature, v. 449, p. 361-+.

Tsukamoto, K., H. Hayakawa, A. Sato, K. Chida, H. Nakamura, and K. Miura, 2000, Involvement of Epstein-Barr virus latent membrane protein 1 in disease progression in patients with idiopathic pulmonary fibrosis: Thorax, v. 55, p. 958-961.

Uhal, B. D., I. Joshi, W. F. Hughes, C. Ramos, A. Pardo, and M. Selman, 1998, Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung: American Journal of Physiology-Lung Cellular and Molecular Physiology, v. 275, p. L1192-L1199.

van Meerloo, J., G. J. L. Kaspers, and J. Cloos, 2011, Cell Sensitivity Assays: The MTT Assay: Cancer Cell Culture: Methods and Protocols, Second Edition, v. 731, p. 237-245.

Vancheri, C., C. Mastruzzo, M. A. Sortino, and N. Crimi, 2004, The lung as a privileged site for the beneficial actions of PGE(2): Trends in Immunology, v. 25, p. 40-46.

Vancheri, C., M. A. Sortino, V. Tomaselli, C. Mastruzzo, F. Condorelli, G. Bellistri, M. P. Pistorio, P. L. Canonico, and N. Crimi, 2000, Different expression of TNF-alpha receptors and prostaglandin E-2 production in normal and fibrotic lung fibroblasts - Potential implications for the evolution of the inflammatory process: American Journal of Respiratory Cell and Molecular Biology, v. 22, p. 628-634.

Vassallo, R., E. Matteson, and C. F. Thomas, 2002, Clinical response of rheumatoid arthritis-associated pulmonary fibrosis to tumor necrosis factor-alpha inhibition: Chest, v. 122, p. 1093-6.

Vaughan, M. B., E. W. Howard, and J. J. Tomasek, 2000, Transforming growth factor-beta 1 promotes the morphological and functional differentiation of the myofibroblast: Experimental Cell Research, v. 257, p. 180-189.

Verrecchia, F., and A. Mauviel, 2002, Transforming growth factor-beta signaling through the Smad pathway: Role in extracellular matrix gene expression and regulation: Journal of Investigative Dermatology, v. 118, p. 211-215.

Vignaud, J. M., M. Allam, N. Martinet, M. Pech, F. Plenat, and Y. Martinet, 1991, Presence of platelet-derived growth factor in normal and fibrotic lung is specifically associated with interstitial macrophages, while both interstitial macrophages and alveolar epithelial cells express the c-sis proto-oncogene: Am J Respir Cell Mol Biol, v. 5, p. 531-8.

Vinals, F., and J. Pouyssegur, 2001, Transforming growth factor beta 1 (TGF-PI) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling: Molecular and Cellular Biology, v. 21, p. 7218-7230.

Wallace, W. A. H., and S. E. M. Howie, 1999, Immunoreactive interleukin 4 and interferon-gamma expression by type II alveolar epithelial cells in interstitial lung disease: Journal of Pathology, v. 187, p. 475-480.

Wang, Q. J., Y. J. Wang, D. M. Hyde, P. J. Gotwals, V. E. Koteliansky, S. T. Ryan, and S. N. Giri, 1999, Reduction of bleomycin induced lung fibrosis by transforming growth factor beta soluble receptor in hamsters: Thorax, v. 54, p. 805-812.

Wang, Z. L., 2009, Advances in understanding of idiopathic pulmonary fibrosis: Chinese Medical Journal, v. 122, p. 844-857.

Wang, Z. P., T. J. Dillon, V. Pokala, S. Mishra, K. Labudda, B. Hunter, and P. J. S. Stork, 2006, Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation: Molecular and Cellular Biology, v. 26, p. 2130-2145.

Watanabe, H., M. P. de Caestecker, and Y. Yamada, 2001, Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecangene expression in chondrogenic ATDC5 cells: Journal of Biological Chemistry, v. 276, p. 14466-14473.

Wells, A. U., N. Hirani, I. British Thoracic Soc, S. British Thoracic Soc, Z. Thoracic Soc Australia New, and S. Irish Thoracic, 2008, Interstitial lung disease guideline: the British Thoracic Society in collaboration with the Thoracic Society of Australia and New Zealand and the Irish thoracic society: Thorax, v. 63.

White, E. S., 2008, Prostaglandin E-2 inhibits fibroblast migration by E-prostanoid 2 receptor-mediated increase in PTEN activity (vol 32, pg 135, 2005): American Journal of Respiratory Cell and Molecular Biology, v. 39, p. 252-252.

Whitman, M., and D. A. Melton, 1992, INVOLVEMENT OF P21RAS IN XENOPUS MESODERM INDUCTION: Nature, v. 357, p. 252-254.

Whyte, M., R. Hubbard, R. Meliconi, M. Whidborne, V. Eaton, C. Bingle, J. Timms, G. Duff, A. Facchini, A. Pacilli, M. Fabbri, I. Hall, J. Britton, I. Johnston, and F. Di Giovine, 2000, Increased risk of fibrosing alveolitis associated with interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphisms: American Journal of Respiratory and Critical Care Medicine, v. 162, p. 755-758.

Wilborn, J., L. J. Crofford, M. D. Burdick, S. L. Kunkel, R. M. Strieter, and M. Petersgolden, 1995, CULTURED LUNG FIBROBLASTS ISOLATED FROM PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS HAVE A DIMINISHED CAPACITY TO SYNTHESIZE PROSTAGLANDIN E(2), AND TO EXPRESS CYCLOOXYGENASE-2: Journal of Clinical Investigation, v. 95, p. 1861-1868.

Willis, B. C., J. M. Liebler, K. Luby-Phelps, A. G. Nicholson, E. D. Crandall, R. M. du Bois, and Z. Borok, 2005, Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-ss 1 - Potential role in idiopathic pulmonary fibrosis: American Journal of Pathology, v. 166, p. 1321-1332.

Wipff, P.-J., and B. Hinz, 2008, Integrins and the activation of latent transforming growth factor beta 1 - An intimate relationship: European Journal of Cell Biology, v. 87, p. 601-615.

Wojnowski, L., A. M. Zimmer, T. W. Beck, H. Hahn, R. Bernal, U. R. Rapp, and A. Zimmer, 1997, Endothelial apoptosis in Braf-deficient mice: Nature Genetics, v. 16, p. 293-297.

Wu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill, 1993, INHIBITION OF THE EGF-ACTIVATED MAP KINASE SIGNALING PATHWAY BY ADENOSINE-3',5'-MONOPHOSPHATE: Science, v. 262, p. 1065-1069.

Wynn, T. A., 2008, Cellular and molecular mechanisms of fibrosis: Journal of Pathology, v. 214, p. 199-210.

Xia, H., W. Khalil, J. Kahm, J. Jessurun, J. Kleidon, and C. A. Henke, 2010, Pathologic Caveolin-1 Regulation of PTEN in Idiopathic Pulmonary Fibrosis: American Journal of Pathology, v. 176, p. 2626-2637.

Xu, M. Y., J. Porte, A. J. Knox, P. H. Weinreb, T. M. Maher, S. M. Violette, R. J. McAnulty, D. Sheppard, and G. Jenkins, 2009, Lysophosphatidic Acid Induces alpha v beta 6 Integrin-Mediated TGF-beta Activation via the LPA2 Receptor and the Small G Protein G alpha(q): American Journal of Pathology, v. 174, p. 1264-1279.

Yamaguchi, K., S. Nagai, J. Ninomiya-Tsuji, M. Nishita, K. Tamai, K. Irie, N. Ueno, E. Nishida, H. Shibuya, and K. Matsumoto, 1999, XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway: Embo Journal, v. 18, p. 179-187.

Yan, J., S. Roy, A. Apolloni, A. Lane, and J. F. Hancock, 1998, Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase: Journal of Biological Chemistry, v. 273, p. 24052-24056.

Yang, I. V., and D. A. Schwartz, 2011, Epigenetic Control of Gene Expression in the Lung: American Journal of Respiratory and Critical Care Medicine, v. 183, p. 1295-1301.

Yokomizo, T., K. Kato, K. Terawaki, T. Izumi, and T. Shimizu, 2000, A second leukotriene B-4 receptor, BLT2: A new therapeutic target in inflammation and immunological disorders: Journal of Experimental Medicine, v. 192, p. 421-431.

Yokoyama, U., H. H. Patel, N. C. Lai, N. Aroonsakool, D. M. Roth, and P. A. Insel, 2008, The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals: Proceedings of the National Academy of Sciences of the United States of America, v. 105, p. 6386-6391.

Yoshida, K., K. Kuwano, N. Hagimoto, K. Watanabe, T. Matsuba, M. Fujita, I. Inoshima, and N. Hara, 2002, MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis: Journal of Pathology, v. 198, p. 388-396.

Young, S. W., M. Dickens, and J. M. Tavare, 1994, DIFFERENTIATION OF PC12 CELLS IN RESPONSE TO A CAMP ANALOG IS ACCOMPANIED BY SUSTAINED ACTIVATION OF MITOGEN-ACTIVATED PROTEIN-KINASE - COMPARISON WITH

THE EFFECTS OF INSULIN, GROWTH-FACTORS AND PHORBOL ESTERS: Febs Letters, v. 338, p. 212-216.

Yu, H. Y., M. Konigshoff, A. Jayachandran, D. Handley, W. Seeger, N. Kaminski, and O. Eickelberg, 2008, Transgelin is a direct target of TGF-beta/Smad3-dependent epithelial cell migration in lung fibrosis: Faseb Journal, v. 22, p. 1778-1789.

Yu, L., M. C. Hebert, and Y. E. Zhang, 2002, TGF-beta receptor-activated p38 MAP kinase mediates smad-independent TGF-beta responses: Embo Journal, v. 21, p. 3749-3759.

Yue, J. B., and K. M. Mulder, 2000, Activation of the mitogen-activated protein kinase pathway by transforming growth factor-beta: Transforming Growth Factor-Beta Protocols, v. 142, p. 125-131.

Zhang, A. H., M. H. Wang, Z. Dong, and T. X. Yang, 2006, Prostaglandin E-2 is a potent inhibitor of epithelial-to-mesenchymal transition: interaction with hepatocyte growth factor: American Journal of Physiology-Renal Physiology, v. 291, p. F1323-F1331.

Zhang, H. Y., M. GharaeeKermani, K. Zhang, S. Karmiol, and S. H. Phan, 1996, Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis: American Journal of Pathology, v. 148, p. 527-537.

Zhang, K., M. Gharaee-Kermani, B. McGarry, D. Remick, and S. H. Phan, 1997, TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis: J Immunol, v. 158, p. 954-9.

Zhang, K., M. D. Rekhter, D. Gordon, and S. H. Phan, 1994, MYOFIBROBLASTS AND THEIR ROLE IN LUNG COLLAGEN GENE-EXPRESSION DURING PULMONARY FIBROSIS - A COMBINED IMMUNOHISTOCHEMICAL AND IN-SITU HYBRIDIZATION STUDY: American Journal of Pathology, v. 145, p. 114-125.

Zhang, Y., T. C. Lee, B. Guillemin, M. C. Yu, and W. N. Rom, 1993, Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure: J Immunol, v. 150, p. 4188-96.