

Tissue engineering of lung organoids with advanced biomaterials for in-vitro disease modelling

**A thesis submitted for the degree of
Doctor of Philosophy (PhD)**

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Statement of originality

I, Isabel Uwagboe, declare that I wrote this thesis and that all work and results described within, were performed by myself. Any work, data or information from others have been declared and appropriately referenced when required.

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Abstract

The pathophysiological nature of extracellular matrix (ECM) in pulmonary fibrosis is often overlooked. Chronic Obstructive Pulmonary Disease (COPD), hallmarked by loss of alveolar architecture, thickening of the small airways and mucosal hypersecretion is associated with remodelled lung following continuous exposure to noxious particles and chronic inflammation. The degree of matrix stiffness is amplified in Idiopathic Pulmonary Fibrosis (IPF), a disease linked to fibroblast foci in collapsed alveoli. In both, the microenvironment governed by the ECM deviates from the norm, including reduction of elastin (COPD), increased presence of collagen and other ECM proteins (IPF), becoming stiffer. Within this PhD project, a photo-inducible biosynthetic hydrogel (PMET) containing photoinitiator Eosin Y and co-initiator Triethanolamine was generated, recapitulating the range of stiffnesses seen in these diseases with dynamic control following green light exposure. Encapsulated bronchospheres derived from healthy (NHBEs) and COPD (CHBEs) bronchial epithelial cells were used to investigate the influence of fibrotic-like matrix on normal and diseased cellular phenotype and functionality. RT-qPCR analysis showed upregulation of MUC5AC mRNA expression in NHBE bronchospheres as the PMET matrix became stiffer, with greater increase seen with MUC5B. CHBE models also showed enhanced MUC5AC mRNA expression upon stiffening. Increased expression of cell proliferation marker SOX2 was noted in both models. Single cell RT-qPCR analysis, immunocytochemistry and western blotting explored the expression of key mechanosensitive proteins e.g., YAP1 with upregulation rapidly seen following photo-induction. The magnitude and timing of the mechanoresponse was altered in CHBEs with respect to the healthy donors. In this model, I demonstrate markers of respiratory fibrotic disease including goblet cell hyperplasia, mucin overexpression and cell proliferation are stimulated by increased matrix stiffness. These results highlight the important role of Mechanobiology, with further studies needed to examine the role of YAP signalling in this process, elucidating the bidirectional talk between matrix and cell.

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Table of Contents

Chapter 1: Introduction

1.1 The Extracellular Matrix.....	23
1.2 Respiratory diseases	28
1.2.1 Chronic Obstructive Pulmonary Disease	28
1.2.2 Current treatment options for COPD	37
1.2.3 Idiopathic Pulmonary Fibrosis (IPF).....	38
1.2.4 Combined Pulmonary Fibrosis and Emphysema- CPFE.....	42
1.3 Stiffness as a pathological feature?.....	43
1.4 Mechanotransduction pathways.....	49
1.5 Organoids	53
1.6 Overarching Hypothesis	57

Chapter 2: Methods

2.1 Cell culture	60
2.1.1 Bronchial epithelial cell culture	60
2.1.2 Cell Stocks.....	61
2.2 Passaging of cells	63
2.2.1 3T3s.....	63
2.2.2 Bronchial epithelial cells.....	63
2.3 Bronchosphere culture.....	64
2.4 PMET (PEGDA-Matrigel®- Eosin Y and Triethanolamine) gel fabrication and encapsulation of bronchospheres.....	66
2.5 RNA extraction.....	68
2.5.1 Bronchosphere homogenisation	68
2.5.2 Phenol-Chloroform extraction.....	69
2.5.3 cDNA synthesis.....	69
2.5.4 Bench RT-qPCR.....	70
2.6 Single -cell genomics.....	71
2.6.1 Harvesting of bronchospheres from 25% Matrigel® culture/uncured PMET gel	71
2.6.2 Harvesting of bronchospheres from PMET gel photocured for 1 minute and 5 minutes	72
2.6.3 Amplification of Single cell RNA and cDNA synthesis	72

2.6.4	<i>Single cell (sc)RT-qPCR</i>	74
2.7	<i>Bronchosphere cell viability</i>	75
2.7.1	<i>2D cell viability assay</i>	75
2.7.2	<i>3D cell structural integrity assay</i>	76
2.8	<i>Gel characterisation</i>	77
2.8.1	<i>Compression testing for Young's Moduli values</i>	77
2.9	<i>Immunocytochemistry (ICC) and imaging of bronchospheres</i>	80
2.9.1	<i>Whole mount immunofluorescence staining</i>	80
2.9.2	<i>Time-lapsed live imaging of bronchospheres (from 25% Matrigel® culture)</i>	82
2.10	<i>Zymography</i>	83
2.11	<i>Western Blot proteomics</i>	84
2.11.1	<i>Total protein normalisation</i>	84
2.12	<i>Inverted bronchospheres</i>	85

Chapter 3: Development of photocured culture matrix gel as a model for varying matrix stiffness

3.1	<i>Introduction</i>	87
3.2	<i>Hypothesis</i>	89
3.3	<i>Aims</i>	89
3.4	<i>Methods</i>	90
3.4.1	<i>Structural components</i>	90
3.4.2	<i>Photoinitiator selection</i>	91
3.5	<i>Results</i>	94
3.5.1	<i>Photocuring times: Camphorquinone (CQ), Riboflavin (RF) and Fluorescein (FR)</i>	94
3.5.2	<i>Photocuring times: Eosin Y (EY) and Triethanolamine (TEAO)</i>	96
3.5.3	<i>2D cell viability assay</i>	98
3.5.4	<i>3D cell structural integrity assay</i>	117
3.5.5	<i>PMET gel characteristics</i>	120
3.6	<i>Summary</i>	123

Chapter 4: Matrix stiffness impacts epithelial cell differentiation markers in encapsulated bronchospheres

4.1	<i>Introduction</i>	126
4.2	<i>Methods</i>	127
4.3	<i>Results</i>	130
4.3.1	<i>NHBE Mucins</i>	130

4.3.1.1	<i>Immunocytochemistry (ICC) images of NHBE bronchospheres</i>	131
4.3.2	<i>CHBE Mucins</i>	134
4.3.2.1	<i>Immunocytochemistry (ICC) images of CHBE bronchospheres</i>	135
4.3.4	<i>CHBE cell cycle promoters and inhibitors</i>	140
4.3.5	<i>NHBE TP63, FOXJ1 and TGFβ1</i>	142
4.3.6	<i>CHBE TP63, FOXJ1 and TGFβ1</i>	143
4.4	<i>Discussion</i>	144
4.4.1	<i>MUC5AC AND MUC5B</i>	144
4.4.2	<i>KI67, SOX2, CDKN1A AND CDKN2A</i>	147
4.4.3	<i>TP63, FOXJ1 AND TGFβ1</i>	150
4.5	<i>Summary</i>	152

Chapter 5: Mechanisms and impact of Mechanotransduction

5.1	<i>Single cell RT-qPCR</i>	157
5.1.1	<i>Introduction</i>	157
5.1.2	<i>scRT-qPCR methods</i>	158
5.1.3	<i>Results</i>	161
5.1.3.1	<i>scRT-qPCR of NHBE bronchospheres</i>	161
5.1.3.2	<i>Single cell RT-qPCR of CHBE Bronchospheres</i>	166
5.1.4	<i>Discussion</i>	170
5.2	<i>Proteomic analysis</i>	178
5.2.1	<i>Introduction</i>	178
5.2.2	<i>Methods</i>	181
5.2.3	<i>Results</i>	182
5.2.3.1	<i>Total Protein Normalisation- NHBEs</i>	182
5.2.3.2	<i>Total Protein Normalisation- CHBEs</i>	183
5.2.3.3	<i>Chemiblot results- NHBE YAP1</i>	184
5.2.3.4	<i>Chemiblot results- CHBE YAP1</i>	186
5.2.3.5	<i>Chemiblot results- TAZ</i>	188
5.2.3.6	<i>Chemiblot results- NF-κB</i>	190
5.2.3.7	<i>Chemiblot results – ACE2</i>	191
5.2.4	<i>Discussion</i>	192
5.3	<i>YAP1 Immunocytochemistry</i>	197
5.3.1	<i>Introduction</i>	197
5.3.2	<i>Methods</i>	198
5.3.3	<i>Results</i>	199

5.3.3.1 Confocal Imaging – Effect of photocuring on Normal Human Bronchial Epithelial cells	199
5.3.3.2 Confocal Imaging – Effect of photocuring on COPD Human Bronchial Epithelial cells	202
5.3.4 Discussion.....	205
5.4 Zymography.....	208
5.4.1 Introduction.....	208
5.4.2 Methods.....	209
5.4.3 Results.....	210
5.4.3.1 PMET gel (1 minute photocure) results.....	210
5.4.3.2 PMET gel (5 minute photocure) results.....	213
5.4.4 Discussion.....	216
5.5 Bronchospheres and COVID-19.....	218
5.6 Summary.....	223
 Chapter 6: Conclusion	
6.1 Introduction.....	226
6.2 Gel matrix synthesis	226
6.3 Effects of stiffness on epithelial mRNA from healthy and COPD donors.....	229
6.4 Mechanisms of mechanotransduction via single cell (sc) analysis.....	231
6.5 Hypothesis validation and summary	234
6.6 Outlook of future work.....	236
 Chapter 7: References	
7.1 References (list of).....	240
 Chapter 8: Appendix	
8.1 Appendix (licences and granted permissions).....	268

List of Figures

Chapter 1: Introduction

Figure 1.1: Illustration showing the structural changes in the lung, pertaining to the alveolar walls in COPD.....	30
Figure 1.2: Activation of EMT programme within epithelial cells in the lung.....	31
Figure 1.3: Schematic representation of ciliary dysfunction in COPD.....	33
Figure 1.4: Comparison of pathophysiological ECM structural changes in healthy vs COPD lung.....	34
Figure 1.5: Main pathological features of IPF.....	38
Figure 1.6: Comparison of pathophysiological ECM structural changes in healthy vs IPF lung.....	40
Figure 1.7: Mechanical forces on the airway walls in COPD and effects on surrounding lung parenchyma.....	44
Figure 1.8: Difference in stiffness between healthy human lung and the fibrotic lung.....	47
Figure 1.9: Illustration of converging points of TGF β , YAP/TAZ and WNT pathways.....	51

Chapter 2: Methodology

Figure 2.1: Schematic of bronchial epithelial cell culture and bronchosphere organoids....	65
Figure 2.2: Schematic of photocuring mechanism of PEGDA-Matrigel [®] - Eosin Y and Triethanolamine (PMET) gel and transwell culture.....	67
Figure 2.3: Representative images of PEGDA-Matrigel [®] - Eosin Y and Triethanolamine (PMET) (photocured for 1 minute) gel compression testing.....	77
Figure 2.4: Representative images of PEGDA-Matrigel [®] - Eosin Y and Triethanolamine (PMET) (photocured for 5 minutes) gel compression testing.....	78
Figure 2.5: Representative images of 25% Matrigel [®] gel compression testing attempt.....	79
Figure 2.6: Coverwell [™] Induction Chamber Gasket schematic.....	82
Figure 2.7: Schematic of Bronchospheres cultured in 25% Matrigel and with reversed polarity	85

Chapter 3: Development of photocured culture matrix gel as a model for varying matrix stiffness

Figure 3.1: Jablonski diagram.....	88
Figure 3.2: 2D structure of Polyethylene glycol diacrylate	90

Figure 3.3: Schematic of photochemical reaction between PEGDA and photoinitiators for crosslinked hydrogel production.....	93
Figure 3.4: Photocuring assays of Camphorquinone, Riboflavin and Fluorescein.....	95
Figure 3.5: Photocuring assays of Eosin Y and Triethanolamine.....	97
Figure 3.6: 2D cell viability assay of Normal Human Bronchial Epithelial cells (NHBE) (control).....	100
Figure 3.7: 2D cell viability assay of Normal Human Bronchial Epithelial cells (NHBE) from green light exposure	102
Figure 3.8: 2D cell viability assay of Normal Human Bronchial Epithelial cells (NHBE) from green light and photoinitiator Eosin Y and Triethanolamine exposure.....	104
Figure 3.9: 2D cell viability assay of Normal Human Lung Fibroblast cells (NHLF) (control).....	106
Figure 3.10: 2D cell viability assay of Normal Human Lung Fibroblast (NHLF) cells from green light exposure	108
Figure 3.11: 2D cell viability assay of Normal Human Lung Fibroblast (NHLF) cells from green light and photoinitiator Eosin Y and Triethanolamine exposure	110
Figure 3.12: 2D cell viability assay of Normal Human Airway Smooth Muscle (NHASM) cells (control).....	112
Figure 3.13: 2D cell viability assay of Normal Human Airway Smooth Muscle (NHASM) cells from green light exposure	114
Figure 3.14: 2D cell viability assay of Normal Human Airway Smooth Muscle cells (NHASM) from green light and photoinitiator Eosin Y and Triethanolamine exposure	116
Figure 3.15: 3D cell viability assay of bronchospheres in PMET gel (photocured and uncured).....	119
Figure 3.16: Young's Moduli of photocured PMET gel	121
Figure 3.17: PMET gel compression tests graphs	122

Chapter 4: Matrix stiffness impacts epithelial cell differentiation markers in encapsulated bronchospheres

Figure 4.1: RT-qPCR mRNA expression of mucin markers in Normal Human Bronchial Epithelial (NHBE) cells	130
Figure 4.2: Representative immunocytochemistry confocal images of mucin expression in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells- Control culture (25% Matrigel) vs PMET 1 minute photocure encapsulation.....	132

Figure 4.3: Representative immunocytochemistry confocal images of mucin expression in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells- Control culture (25% Matrigel) vs PMET 5 minutes photocure encapsulation.....	133
Figure 4.4: RT-qPCR mRNA expression of mucin markers in COPD derived Human Bronchial Epithelial (CHBE) cells.....	134
Figure 4.5: Representative immunocytochemistry confocal images of mucin expression in bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells- Control culture (25% Matrigel) vs PMET 1 minute photocure encapsulation.....	136
Figure 4.6: Representative immunocytochemistry confocal images of mucin expression in bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells- Control culture (25% Matrigel) vs PMET 5 minutes photocure encapsulation.....	137
Figure 4.7: RT-qPCR mRNA expression of proliferation markers and cell cycle inhibitors in Normal Human Bronchial Epithelial (NHBE) cells.....	139
Figure 4.8: RT-qPCR mRNA expression of proliferation markers and cell cycle inhibitors in COPD derived Human Bronchial Epithelial (CHBE) cells.....	141
Figure 4.9: RT-qPCR mRNA expression of cell types and profibrotic markers in Normal Human Bronchial Epithelial (NHBE) cells.....	142
Figure 4.10: RT-qPCR mRNA expression of cell types and profibrotic markers in COPD derived Human Bronchial Epithelial (CHBE) cells.....	143

Chapter 5: Mechanisms and impact of mechanotransduction

Figure 5.1: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells, photocured for 1 minute and 5 minutes at 0 hours.....	163
Figure 5.2: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells, photocured for 1 minute and 5 minutes at 24 hours.	164
Figure 5.3: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells, photocured for 1 minute and 5 minutes at 48 hours.	165
Figure 5.4: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells, photocured for 1 minute and 5 minutes at 0 hours.....	167
Figure 5.5: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells, photocured for 1 minute and 5 minutes at 24 hours.	168

Figure 5.6: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells, photocured for 1 minute and 5 minutes at 48 hours.	169
Figure 5.7: Schematic of Hippo pathway.....	178
Figure 5.8: Total protein normalisation staining of bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells	182
Figure 5.9: Total protein normalisation staining of bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells.....	183
Figure 5.10: Western blot of YAP 1 in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells.....	185
Figure 5.11: Western blot of YAP 1 in bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells.....	187
Figure 5.12: Western blot of TAZ in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells and COPD derived Human Bronchial Epithelial (CHBE) cells..	189
Figure 5.13: Western blot of NF- κ B in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells and COPD derived Human Bronchial Epithelial (CHBE) cells	190
Figure 5.14: Western blot of ACE2 in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells and COPD derived Human Bronchial Epithelial (CHBE) cells	191
Figure 5.15: Representative immunocytochemistry confocal images of YAP expression in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells- Control culture (25% Matrigel) vs PMET 1 minute photocure encapsulation.....	200
Figure 5.16: Representative immunocytochemistry confocal images of YAP expression in bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells- Control culture (25% Matrigel) vs PMET 5 minutes photocure encapsulation.....	201
Figure 5.17: Representative immunocytochemistry confocal images of YAP expression in bronchospheres derived from COPD-derived Human Bronchial Epithelial (CHBE) cells- Control culture (25% Matrigel) vs PMET 1 minute photocure encapsulation.....	203
Figure 5.18: Representative immunocytochemistry confocal images of YAP expression in bronchospheres derived from COPD-derived Human Bronchial Epithelial (CHBE) cells- Control culture (25% Matrigel) vs PMET 5 minutes photocure encapsulation.....	204
Figure 5.19: Representative zymograms of supernatant samples from bronchospheres derived from Normal Human Bronchial Epithelial (NHBE) cells.....	211
Figure 5.20: Densitometry data from PMET gel 1 minute photocure encapsulation of Normal Human Bronchial Epithelial (NHBE) cells and COPD derived Human Bronchial Epithelial (CHBE) cells bronchospheres.....	212

Figure 5.21: Representative zymograms of supernatant samples from bronchospheres derived from COPD derived Human Bronchial Epithelial (CHBE) cells.....	214
Figure 5.22: Densitometry data from PMET gel 5 minutes photocure encapsulation of Normal Human Bronchial Epithelial (NHBE) cells and COPD derived Human Bronchial Epithelial (CHBE) cells bronchospheres.....	215
Figure 5.23: Representative ICC image of a Normal Human Bronchial Epithelial (NHBE) cells bronchosphere, differentiated from single cell seeding.....	219
Figure 5.24: Representative ICC image of a Normal Human Bronchial Epithelial (NHBE) cells bronchosphere, inverted after initially differentiated from single cells.....	220
Figure 5.25: Representative ICC image of a nasal epithelial organoid, differentiated from single cell donor brushings.....	221
Figure 5.26: Representative ICC image of a nasal epithelial organoid, inverted after differentiated from single cell brushings.....	222

Supplementary files

Please see attached folders:

- **Figure 4.2E:** Volocity 3D rendering of NHBE bronchosphere embedded in PMET gel (1 minute photocure), stained for MUC5AC and MUC5B
- Video [files](#) of NHBE bronchospheres in media- examples of normal polarity and induced inverted polarity

List of Tables

Chapter 1: Introduction

Table 1.1: List of available matrices for cell culture.....	27
Table 1.2: Classification of COPD stage by airflow limitation after spirometry assessment..	29
Table 1.3: Extracellular matrix (ECM) substrates, precursors, inhibitors and cells associated with Matrix Metalloproteases (MMP) 2 and 9.....	36
Table 1.4: Types of cultured organoids in research.....	55
Table 1.5: Types of cultured lung organoids in research.....	56

Chapter 2: Methodology

Table 2.1: List of reagents for Co-culture medium.....	60
Table 2.2: List of donor details and sources of Normal Human Bronchial Epithelial (NHBE) cells, COPD derived Human Bronchial Epithelial (CHBE) cells, Normal Human Airway Smooth Muscle (NHASM) cells and Normal Human Lung Fibroblast (NHLF) cells.....	61
Table 2.3: List of reagents for 3T3 cell culture medium.....	62
Table 2.4: List of reagents for bronchosphere culture medium.....	64
Table 2.5: List of reagents for PEGDA- Matrigel®- Eosin Y and Triethanolamine (PMET) gel suspension.....	66
Table 2.6: List of TaqMan probes and housekeeping genes used in RT-qPCR.....	70
Table 2.7: List of reagents for single cell dissociation protease solution.....	71
Table 2.8: List of reagents for single cell cDNA amplification master mix.....	72
Table 2.9: List of TaqMan primers (Single cell RT-qPCR).....	73
Table 2.10: List of Live/Dead cell viability stains.....	75
Table 2.11: List of immunocytochemistry primary and secondary antibodies.....	81

Chapter 3: Development of photocured culture matrix gel as a model for varying matrix stiffness

Table 3.1: List of possible photoinitiators for hydrogel formation.....	91
Table 3.2: Young's moduli of PMET gel, photocured at 1 minute and 5 minutes.....	121

Chapter 4: Matrix stiffness impacts epithelial cell differentiation markers in encapsulated bronchospheres

<i>Table 4.1: List of genes investigated in RT-qPCR.....</i>	<i>128</i>
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Chapter 5: Mechanisms and impact of mechanotransduction

<i>Table 5.1: List of genes investigated in single cell RT-qPCR.....</i>	<i>159</i>
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<i>Table 5.2: Summary of Western Blot results from TAZ antibody staining.....</i>	<i>189</i>
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<i>Table 5.3: Summary of Western Blot results from ACE2 antibody staining.....</i>	<i>191</i>
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Chapter 6: Conclusion

<i>Table 6.1: Comparisons between different modelling methods of the lung.....</i>	<i>228</i>
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Abbreviations

ECM	Extracellular Matrix
COPD	Chronic Obstructive Pulmonary Disease
IPF	Idiopathic Pulmonary Fibrosis
PEGDA-Matrigel®-Eosin Y and Triethanolamine	PMET
NHBE	Normal Human Bronchial Epithelial cells
CHBE	Chronic Human Bronchial Epithelial cells
MUC5AC	Mucin 5AC
SOX2	SRY (sex determining region Y)-box 2
YAP1	Yes-associated protein
BM	Basement membrane
SPARC	Secreted protein acidic and rich in cysteine protein
EMILINs	Elastin microfibril interface located proteins
TGFβ	Transforming growth factor beta
EMT	Epithelial-Mesenchymal cell transitions
YM	Young's Modulus
PAM	Polyacrylamide
PEGDA	Polyethylene Glycol Diacrylate
NHS	National Health Service
WHO	World Health Organisation
GOLD	Global Initiative for Chronic Obstructive Lung Disease
FEV1	Forced Expiratory Volume
FVC	Forced vital capacity
MUC5B	Mucin 5B
ZO-1	Zonula occludens-1 tight junction protein
FSP	Fibroblast-specific protein 1
S100A4	Member of the S100 calcium binding protein family

RTK	Receptor tyrosine kinase
SMAD	SAMD transcription factor
WNT	Wingless-related integration site protein
NF-κB	Nuclear factor -kappa B
CTGF	Connective tissue growth factor
EGFR	Epidermal growth factor receptor
α-SMA	α -smooth muscle actin
MMP	Matrix Metalloprotease
CD8+	Cluster of differentiation 8
IL-33	Interleukin 33
TIMPs	Tissue inhibitors of metalloproteinase
SABAs	Short acting β_2 agonists
LABAs	Long-acting β_2 agonists
LAMAs	Long-acting anti-muscarinic
SFTPA2	Surfactant protein 2
UIP	Usual Interstitial Pneumonia pattern
MSCs	Mesenchymal stromal cells
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
PDGF	Platelet-derived growth factor
CPFE	Combined Pulmonary Fibrosis and Emphysema
CT	Computed Tomography
ASM	Airway smooth muscle cells
AT2	Alveoli epithelial cell type 2
AT1	Alveoli epithelial cell type 1
AFM	Atomic force microscopy
TC	Tissue Culture
BLS	Brillouin Light-Scattering
FRET	Fluorescence resonance energy transfer
TAZ	WW-domain-containing transcription regulator 1
TEADs	Transcriptional enhancer factor domains

MST1/2	Serine/threonine kinase 3 and 4
SAV1	Salvador family WW- domain containing protein
LATS1/2	Large tumour suppressor kinase 1/2
MOB1A/1B	MOB kinase activator 1A and 1B
GSK	Glycogen synthase kinase
CK	Casein kinase
FGF10	Fibroblast growth factor 10
EGF	Epidermal growth factor
RA	Retinoic acid
IGF	Insulin-like growth factor
A8301	Activin/NODAL/TGF-beta Pathway Inhibitor
DAPT	Notch (γ -secretase) inhibitor
PGE2	Prostaglandin E2
RSP01	R-spondin 1
FGF2	Fibroblast growth factor 2
N2	N2 supplement
B27	B-27 supplement
SB202190	P38 MAP kinase inhibitor
BME	Basement membrane extract
RT-qPCR	Reverse transcription (quantitative) PCR
Sc RT-qPCR	Single cell reverse transcription (quantitative) PCR
HBE	Human bronchial epithelial cells
DMEM	Dulbecco's Modified Eagle Medium
Y-27632	Y-27632 Rock Inhibitor
NHLF	Normal human lung fibroblasts
3T3	NIH 3T3 mouse embryonic fibroblast cells
ATCC	American Type Culture Collection
EDTA	Ethylenediaminetetraacetic acid
PBS	Phosphate-buffered saline
RT	Room temperature

EY	Eosin Y
TEAO	Triethanolamine
LED	Light-emitting Diode
cDNA	Complementary DNA
KI67	Marker of Proliferation Ki-67
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
TP63	Tumor Protein P63
FOXJ1	Forkhead Box J1
TGFB1	Transforming growth factor beta 1
18S	RNA, 18S Ribosomal
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1
TE	Tris-EDTA
ACTB	Beta Actin
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
TBP	TATA-Box Binding Protein
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
RPL13A	Ribosomal Protein L13a
YAP1	Yes1 Associated Transcriptional Regulator
ACE2	Angiotensin Converting Enzyme 2
MUC2	Mucin 2, Oligomeric Mucus/Gel-Forming
CEACAM5	CEA Cell Adhesion Molecule 5
CHGA	Chromogranin A
KRT4	Keratin 4
SNTN	Sentan
CSF3	Colony Stimulating Factor 3
CXCL10	C-X-C Motif Chemokine Ligand 10
CTNNB1	Catenin Beta 1
CRIM1	Cysteine Rich Transmembrane BMP Regulator 1

DOCK5	Dedicator Of Cytokinesis 5
VCL	Vinculin
DAPI	4',6-diamidino-2-phenylindole
PFA	Paraformaldehyde
BSA	Bovine serum albumin
NGS	Normal Goat Serum
TDE	2'-2'-thio-diethanol
RIPA	Radioimmunoprecipitation assay
UCST	Upper critical solution temperature
LCST	Lower critical solution temperature
S₀	Ground state
S₁	Excited state
CQ	Camphorquinone
FR	Fluorescein
RF	Riboflavin
FITC	Fluorescein isothiocyanate
MUC2	Mucin 2
MUC6	Mucin 6
MUC1	Mucin 1
MUC3	Mucin 3
MUC4	Mucin 4
MUC12	Mucin 12
MUC13	Mucin 13
HK	Housekeeping
OCT4	octamer-binding transcription factor 4
PTCH1	receptor patched homolog 1
TROP2	trophoblast cell surface antigen 2
ICC	Immunocytochemistry
Ct	Cycle threshold
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
TPN	Total protein normalisation
BCA	Bicinchoninic acid

A549	Human alveolar basal epithelial cell line
SAEC	Small airways epithelial cell
ALI	Air-liquid interface
kPa	Kilopascals
GPa	Gigapascals
RGD	Arginine-Glycine-Aspartic acid
LCM	Laser Capture Microscopy
IL-13	T helper (Th) type 2 cytokine IL-13
hMSCs	Human mesenchymal stem cells

Chapter 1: **Introduction**

1.1 The Extracellular Matrix

The composition of the pulmonary extracellular matrix (ECM) matrixome is complex; its architecture serving as a scaffold for cells as a basement membrane as well as an intercellular mesh network which surround the cells and adds to the biophysical properties of the lung (Burgstaller et al., 2017). The glycoproteinous basement membrane (BM) anchors the most superficial cell layers such as epithelial and endothelial on their basal surface via adhesion receptors (Jayadev and Sherwood, 2017). The BM is also found around other cells including adipose and muscle cells, with the matrix protein composition being specific for each cell type (Jayadev and Sherwood, 2017, Randles et al., 2017).

One of the major components in mammalian BM is the heterotrimeric protein laminin. These α , β and γ trimers self-assemble into a network that interacts directly with cell surfaces (Jayadev and Sherwood, 2017, Pozzi et al., 2017). Another stiffer network of collagen IV is present as an overlay; these meshes are covalently bound together and are responsible for the overall tensile strength of the ECM (Wiradjaja et al., 2010). Interactions between the two structural meshes are facilitated by high affinity proteins like nidogen (glycoprotein), perlecan (heparan sulfate proteoglycan), collagen XVIII (proteoglycan), SPARC (secreted protein acidic and rich in cysteine protein) and fibulin-1 (glycoproteins) (Murphy-Ullrich and Sage, 2014, Naba et al., 2016, Jayadev and Sherwood, 2017). Laminins which directly associate with the cells, are linked via integrins and dystroglycan receptors, embedded into the cell's surface (Pozzi et al., 2017). This, in turn, communicates with the α subunit of the trimeric protein where the laminin globular-like domain resides (Mckee et al., 2007, Yurchenco, 2011). The organ-specificity of the BM not only involves the composition and structure (whereby the self-assembly ability can take on multiple arrangements due to the identification of 5 α , 4 β and 3 γ laminin chains in mammals) but also extends to the relative thickness and orientation of end domains (Jayadev and Sherwood, 2017). The difference in these ECM protein networks also determines their structural and mechanical properties and roles within the BM (Randles et al., 2017). For example, laminin is required for cell differentiation and tissue formation and is laid down early during development (Wiradjaja et al., 2010). Type IV collagen on the other hand, provides the structural and mechanical strength to withstand any stressors whilst the polarity within the cell culture is also dictated by the BM (Jayadev and Sherwood, 2017, Boudko et al., 2018, Sekiguchi and Yamada, 2018). Cell-to-matrix communication, especially with laminin via surface bound integrins,

can determine the basal to apical direction of cells aiding the formation of e.g., lumenogenesis (Klein et al., 1988, Murray and Edgar, 2000, O'brien et al., 2001, Jayadev and Sherwood, 2017). In comparison, the interstitial matrix is looser, more fibril-like and found within tissue to aid and maintain the 3D structural integrity of the organ, as well as the dynamic movements required for function. Its composition is mainly collagen type 1 and III but also contains elastin. It is the presence of these elastin fibres which gives rise to the viscoelastic and hysteresis profiles seen in connective tissue, converting the matrix from a typical linear stress versus strain relationship (Humphrey et al., 2014, Burgstaller et al., 2017). In the lung, this is particularly important as lung compliance during breathing is partially dependent on these proteins, and the proteins which facilitate cell-to-ECM adhesions such as elastin and related factors such as elastin microfibril interface located proteins (EMILINS) and fibrillin (Nakamura et al., 2002, Spessotto et al., 2003, Burgstaller et al., 2017).

Although a lot of the research published on the ECM is about its structural properties, it should also be considered that the matrix itself, is not inert. The structural complexity can also act as a reservoir for certain growth factors and ligands that are sequestered until a conformational change takes place. A prime example is Transforming Growth Factor beta ($TGF\beta$) whereby its latent form can exist in the matrix, bound by the microfibrils of fibrillin-1 (Sengle and Sakai, 2015, Haller and Dudley, 2022). Stresses upon the matrix rather than the cells can induce biochemical changes since the matrix itself is mechanosensitive. The resulting strain deforms the microfibrils enough to decrease the protein-to-protein end contacts and increases the bioavailability of the now-free $TGF\beta$. $TGF\beta$ (particularly $TGF\beta 1$) is recognised as a major cytokine for promoting the remodelling of the matrix by stimulating the differentiation of fibroblasts to myofibroblasts as well as contributing to the EMT process (Hinz, 2012). The mechanosensitivity of the matrix is also exhibited by stressed collagen and fibrin. Both proteins in their fibrillar form, align when strained and become more resistant to proteolysis compared to the fibres under less mechanical stress (Haller and Dudley, 2022). Binding sites specific to integrins within fibronectin fibres can also become exposed when stretched. These once-cryptic sites can modulate cellular behaviour by extracellular mechanotransduction by the matrix (Kubow et al., 2015, Haller and Dudley, 2022). Such examples demonstrate the importance of bidirectional communication between cells and the

matrix. Therefore, for *in-vitro* models to try and emulate fibrotic diseases, the influence from the matrix design introduced should also be addressed, not just the cell phenotype.

Matrigel[®] is a common commercial basement membrane matrix used for *in-vitro* modelling, made from extract of mouse (Engelbreth-Holm-Swarm) tumours (Kleinman et al., 1986). There are other basement membrane matrices available such as Cultrex[®] and Geltrex[®], providing the same properties as Matrigel[®] (see **Table 1.1**). The properties of Matrigel[®] such as its composition of basement membrane-associated proteins including laminin, collagen IV, entactin and perlecan, at approximately 60%, 30%, 8% and 2-3% respectively, help to successfully culture cells into 3D organoids (Hughes et al., 2010, Aisenbrey and Murphy, 2020, Corning, 2021). However, the degradability of Matrigel[®] can make it difficult to sustain this 3D cell culture (when long-term matrix structural integrity is required), as the sensitivity of the proteins to *in-vivo* proteolysis e.g. with matrix metalloprotease (MMP)2 and 9 also occurs *in-vitro* (Wong et al., 1992, Dolo et al., 1999, Aisenbrey and Murphy, 2020).

A maturing organoid remodels the proximal environment to allow for growth and differentiation (Loebel et al., 2019, Blache et al., 2020). However, in doing so, this loosens the scaffold's integrity and even though new native ECM is produced, the rate of production versus the rate of degradation of the old ECM is often mismatched increasing the risk of the gel matrix collapsing. Alongside this, Matrigel[®]'s batch-to-batch similarity can vary greatly. There are reportedly 424 identified proteins within the growth-factor reduced version of Matrigel[®] but its homogeneity is capped at approximately at 53% (Hughes et al., 2010, Aisenbrey and Murphy, 2020).

This heterogeneity may also lead to the differences seen in the Young's Modulus (YM) values regarding stiffness of various Matrigel[®] models. Different models state values ranging from 400-840 Pa, with the higher values measured in non-uniform regions of density (Soofi et al., 2009, Aisenbrey and Murphy, 2020). The xenogeneic factor is also taken into consideration when the desired culture is to be human-derived and have no animal contaminants present. As a result, alternative synthetic matrices have been developed, mostly to try and address the strength of the scaffold produced and the durability of its architecture in long-term culture, specifically concerning the stiffness and especially if required as a constant variable. They are often presented as hydrogels, tuneable to the desired stiffness

needed, rate of degradability can be controlled and chemically defined (Aisenbrey and Murphy, 2020). Examples include functionalised monomers of PAM (polyacrylamide) (Wang and Pelham Jr, 1998, Fischer et al., 2012) and PEG (polyethylene glycol) which can be crosslinked to undergo polymerisation through a variety of means (Krsko and Libera, 2005, Zhu, 2010). PEG is often preferred as a monomer as it has lower toxicity during fabrication and can be used in various encapsulation assays (Burdick and Anseth, 2002, Roberts and Bryant, 2013, Aisenbrey and Murphy, 2020).

List of extracellular matrices than can be used for cell culture

Matrices	Composition	Biological source
<i>Non-synthetic</i>		
Matrigel [®] Geltrex [®] Cultrex [®]	Laminin, Collagen IV, Entactin, Heparin Sulfate, Proteoglycans, Growth Factors	Mouse Engelbrecht-holm- swarm tumor
PureCol [®]	97% Type I atecollagen, 3% Type III collagen	Bovine hide
Type I Collagen	Collagen I	Rat tail
Type IV Collagen	Collagen IV	Human cell culture
Fibronectin	Fibronectin (Human plasma)	Human plasma
Laminin	Laminin	Human placenta, human fibroblasts, murine Engelbrecht-holm-swarm tumor
Silk	Protein polymers	Insects, worms (lepidoptera)
Gelatin	Denatured protein	Collagen
Hyaluronan	Non-sulfated glycosaminoglycan	Connective tissue e.g., cartilage
Alginate	Polysaccharide block copolymers- 1,4- β -d- mannuronic acid (M) and 1,4- α -l-guluronic acid (G) monomers	Marine brown algae
Agarose	Galactose-based backbone polysaccharide	Red algae and seaweed
Chitosan	Chitin partial deacetylation 2- acetamido-2-deoxy- β -D- glucose	Marine crustaceans, insects and fungi walls
<i>Synthetic</i>		
Polyethylene glycol (PEG) - based hydrogels	Monomers of functionalised PEG	-
Poly(lactic-co-Glycolic Acid)	Poly(lactic and polyglycolic acid copolymers	-
Nanocellulose-Based Hydrogels	cellulose nanocrystals, cellulose nanofibers, and bacterial nanocellulose	-
Polyacrylamide (PAM)	acrylamide monomers and bis-acrylamide crosslinkers	-

Table 1.1: List of some of the matrices available to use within cell culture, both non-synthetic and synthetic. Examples from references (Malafaya et al., 2007, Aisenbrey and Murphy, 2020, Busch et al., 2021, Poudel et al., 2022).

1.2 Respiratory diseases

1.2.1 Chronic Obstructive Pulmonary Disease

The impact of COPD (Chronic Obstructive Pulmonary Disease) is global, adversely affecting respiratory health and equally bearing economic pressure on healthcare systems to effectively treat it. According to 2019's World Health Organization (WHO) Global Health Estimates factsheet, COPD is the 3rd leading cause of death worldwide with around 3.23 million deaths annually (World Health Organization, 2020, World Health Organization, 2021). In 2017, the cost to the NHS of COPD in the UK was £1.9 billion per year and there is currently no cure, only treatments being palliative and directed towards improving lung function with minimal effect on the chronic inflammation associated with the disease (British Lung Foundation, 2017).

COPD is regarded as an umbrella term, encompassing respiratory conditions such as emphysema, chronic bronchitis, chronic inflammation and remodelling of the airways particularly the small airways (World Health Organization, 2021). COPD is caused by continuous smoke inhalation as well as exposure to harmful, noxious airborne substances like smoke from indoor biomass combustion (Vestbo et al., 2013, Barnes et al., 2015, World Health Organization, 2021). The resulting airflow obstruction is due to the constant remodelling occurring in the small airways (Churg et al., 2006). The emerging vacuolar nature of the damaged alveoli within the emphysematous lung impacts upon the main function of the lung i.e. gas exchange and respiration (Black et al., 2008) (**Figure 1.1**). Alongside the inefficient gaseous exchange seen in COPD patients with emphysema, the loss of alveolar attachments results in a reduction in the recoil nature of breathing (Black et al., 2008). These structural changes, together with the increased number of exacerbations caused by bacterial or viral infection or exposure to pollution, lower the patients' quality of life. **Table 1.2** shows the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines for diagnosing the severity of COPD patients' airflow limitation, which is used to subsequently advise treatment depending on the symptoms and risks experienced. Originally created in 2001 as the '*Global Strategy for the Diagnosis, Management, and Prevention of COPD*' consensus report, this advice is constantly updated and is implemented in multiple regions as part of national guidelines (Vestbo et al., 2013).

Grading of severity of airflow limitation in COPD (based on post-bronchodilator FEV1)				
<i>In patients with FEV1/FVC < 0.70:</i>				
GOLD 1:	Mild	FEV1 $\geq$ 80% predicted	Patient group A —low risk, less symptoms	0-1 exacerbations per year
GOLD 2:	Moderate	50% $\leq$ FEV1 < 80% predicted	Patient group B —low risk, more symptoms	
GOLD 3:	Severe	30% $\leq$ FEV1 < 50% predicted	Patient group C —high risk, less symptoms	and/or $\geq$ 2 exacerbations per year and/or $\geq$ 1 hospitalized exacerbation per year
GOLD 4:	Very severe	FEV1 < 30% predicted	Patient group D —high risk, more symptoms	

Table 1.2: Classification of COPD stage by airflow limitation after spirometry assessment. Table adapted from reference (Vestbo et al., 2013).

Although the transpulmonary pressure is reduced owing to the degradation of the alveoli septum, the remaining interconnecting stromal framework and extracellular matrix (ECM) is rendered stiffer (due to high tensile stress on the remaining alveolar walls) and thicker than in healthy subjects, particularly in the small airways (Suki et al., 2013, Bidan et al., 2015, Jones et al., 2016, Burgess and Harmsen, 2022). The pathogenic remodelling also influences the presence of goblet cells and hypersecretion of mucins, adding to the obstructive nature of COPD. Goblet cells are a subtype of epithelial cell found in the respiratory epithelium, which secrete mucins as a first-line defence against inhaled microparticles which are cleared by the mucociliary escalator (Zanin et al., 2016, Walentek, 2021). This is reduced in COPD due to the excess mucus and the reduced ciliary beat frequency, cilia length and potentially loss of ciliated cells (Yaghi and Dolovich, 2016, Thomas et al., 2021) (described in more detail, pages 30-32). Mucins also function to maintain the osmotic nature of the secreted barrier or periciliary layer which is composed of membrane-bound mucin and the superficial gel forming layer (Ma et al., 2018, Wagner et al., 2018). Goblet and club cells, the latter found in the small airways that predominantly do not secrete mucins, constitute the major secretory cells present in the airways (Hadzic et al., 2020). Club cells can act as progenitor cells for goblet and ciliated epithelial cells and release

secretoglobins into the lumen, stored in granular form (Davies et al., 2002, Reynolds and Malkinson, 2010, Walentek, 2021).

At least 21 human mucosal genes have been identified and 2 mucins have been identified in association with COPD (Whitsett, 2018). MUC5AC is localised to the goblet cells in the superficial region of the large airways, whereas MUC5B is found both at the superficial layer as well the submucosal glands. Mucus obstruction is a hallmark pathology of COPD, with MUC5AC upregulation due to persistent stimulation by chronic inflammation, the presence of harmful substances such as cigarette smoke extract and inhaled environmental pollutants and tissue remodelling. Increased MUC5AC expression also results from the increased presence and growth of goblet cells indicative of goblet cell hyperplasia (Fahy and Dickey, 2010, Ma et al., 2018). MUC5B, a mucin paralog, is also upregulated in COPD but its function is unclear, perhaps rather due to the paralog being the dominant gel forming mucin to help trap and clear inhaled pathogens (Lu and Zheng, 2013, Okuda et al., 2019, Radicioni et al., 2021). Intriguingly, MUC5B is genetically associated with the incidence of idiopathic pulmonary fibrosis (IPF) (Conti et al., 2016b, Hancock et al., 2018) although those with reduced expression have milder disease and improved survival,

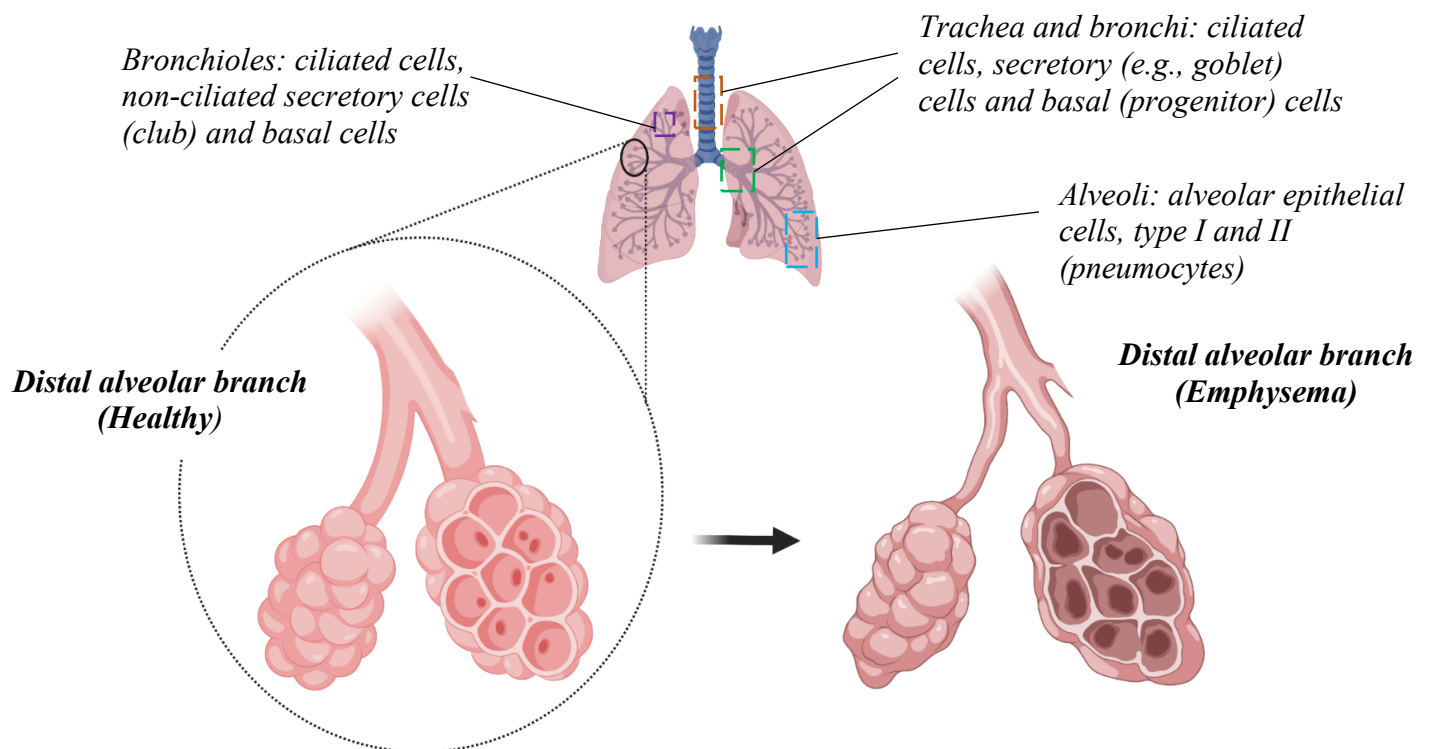


Figure 1.1: Illustration showing the structural changes in the lung, pertaining to the alveolar walls in COPD. The loss of the typical elastic nature for lung compliance is a hallmark trait of Emphysema. Efficient gaseous exchange is reduced and is accompanied with mucus hypersecretion. Created on Biorender.com.

particularly with the presence of MUC5B polymorphism rs35705950 (Peljto et al., 2013). The reason for this is still under investigation.

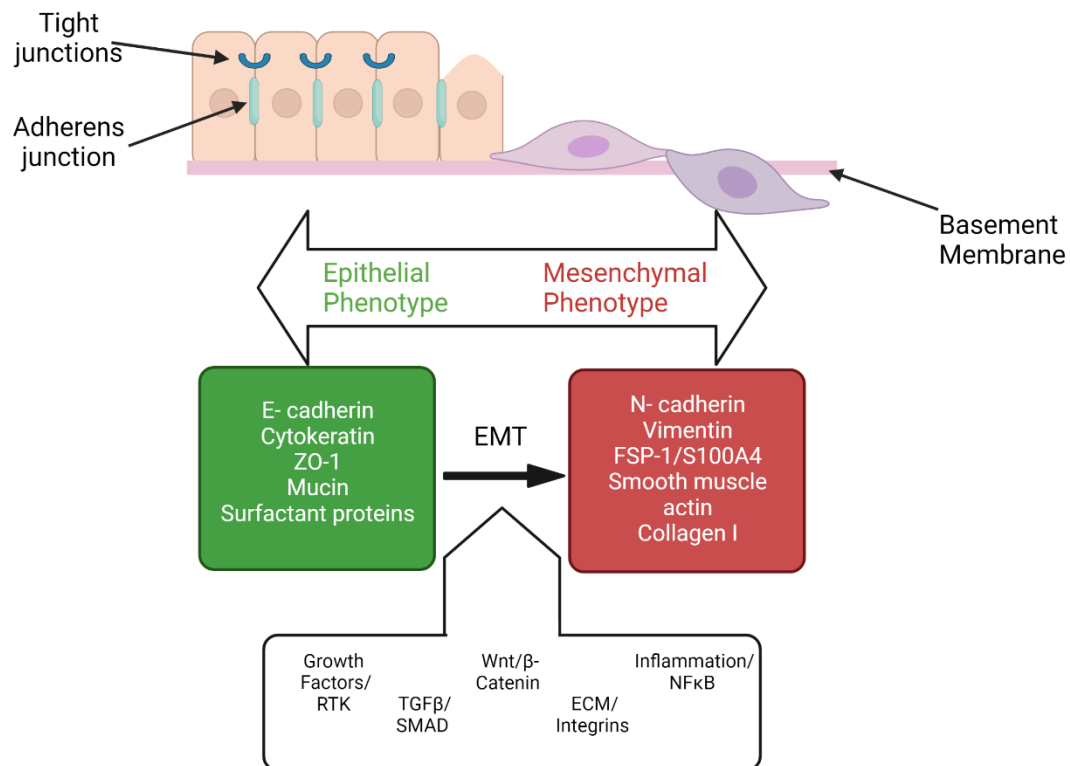


Figure 1.2: Many pathways (in particular, $TGF\beta$ signalling) can lead to activating the EMT programme within epithelial cells, causing features in the epithelial phenotype to be lost, such as E-cadherin and cytokeratin expression. Instead, the mesenchymal phenotype is favoured with the expression of N-cadherin and vimentin upregulated. Figure reproduced from (Bartis et al., 2014) and created on Biorender.com.

Another pathological feature of COPD is epithelial -mesenchymal cell transition (EMT) resulting from the presence of persistent inflammation that creates a microenvironment that drives the phenotypic change of resident epithelial cells through de-differentiation (Gohy et al., 2015). This process normally occurs during tissue damage-repair in response to infection or exposure to noxious agents. However, the enhanced inflammation/remodelling conditions present in the COPD peribronchial, and alveoli space causes neighbouring epithelial cells to lose their normal phenotype and become more mesenchymal- or fibroblast-like. The backlash of such a phenomenon will add to the continuous cycle of remodelling, due to the release of pro-fibrotic factors such as $TGF\beta$ and CTGF (Connective tissue growth factor) (Khalil et al., 1991, Pan et al., 2001, Pain et al., 2014).

EMT can be functionally categorised into 3 types which are present in many fibrotic stages and across all organs due to the activation of different pathways (**Figure 1.2**). Type I EMT is associated with the embryonic development stage of the lung, allowing for tissue and organ modelling. The reverse process can then be initiated for further cell differentiation and organ dimensionality (Kalluri, 2009, Pain et al., 2014). Type III EMT is associated with cancerous mechanisms and activities to promote cellular invasiveness. Epithelial cell polarity is lost aiding invasion through the basal membrane and subsequent metastasis (Yang and Weinberg, 2008). This EMT category is associated with the presence of increased fibroblast-associated markers such as vimentin and α -smooth muscle actin (α -SMA) indicating cytoskeletal and mesenchymal changes respectively (Pain et al., 2014). α -SMA is produced by smooth muscle cells and by myofibroblasts, which also contributes to the remodelling seen in COPD. COPD is linked with Type II EMT, which is exacerbated by the presence of chronic inflammation, whereby fibroblasts become primed to secrete excess ECM proteins including collagen and fibronectin, an unregulated and enhanced version of the remodelling and repair function of tissue regeneration (Kim et al., 2006). Type I EMT factors are also displayed in COPD; one study showed clefts within the reticular basement membrane of patients (ex/current smokers and/or with COPD) exhibiting increased expression of vimentin, the tissue modelling factor matrix metalloprotease 9 (MMP9), epidermal growth factor receptor (EGFR) and S100A4 (fibroblast-specific protein 1) compared to healthy control subjects (Bartis et al., 2014). The up-regulation of these markers reflect the occurrence of ongoing EMT (Sohal et al., 2010).

The presence of myofibroblasts is another hallmark feature of pulmonary fibrosis. This cell subtype behaves as an intermediate between fibroblasts and smooth muscle cells and are normally present during tissue repair in response to an insult and are then removed from healthy tissue (Hinz, 2012). However, this controlled regulation is lost in respiratory diseases such as COPD and IPF and the 'repair' function becomes excessive (Kis et al., 2011). Persistent cigarette/noxious particle inhalation results in continuous damage to the airways, and as a result, drives a positive feedback loop of dense collagen ECM secretion. In the case of a fibrotic lung, the origin of myofibroblasts is believed to be resident fibroblasts driven by EMT changes and TGF β activation (Sheppard, 2006, Hinz, 2012). The change in mechanical stress experienced by the cell is also a contributing factor and will be discussed further in detail (see page 43).

A prominent contributor to mucus obstruction and progressive inflammation in COPD is cilia motor dysfunction at the epithelial surface layer. 50-80% of the upper and lower respiratory tract is ciliated and therefore becomes clogged with excess mucus and accumulated inhaled pathogens, due to the lack of the dedicated mucociliary transport mechanism (Patton and Byron, 2007, Yaghi and Dolovich, 2016). Also, the maintenance of the mucosal layer composition becomes lost as its initial thickness is determined by the location within the lung e.g., the mucosal barrier layer which acts as a physical protective layer to microscopic inhalants, thins out to $<0.1\mu\text{m}$ at the alveolar region to ensure no risk of plugging or blocking (Patton and Byron, 2007). Such regulation is lost in respiratory disease (**Figure 1.3**) (Yaghi and Dolovich, 2016). Studies have also reported modifications of the cilia structures themselves, with differences such as loss of or shorter ultra-filaments which further attenuate mucosal clearance (Thomas et al., 2021).

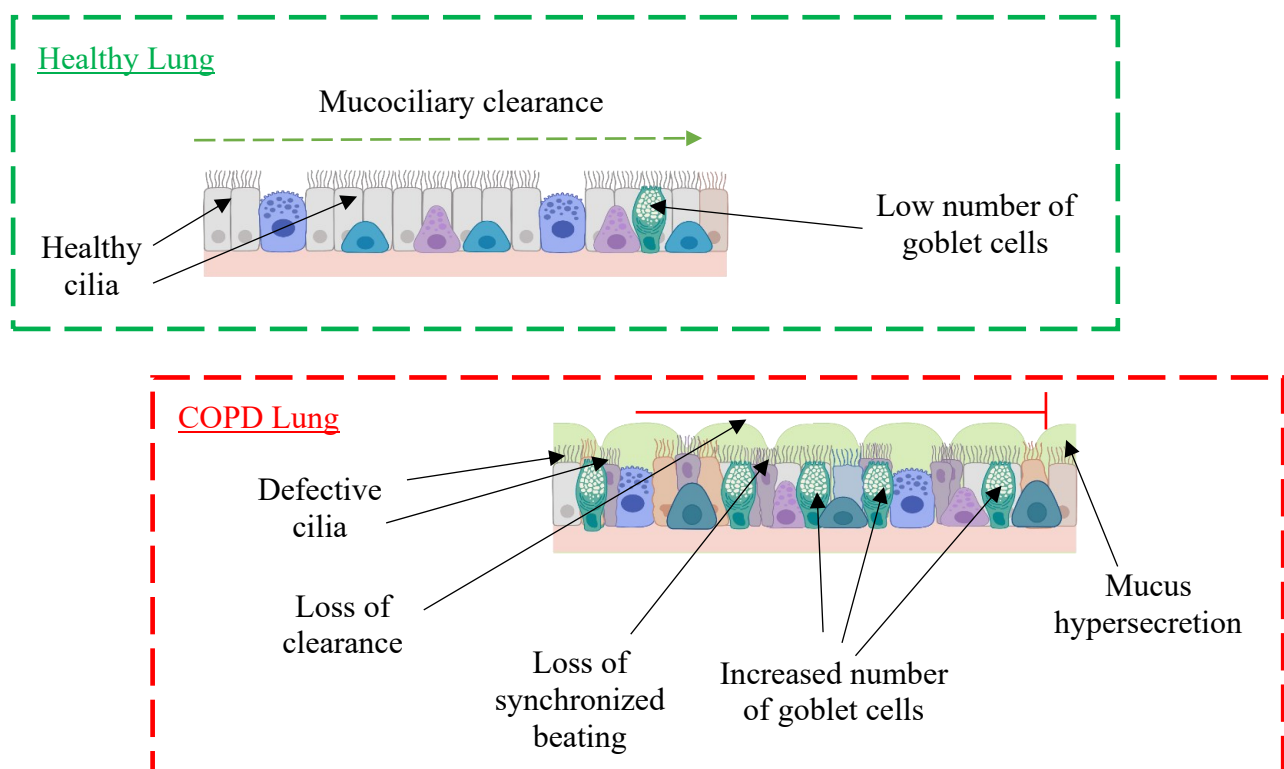


Figure 1.3: Schematic representation of how ciliary dysfunction can worsen COPD as fluid flow and mucociliary clearance are also compromised. The increase in goblet cells adds to the gel-like layer on the apical surface, disrupting the balance found in healthy lungs and resulting in the accumulation of pathogens and contaminants inhaled (Walentek, 2021). Created on Biorender.com.

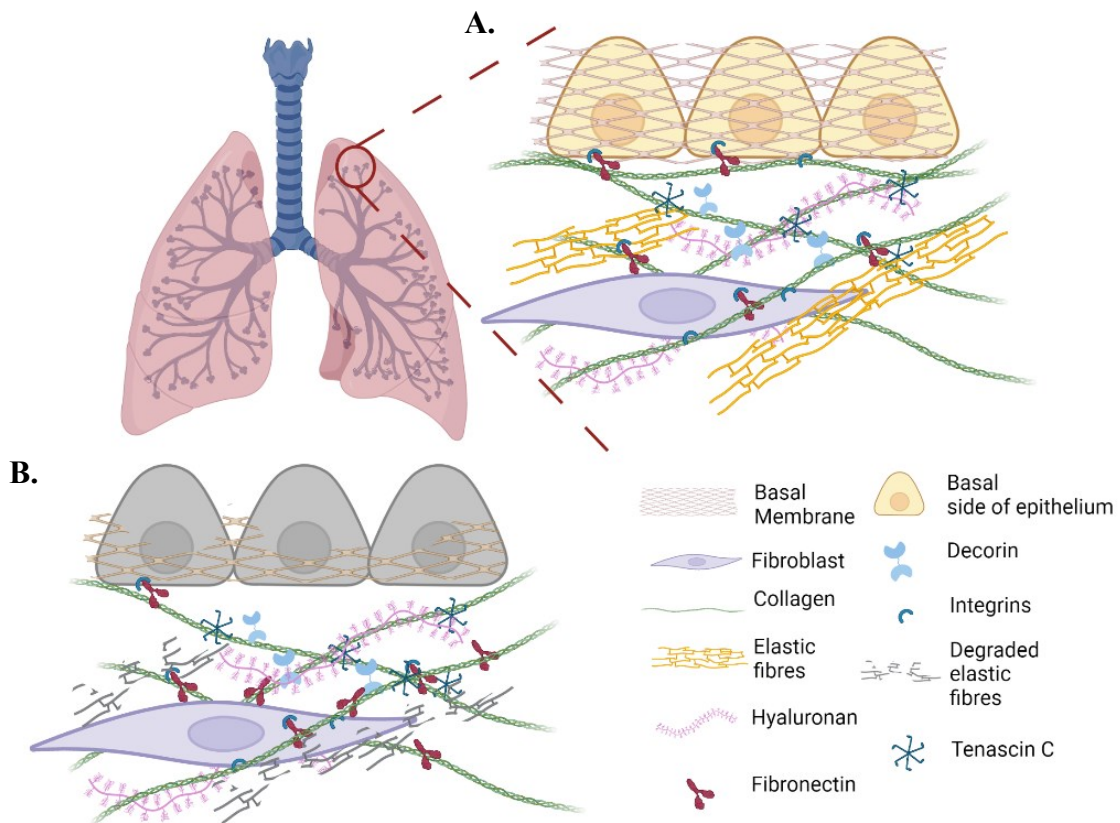


Figure 1.4: Image comparison of pathophysiological ECM structural changes from (A) healthy to a (B) COPD lung. Degradation of elastic fibres are depicted, alongside the increased presence of ECM proteins like fibronectin and tenascin C. Reproduced schematic from reference (Burgstaller et al., 2017), with granted permission of the ©ERS 2022 and created in Biorender.com.

Thickening of the bronchial airway walls is also due to upregulated proliferation and possibly hyperplasia of airway smooth muscle cells physically limiting airflow. These cells also undergo phenotypic changes to become more Ca^{2+} sensitive which promotes bronchoconstriction (Kume, 2021). Such inflated responsiveness is also accompanied by the presence of inflammatory cells such as innate immune-associated mast cells and macrophages influencing the level of protease activity, contributing to the loss of protease homeostasis and the activation of MMPs (Hadzic et al., 2020).

Adaptive immunity associated CD8^+ T lymphocytes also help to create a pro-inflammatory environment. The secretion of interleukin (IL)-33, an alarmin, from epithelial and endothelial cells is further favoured in such an environment, which, together with excessive oxidative stress, have the potential to result in the generation of more autoantibodies in the respiratory tract and lung which target alveolar epithelial cells, recruit

complements and induce cell death (Kirkham et al., 2011, Hadzic et al., 2020). Oxidative and nitrosative stress have been ‘linked’ to COPD prognosis. Reactive oxygen species (ROS) can trigger immune responses and aid in the overall progression with COPD by protein modification. Further modification can occur, resulting in the nitration of tyrosine residues, feeding the COPD pathogenesis by causing further cell signalling alterations, inflammation and breaking self-tolerance (Hadzic et al., 2020).

Alongside structural changes to lung resident cells, the composition of the ECM also becomes modified in COPD (**Figure 1.4**). In emphysema, the loss of the alveoli recoil nature is due to the degradation of the elastin protein in the elastin fibres by proteases and its regulators (α 1-antitrypsin is a regulator of elastase). Collagen deposition is also increased together with that of fibronectin, tenascin-C and versican; all contributing to COPD remodelling (Burgstaller et al., 2017).

MMP2 and MMP9 (gelatinases A and B respectively) are also upregulated in COPD and are associated with tissue modelling (**Table 1.3**) (Chakrabarti and Patel, 2005). Briefly referred to in the previous sections, these endopeptidases are naturally present in the lung, degrading proteins in the ECM but under a tightly controlled manner by tissue inhibitors of metalloproteinase (TIMPs) and exist in their pro-peptide form before activation. Gelatinases are a subclass of MMPs, alongside collagenases (MMP1, 8 and 13), stromelysins (MMP3, 10 and 11), matrilysin (MMP7), elastase (MMP12) and membrane-type MMPs (MMP14, 15, 16 and 17) (Chakrabarti and Patel, 2005).

MMP2 and MMP9

Enzyme	Substrates	Inhibitors	Associated cells
MMP-2	Gelatin, Collagen types: I, IV, V, VII, X, XI, Elastin, Fibronectin, Laminin-5, Aggrecan, Brevican, Vitronectin, Decorin	TIMP-2, -3, -4, α_2 -Macroglobulin, α_1 -anti-trypsin	Activated macrophages Monocytes, Endothelial cells
MMP-9	Gelatin, Collagen types: I, IV, V, VII, X, XI Elastin, Fibronectin, Laminin, Aggrecan, Vitronectin	TIMP-1, -3, -4, α_2 - Macroglobulin, α_1 -anti-trypsin	Activated macrophages, Neutrophils, Monocytes, Eosinophils, Bronchial epithelial cells, AT2 cells, Smooth muscle cells, Fibroblasts

Table 1.3: Summarised list of Matrix Metalloprotease 2 and 9, their substrate in the ECMs, inhibitors and cellular sources. Information adapted from reference (Chakrabarti and Patel, 2005).

1.2.2 Current treatment options for COPD

There are few available options for the treatment of COPD, and none prevent COPD inflammation or remodelling. There is no cure for COPD and the opportunities for lung transplantation is rare. Current treatments are reliant on both pharmacological and non-pharmacologic approaches. Treatment is based on the regularly updated GOLD (Global Initiative for Chronic Obstructive Lung Disease) guidelines which is used to aid physicians in prescribing the right method of treatment (Vestbo et al., 2013). Smoking cessation is normally combined with therapies such as bronchodilators; short acting and long acting β_2 agonists (SABA and LABA) to help relax smooth muscle during exacerbations caused by COPD (Vogelmeier et al., 2017, Hadzic et al., 2020). In addition, short-acting and preferably long-acting anti-muscarinic (LAMAs) treatments are the preferred first line bronchodilator therapy to improve COPD lung function. Bronchodilator polytherapy is preferred and for patients who suffer from severe COPD, options also include oxygen therapy, inhaled corticosteroids to improve lung function, even though such medication has its own plethora of side effects (Vogelmeier et al., 2017).

A revision of the GOLD strategy in 2019, fine-tuned COPD diagnosis by adding on a follow-up assessment of the patient to consider symptoms and exacerbation risk (Singh et al., 2019). Newer treatment options are being further explored, with considerations towards treatment of selected patient subgroups with inhaled glucocorticoids or monoclonal antibodies used in the treatment of asthma to target inflammation and new protease inhibitors e.g. intravenous and inhaled recombinant human α_1 -antitrypsin inhibitors to combat the imbalance of protease presence and their contribution to cellular scaffold degradation (Hadzic et al., 2020, Uwagboe et al., 2022). Severe cases may consider surgical interventions like lung volume reduction surgery, bullectomy and lung transplantation to improve overall lung health status (Vogelmeier et al., 2017).

1.2.3 Idiopathic Pulmonary Fibrosis (IPF)

IPF is a debilitating respiratory disease that is characterised by severe forms of peripheral lung fibrosis, heavily impacting the resulting quality of life for the patient (**Figure 1.5**). IPF, in contrast to COPD, is considered as a rare interstitial lung disease (Maher et al., 2021). In comparison, lung remodelling also occurs in IPF but with a greater contribution of increased lung fibrosis as opposed to emphysema (Ats/Ers, 2000, Hogg and Timens, 2009, Chilosi et al., 2012). Whilst the hallmarks of COPD include excess secretion of MUC5AC, due to goblet cell hypersecretion and hyperplasia, an increased production of MUC5B is frequently seen in IPF patients (Conti et al., 2016a). This alters the thickness of the submucosal glands layer, of the superficial epithelium and increases the likelihood of mucosal plugs forming (Okuda et al., 2019). Research has also linked the MUC5B promoter variant rs35705950 to the increased MUC5B production in IPF (Nakano et al., 2016). It is considered that this promoter is associated with IPF only, with recent published papers

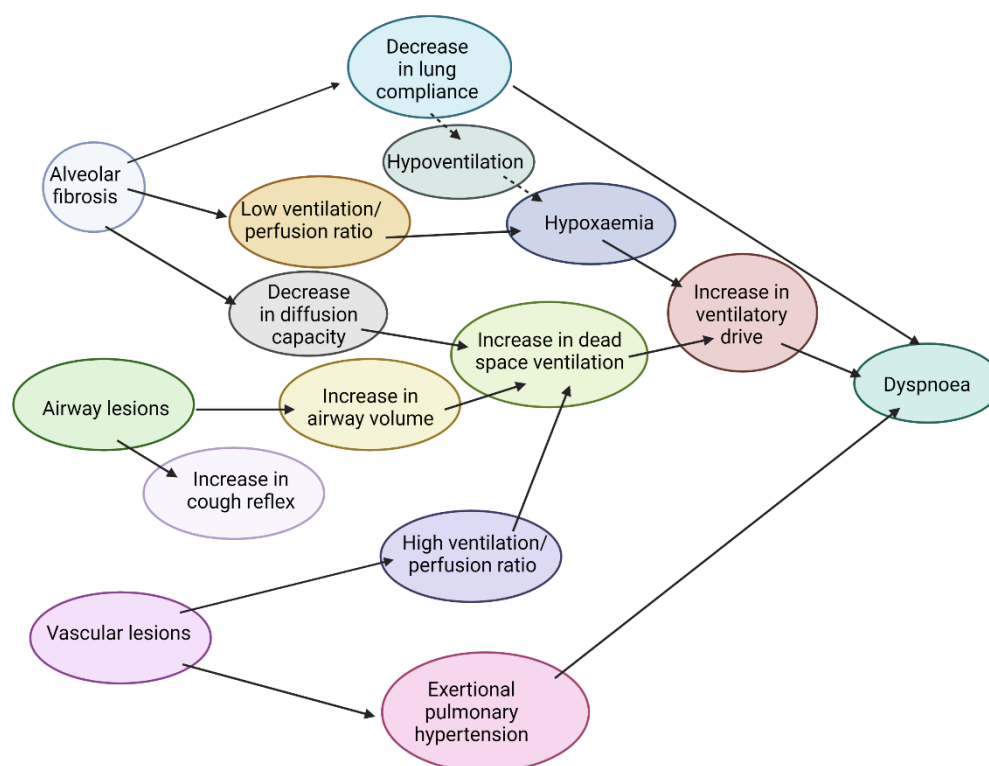


Figure 1.5: Some of the pathological features of IPF and the main associations between them. The links between decreased lung compliance, hypoventilation and hypoxaemia are reported at end -stage disease state (IPF) (dotted arrows). Reproduced from (Plantier et al., 2018), with permission of the ©ERS 2022. Created in Biorender.com.

establishing low prevalence of the polymorphism in other non-Interstitial lung diseases including COPD (Goos et al., 2022). Other mutants may include variants in the usual surfactant proteins found in the lung, e.g. SP-A (SFTPA2), contributing to the loss of alveolar architecture as the tightly controlled surfactant composition has been altered (Borie et al., 2012, Plantier et al., 2018, Snijder et al., 2019).

The main hallmarks of IPF include the increased pathological presence of collagen, an important protein in the lung extracellular matrix, which is integral to its structural properties. The hyper-deposition of collagen affects the overall stiffness of the lung (Thannickal et al., 2014). Whereby the lung is required to have some elastic recoil to ensure adequate compliance for breathing; the increase in collagenous filaments works against this, increasing the mechanical tension and making gaseous exchange less effective (Plantier et al., 2018, Maher et al., 2021). Patients often complain about dyspnoea during early stages of disease and if no pharmaceutical intervention takes place, this can lead to end point respiratory failure with an average survival of 2-3 years (Sharif, 2017, Plantier et al., 2018, Maher et al., 2021). Diagnosis is usually confirmed by high-resolution computed tomography of the lung and further biopsies are taken if required (Ats/Ers, 2002). Recent analysis of epidemiological data shows the age range of IPF prevalence is between 65-70 with a predominance in males and the risk of a positive diagnosis occurring is greater with ageing (Collard, 2010, Jo et al., 2016, Maher et al., 2021).

The deposition of collagen in the lung is due to the increased presence of fibroblasts and myofibroblasts within fibroblastic foci (Cool et al., 2006) (**Figure 1.6**). The tell-tale peripheral ‘honeycomb’-like framework caused from pulmonary lesions is due to these intense patches of enhanced acellular collagen interspersed with alveolar collapse (Plantier et al., 2018, Snijder et al., 2019). These dilated air pockets result from the increased surface tension due to dysfunctional surfactant production and reflect the Usual Interstitial Pneumonia pattern (UIP) used in the definition of IPF- along with lung remodelling and fibrosis due to collagen deposition (Johkoh et al., 2014, Plantier et al., 2018). In addition, lung fibroblasts are activated to change their phenotype and become myofibroblasts (Cool et al., 2006, Bartis et al., 2014, Burgstaller et al., 2017, Snijder et al., 2019). Suggestions of Type II EMT in IPF may be observed, with the production of fibroblastic foci. These contractile transdifferentiated cells secrete ECM proteins including collagen and feed the perpetuation of fibrosis (Zhou et al., 2013, Kulkarni et al., 2016). However, several studies

report contradicting evidence regarding their cell lineage which makes this theory controversial at the present time (Bartis et al., 2014).

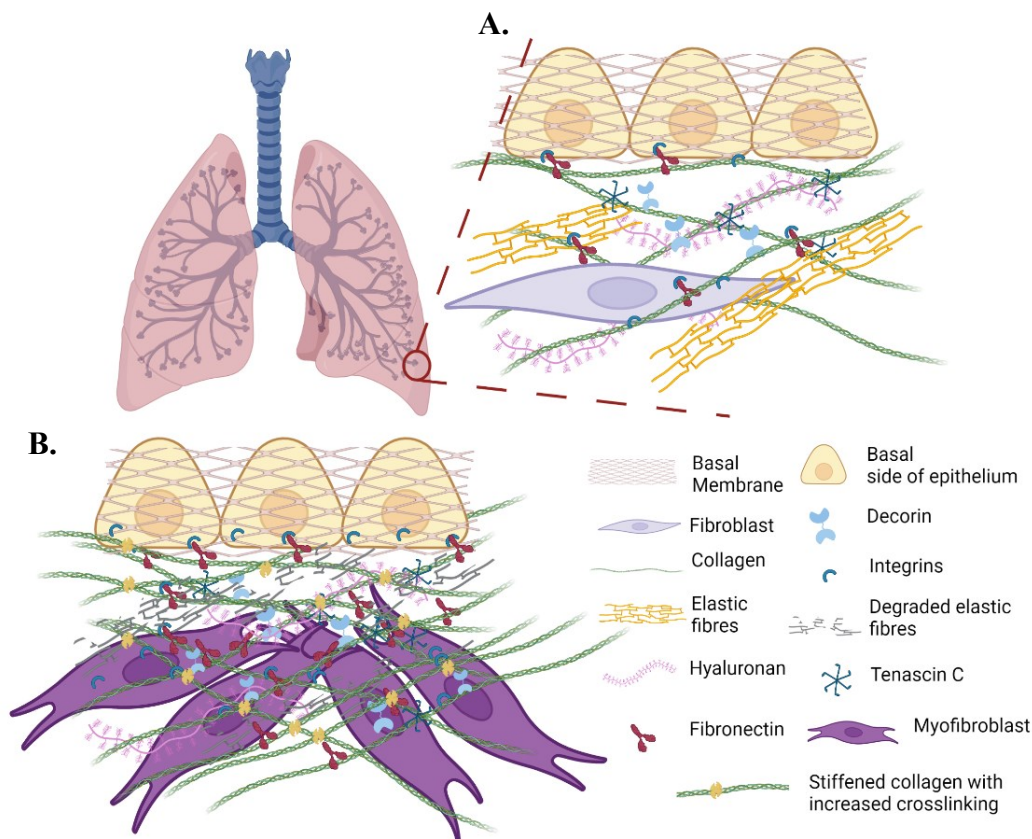


Figure 1.6: Image comparison of pathophysiological ECM structural changes from (A) healthy to a (B) IPF lung. Increased deposition of fibrillar collagen is seen, as well as the presence of myofibroblasts and other ECM associated proteins. Reproduced schematic from (Burgstaller et al., 2017), with permission of the ©ERS 2022 and created in Biorender.com.

The composition of the ECM proteins produced by fibroblasts and myofibroblasts varies as IPF progresses. Collagen III is the main type of fibrillary collagen produced during the early stage of the disease. However, this is then overtaken by the secretion and crosslinking of collagen I during progression to late-stage disease (Rock et al., 2011). This is accompanied by an increase in elastic fibre crosslinking from the developed fibroblast foci, making the lung stiffer than the normal ‘healthy’ lung (Burgstaller et al., 2017, Snijder et al., 2019). Collagen IV is another component of the IPF ECM together with proteoglycans and glycoproteins (Decaris et al., 2014). The mesenchymal cells involved in laying down the IPF matrix originate from multiple sources in addition to EMT such as fibrocytes, pericytes and mesenchymal stromal cells (MSCs) (Rock et al., 2011, Giménez et al., 2017, Snijder et al.,

2019). These progenitor cells, whether originating in the perivascular space or the bone marrow, are associated with further fibrosis and re-enforce the constant cycle of tissue remodelling. The newly remodelled ECM gradually becomes more ‘resistant’ to degradation via regular proteolysis homeostasis. An increase of hydroxyl-bridge crosslinking in collagen I is due to the increase of lysyl oxidase and transglutaminase activity reinforcing the parenchyma’s architecture with more protein interactions like decorin and biglycan (Aumiller et al., 2017, Tjin et al., 2017, Snijder et al., 2019).

Environmental factors can also exacerbate IPF including a failure to stop smoking or exposure to passive cigarette smoke as well as continuous exposure to dust/wood/metal dust that increases the risk of inhalation and therefore cell microinjury within the alveoli (Plantier et al., 2018, Maher et al., 2021). The low survival rate can be ameliorated with the use of some antifibrotic/adjacent drugs. Current options such as FDA approved Pirfenidone[®] and Nintedanib[®] may help to mitigate further exacerbations and the rate of hospitalisation whilst also limiting the presence of the highlighted ECM proteins (Richeldi et al., 2014, Burgstaller et al., 2017, Ley et al., 2017), however novel drugs and therapies are constantly being explored for potential cures, not just palliative treatment with moderate side effects. Pirfenidone is an antifibrotic therapeutic drug, targeting fibrotic characteristics e.g., increased collagen deposition, fibroblast proliferation and TGF- β activity (Ley et al., 2017). However, clinical trials with Nintedanib[®] show reduced efficacy at early-stage disease but helped those disease had further progressed (Kolb et al., 2017, Snijder et al., 2019). As Nintedanib[®] targets the tyrosine kinase activity in VEGF, FGF, PDGF receptors, it can be considered that the inhibitory effect on more active (and dysregulated) growth factor pathways seen in severe cases of IPF from lung remodelling, is therefore more pronounced (Richeldi et al., 2014, Lederer and Martinez, 2018).

1.2.4 Combined Pulmonary Fibrosis and Emphysema- CPFE

An additional pathology has been recognised, embodying the certain phenotypes of both COPD (emphysema) and IPF and identified as Combined Pulmonary Fibrosis and Emphysema (CPFE) (Zhou et al., 2018). In patients with dyspnoea, the upper lobe exhibits hyperinflated emphysematous tissue and the lower lobe and periphery exhibits fibrosis (Cottin and Cordier, 2005, Sugino et al., 2014). The disease can also be accompanied with other co-morbidities such as lung cancer and pulmonary hypertension and is associated with heavy smoking and is more prevalent in males (Hage et al., 2021). Its diagnosis is via CT and its morality rate is high (Sugino et al., 2014). Treatment for this syndrome utilises what is available for IPF and COPD with an emphasis on the need to find new effective anti-fibrotic drugs (Hage et al., 2021).

1.3 Stiffness as a pathological feature?

In both COPD and IPF, changes in the stiffness of the matrix are often regarded as just a by-product of their pathologies. As a result, cell-to-cell interaction has normally taken precedence in research. However, this should not be the case. The stiffness of different tissues is measured in Pascals and the normal lung parenchyma's stiffness sits within a range of 1-7.5 kPa* (*approximation) depending on the location measured e.g. epithelium vs alveoli, large vs small airway walls (Liu et al., 2010b, Booth et al., 2012) and technique used (Polio et al., 2018). In COPD, a spectrum of stiffness is suggested (as discussed further on in this chapter), due to the pathological mechanisms and biomechanics of the disease. The nature of the changes to the alveolar compartment in emphysema is largely due to the loss of elastin and therefore compliance. When breathing, lung recoil of the contractile airways is required to allow for gaseous exchange to take place after regular broncho-contraction and before broncho-relaxation of the airway smooth muscles during cyclic breathing (Suki and Bates, 2008, Suki et al., 2011, Bidan et al., 2015, Zhou et al., 2018).

An interplay between the ECM and airway smooth muscle cells (ASM) occurs as the lung parenchyma including the alveoli stretch in accordance with the transmission of forces from mechanical stimulation (An et al., 2007). In COPD, this chain of events becomes laboured and extended. The viscoelastic nature of the ECM becomes compromised, both at where the ASM cells reside as well as at the alveolar tissue (Bidan et al., 2015). In emphysematous lung, the thickened ASM wall as well as the overall reduction in elastin, the decrease in proteoglycans like biglycan and decorin but an increase in hyaluronan feed the remodelling cycle and exert additional mechanical strain on the parenchyma (Bidan et al., 2015) (**Figure 1.7**). Airway mechanics is, therefore, an important feature in driving the pathology of the disease.

Within the airway

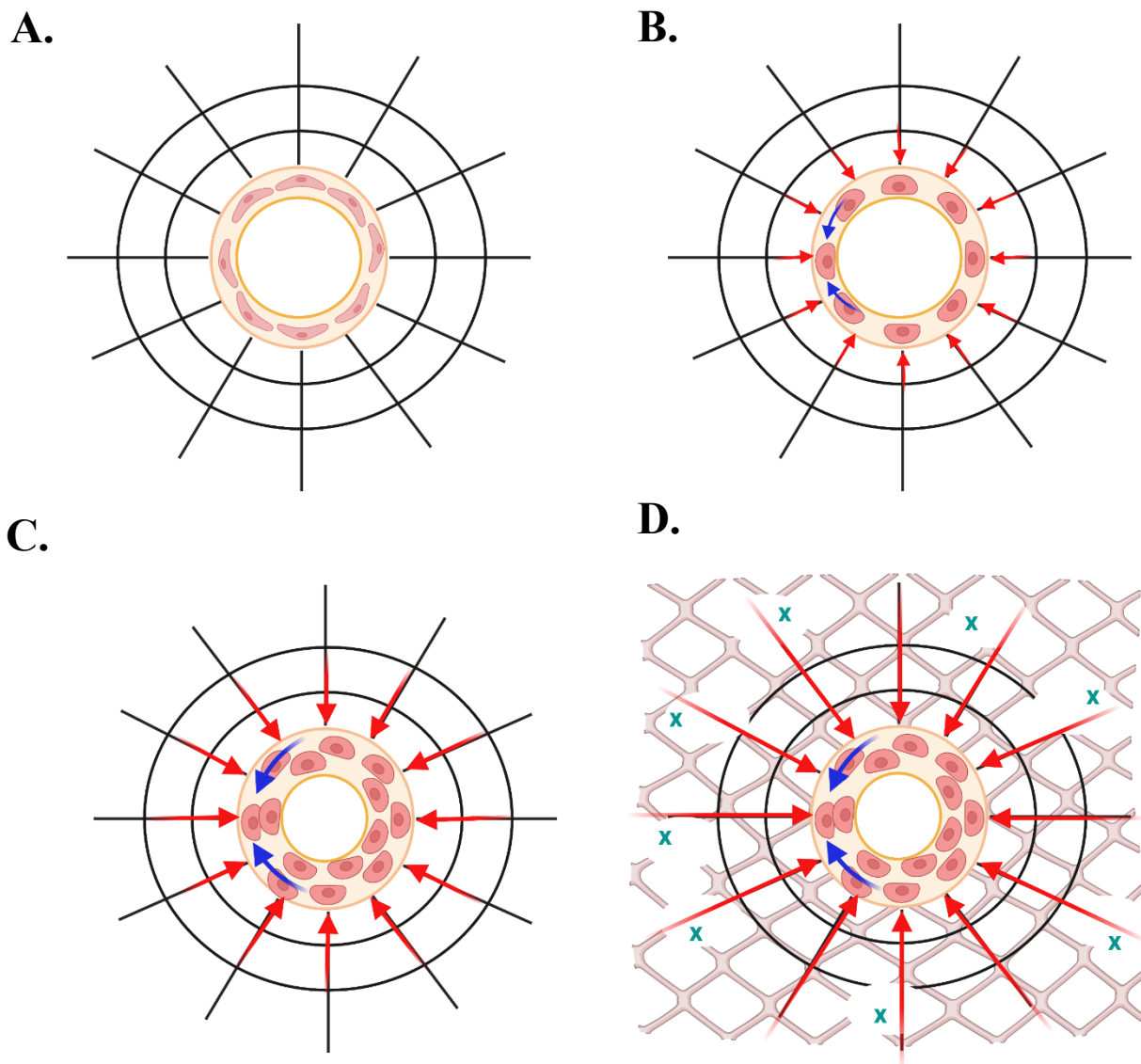


Figure 1.7: Illustration of the mechanical forces on the airway walls in COPD and the effects on the surrounding lung parenchyma. (A) and (B) show the differences between the contractility of breathing (B) and the relaxed nature before and afterwards (A). Airway smooth muscle cells constrict, and the resulting effect is limited to the lung parenchyma in direct radial proximity. Any force experienced and the airway deformation is transient in health but in mild COPD (C) thickened airways from airway smooth muscle hyperplasia and unregulated ECM remodelling is present. Chronic inflammation can also cause sustained hyperresponsiveness and therefore prolonged narrowing of the airways and the broncho-constriction state is constant. (D) displays the sustained mechanical force on the lung parenchyma in severe COPD associated with destruction of the alveolar wall structure or emphysema. With the loss of recoil from elastic fibre degradation, airspace enlargement can develop from architectural airway failure. Red arrows represent the mechanical load exert on the lung parenchyma, blue arrows represent the constriction of the airway smooth muscles, green crosses highlight the loss of the alveolar walls. Created on Biorender.com and reproduced from (Bidan et al., 2015).

Continuous insult to the airways, not only promotes chronic inflammation but also more frequent episodes of bronchoconstriction due to hyperresponsiveness. The airway constriction or deformation caused by the prolonged ASM contraction plus the additional ECM changes ultimately compromises the alveolar wall as the mechanical force is propagated down to the peripheral parenchyma (Yuan et al., 1997, Cavalcante et al., 2005). Structural failure of these walls is caused by the alveolar parenchyma not being able to recover as normal from being stretched during ASM contraction, especially if the stretch is prolonged (Bidan et al., 2015). The ECM as a whole, constantly experiences mechanical loading and normally recovers due to its intrinsic viscoelastic nature (Humphrey et al., 2014). The compromised ECM seen in the alveolar parenchyma in COPD patients subsequently fails, rupturing the alveolar tissue and leading to the hallmark enlarged alveolar spaces in emphysema (Suki et al., 2013, Bidan et al., 2015). A gradient of stiffness is now formed; with a stiffer airway wall from the positive feedback loop of ECM repair and destruction from immune cell activity e.g., macrophages and the presence of MMPs (2, 9, 12). This together with ASM hyperplasia, already narrowing the airway whilst at the distal end, the alveolar parenchyma is looser, less stiff with a modified, non-complicit end composition and weaker structure.

This heterogeneity of suggested stiffness in COPD is also reflective/indicative of changes seen in the ECM composition. Conflicting studies report differences of elastin and collagen content in mild to moderate COPD, with data describing a decrease in both components (Black et al., 2008, Annoni et al., 2012), whilst others demonstrated an inverse trend (Vlahovic et al., 1999, Martin-Mosquero et al., 2006). Severe COPD data has even more of a discordance, with trends of decreased collagen and increased elastin production (although allegedly non-functional) (Deslee et al., 2009, Hogg et al., 2009). There are very few published studies regarding lung stiffness. Assuming the general tenet of the emphysematous tissue decreases the elastic and structural integrity of the alveolar space, computed tomography measurements of the COPD airway wall stiffness during inflation have been reported to be reduced as the severity of the disease increases, compared to healthy subjects (Scichilone et al., 2008). However, the increased mechanical force provided by the now thickened small airways, where the ASM reside, may increase stiffness in its location - with past findings corroborating with such theory (Saez et al., 2000, Bidan et al., 2015).

The difference between IPF and healthy tissue stiffness is vast, with references plotting values in a range of ~10 – 200 kPa (Booth et al., 2012). As fibroblast foci are a distinct feature of IPF and mainly produce fibrillar type I, III and IV collagen, the tensile stiffness of the lung increases with the additional increase of elastic fibres, enhancing and upsetting the normal balance of the lung compliance (Blaauboer et al., 2014). This would presumably result in the requirement for more work to expand the lungs upon inhalation.

Type 2 alveoli epithelial (AT2) cells aid the repopulation of the more dominant type 1 alveolar epithelial (AT1) cells. When there are reduced numbers of AT2 cells, this replenishing cannot occur, resulting in the collapse of the alveolar airspace that will eventually lead to the presence of interstitial fibrosis. This process was first proposed by *Witschi* and colleagues to explain the enhanced fibrosis observed in IPF (Haschek and Witschi, 1979, Snijder et al., 2019). The deposition of collagen by invading fibroblasts and myofibroblasts into the now available airspace increases the stiffness within the alveoli septal wall and hardens the basal lamina in a process called collagen induration (Lutz et al., 2015, Snijder et al., 2019).

Continued remodelling and myofibroblasts' resistance to apoptosis over time ultimately increases the stiffness of the IPF lung with the range well past the upper limit of the young's modulus (YM) of a normal healthy lung (~7.5kPa) (Thannickal and Horowitz, 2006, Kulkarni et al., 2016, Snijder et al., 2019). YM values reported span a broad range according to the exact area of the lung being measured. Probing at the periphery would generate much lower YM values representing a softer lung, if near a collapsed alveolar space prior to collagen invasion than measuring near fibroblastic foci. YM values range from 20kPa to 100kPa or even more, in very extreme cases (Goffin et al., 2006, Hinz, 2010, Booth et al., 2012, Hinz, 2012). These high YM values are not restricted to the lung and the human cornea has YM values quoted as ranging from 2.9kPa to 19MPa using a variety of force measuring techniques including atomic force microscopy (AFM), tensile testing and tonometry (McKee et al., 2011). It is important, therefore, to replicate the same range of YM within *in-vitro* models. Common cell culture techniques employ the use of tissue culture grade (TC) plates and flasks but the corresponding YM of the plastic is not biologically relevant, at 2-4 GPa (**Figure 1.8**) (Butcher et al., 2009, White, 2015). As alluded to above and throughout the rest of this thesis, cells take their cues from their microenvironment and adapt their phenotype accordingly. Typical examples are mesenchymal stem cells, culturing them

on a soft substrate will drive their lineage towards adipocytes but increasing the stiffness of the substrate's YM will switch cellular fate towards chondrocytes or even osteocyte lineage.

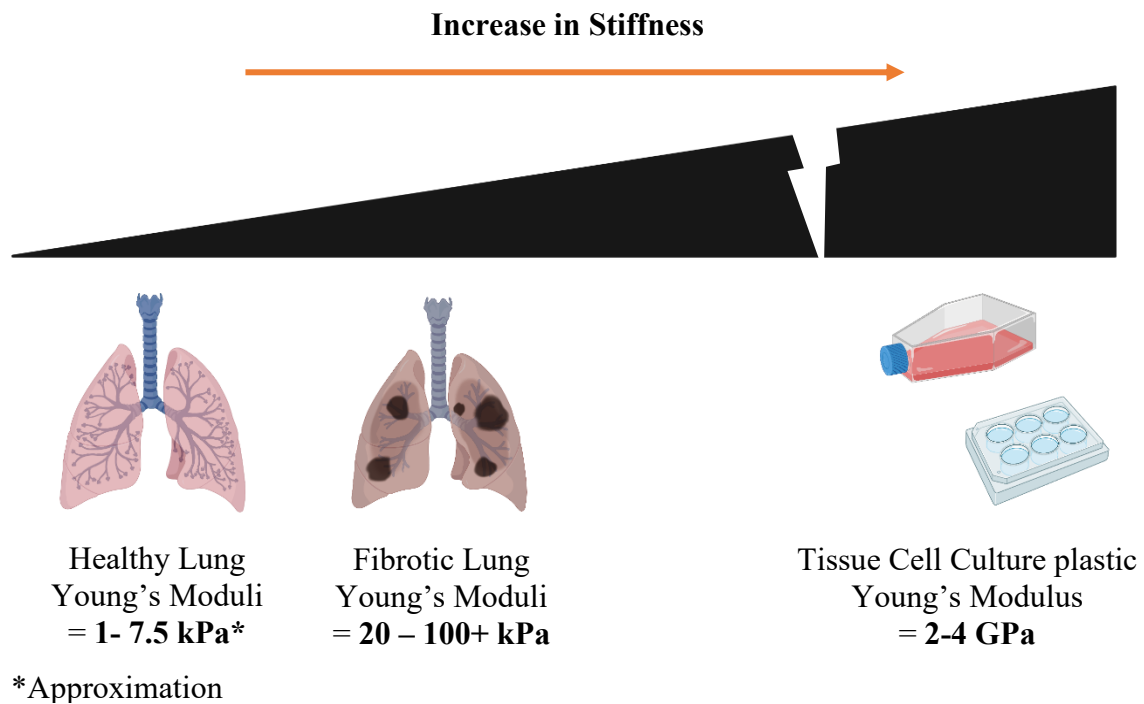


Figure 1.8: Schematic showing the difference in stiffness (Young's modulus, YM) between healthy human lung versus the lungs undergoing fibrosis. A mismatch is seen with the typical tissue culture plastic used, as the YM is several orders of degrees out and therefore not as biologically relevant (Goffin et al., 2006, Hinz, 2009, Hinz, 2010, Liu et al., 2010b, Booth et al., 2012, Hinz, 2012, White, 2015, Huethorst et al., 2022). Created on Biorender.com.

Stiffness, in this project, is measured as a linear relationship between stress (force exerted) over strain (deformation) and reported as the YM (Rodarte, 1982, Greenleaf, 2003, Cavalcante et al., 2005). This can be determined through several means. Within tissue, the stress is labelled as mechanical stress with force like tension, shear or compression experienced over a said area (Sugimura et al., 2016). The deformation is then calculated as change to the specified section as a ratio to the original shape. Although the term YM is used in this chapter and thereafter, the term stiffness should, in reality, be much preferred as a term to broadly describe the forces experienced on tissue/cells/ECM and the mechanical properties inhabited. There are a multitude of ways to determine stiffness and as a result, there are differing values according to the methods used as highlighted by Polio and colleagues (Polio et al., 2018).

As mechanobiology is recognised as an important contributor to disease, AFM is often used, alongside bulk mechanical property testing, like compression testing via rheometer parallel plates. But bulk testing can miss nuances in the material tested. Other methodologies include optical tweezers, optical magnetic twisting cytometry, micropipette aspiration, 3D traction force microscopy (Polacheck and Chen, 2016, Sugimura et al., 2016) and more complex practices; like Brillouin Light-Scattering (BLS) spectroscopy which involves the inelastic scattering of light and the interaction between photons and phonons/magnons (Kargar and Balandin, 2021). There are different techniques for different types (scale) of force e.g. nano and microscale AFM, measurements with fluorescence resonance energy transfer (FRET) based force sensors and measuring tension in organisms or monolayers via laser cutting tissue ablation/tissue resection; considering what each cell feels (at nanoscale) (Polacheck and Chen, 2016, Sugimura et al., 2016, Kargar and Balandin, 2021). With all measurements, it should be considered that most biological tissues are also viscoelastic and therefore the frequency- and strain-dependent values calculated should also include this assumption.

In both COPD and IPF, there is a positive feedback loop whereby the ongoing fibrosis feeds and begets more fibrosis (Parker et al., 2014, Burgess et al., 2016, Snijder et al., 2019). After the initial remodelling occurs, progression of aberrant cell behaviour, phenotype and growth cycle is displayed whilst adaptation to the altered ECM occurs (Snijder et al., 2019).

1.4 *Mechanotransduction pathways*

As one of the lung's major mechanical properties involves recoil to enable breathing to take place, there are many force-sensing proteins present to transduce these stimuli (Suki et al., 2011, Marchioni et al., 2021). These proteins can also be independently regulated, particularly if homeostasis is disrupted via extracellular forces. This section will discuss some of the few proteins and pathways implicated in this phenomenon.

YAP (Yes-associated protein) and TAZ (WW-domain-containing transcription regulator 1) are paralogous transcriptional coactivators, which are part of the hippo signalling pathway. YAP/TAZ bind to transcriptional enhancer factor domains (TEADs) located within the nucleus of a cell (Pocaterra et al., 2020). Once bound to these DNA binding factors, YAP/TAZ can induce proliferation and cell repair/regeneration (Moya and Halder, 2019). Such processes are important during various stages of development including embryogenesis as well as in the response after injury. These coactivators are kept in check through feedback regulation to maintain homeostasis and limit the risk of dysregulated expression resulting in organ hypergrowth and oncological-related pathologies (Camargo et al., 2007). Within the hippo pathway, YAP/TAZ activity is regulated via phosphorylation by a dedicated kinase cascade (serine/threonine kinase 3 and 4 (MST1 and 2) and scaffolding protein salvador family WW- domain containing protein (SAV1) which phosphorylates the large tumor suppressor kinase 1/2 (LATS 1/2). This kinase, alongside its co-activator scaffolding proteins MOB kinase activator 1A and 1B (MOB1A and MOB1B), phosphorylates YAP/TAZ, (Ma et al., 2019, Pocaterra et al., 2020). This phosphorylation tag earmarks the complex for degradation and stops nuclear shuttling when the pathway is switched on (**Figure 1.9**).

However, the same coactivators can be activated via other means, particularly pertaining to cell mechanics. The force the cell experiences can influence YAP/TAZ activity, communicating via cell-to-cell interactions at adherens and tight junctions and by cell-to-matrix interactions by integrins acting as adhesion receptors (Halder et al., 2012, Pocaterra et al., 2020). These examples show that YAP/TAZ are important components of the cell's mechanobiology. Changes in cellular and matrix crosstalk following alterations in the stiffness of the substrate the cells have been cultured on and therefore the microenvironment, or in cells undergoing EMT which disrupts the tight junctions between cells may lead to

increased activation (Cordenonsi et al., 2011, Dupont, 2016). Fibrosis could therefore play a big factor in continuous activation, maintaining the change in stiffness difference.

Vinculin is a cytoplasmic actin binding force-associated protein located within cell-cell and cell-matrix adhesion receptors. Activation of these adhesion receptors can stimulate actin polymerisation as well as recruit further actin proteins resulting in further remodelling (Bays and Demali, 2017). Vinculin itself, is influenced by biomechanical changes from outside of the cell. If dysregulated by changes in ECM mechanics (stiffer ECM), vinculin can influence cellular behaviour further (alongside other actin binding proteins like Talin), having a direct impact on the cellular machinery by propagating any mechanical force sensed (Sun et al., 2016, Goult et al., 2018).

The transcription factor nuclear factor κ B (NF- κ B) is part of a proinflammatory canonical signalling pathway, that is activated in COPD and associated with the increase in chronic inflammation and the drive towards small airway remodelling through fibrotic thickening seen in the disease (Zaynagetdinov et al., 2016). NF- κ B can be influenced via changes in the cytoskeleton as well as the stiffness of the substrata (Zaynagetdinov et al., 2016). The β -catenin protein can also be complexed with E-cadherin. However, loss of E-cadherin during myo/fibroblast *de novo*-synthesis during EMT as well as the occurrence of pro-fibrotic events can lead to free β -catenin and its translocation into the nuclei. This drives the transcription of genes encoding growth factors involved in proliferation, repair, cell polarity and migration (Tian et al., 2011).

β -catenin is linked to the canonical Wnt signalling pathway, with Wnt binding to the transmembrane receptor Frizzled for β -catenin nuclear localisation (Piersma et al., 2015). When the pathway is switched off, dedicated kinases including glycogen synthase kinase (GSK) and casein kinase (CK) regulate the phosphorylation of β -catenin for subsequent degradation (Piersma et al., 2015). However, like the hippo signalling pathway, this pathway can also be stimulated independently by increased contractility and thus, must be considered as part of the mechanobiology of the cell (**Figure 1.9**).

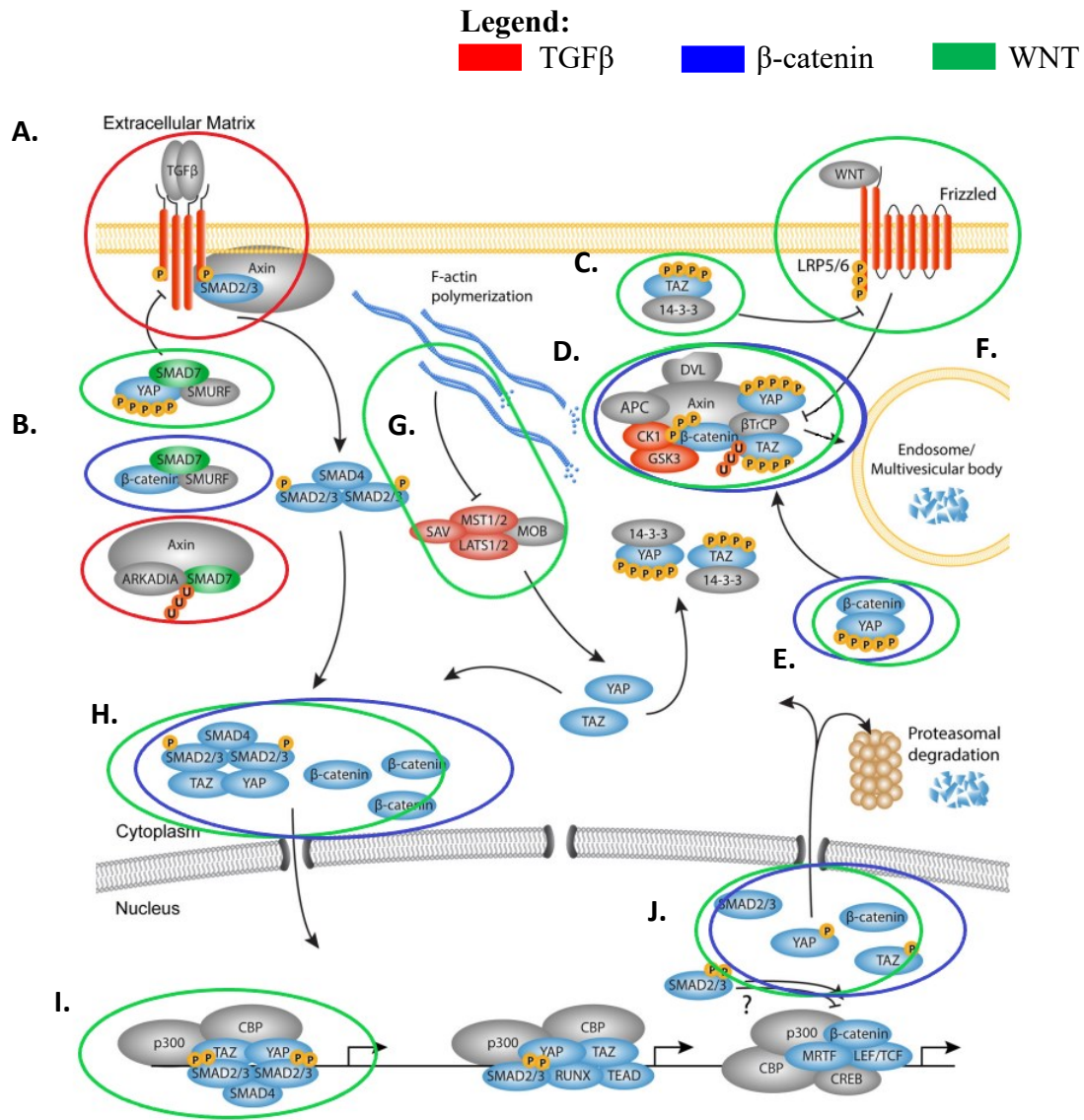


Figure 1.9: Illustration of the many converging points of TGFβ, YAP/TAZ and WNT pathways. Legend colours depict where each transcription or growth factor is present and/may overlap. At (A) and (B), TGFβ stimulation can bind to Axin, leading on to the phosphorylation of the SMAD3 tail and the degradation of SMAD7. YAP and β-catenin can also bind to SMAD7, allowing for the progression of TGFβ signalling and the stabilisation of β-catenin. At (C), TAZ can interact with the WNT pathway by stopping the phosphorylation of DVL (dishevelled) by kinase casein kinase 1 (CK1). At (D), The formation of the phosphodegion in the Hippo signalling pathway occurs, whereby YAP/TAZ is phosphorylated. This complex can also interact with the β-catenin destruction complex. At (E), Phosphorylated YAP can negatively interact with β-catenin, stopping its nuclear localisation. At (F), the WNT signalling pathway can negatively affect the related destruction complex and YAP/TAZ dissociation. Degradation of the same complex then occurs, segregated within multivesicular bodies. At (G), the polymerisation of F-actin can interfere with the dedicated kinases in the Hippo signalling pathway, with YAP/TAZ becoming dephosphorylated. β-catenin also evades destruction and becomes free to act. At (H), the SMAD complex facilitates the translocation of YAP/TAZ, with β-catenin following also from accumulating in the cytoplasm. At (I), transcription factors YAP/TAZ and β-catenin can all aggregate and bind to their specific binding sites. At (J), transcription factors are marked for degradation (phosphorylation) and move back into the cytoplasm. Schematic adapted from reference (Piersma et al., 2015).

Pathways such as TGF β 1, YAP/TAZ and Wnt signalling- are not mutually exclusive and work in tandem to promote fibrogenesis indirectly through stresses experienced by the lung and increased deposition of ECM proteins (*Figure 1.9*). Therefore, studies must consider these components when modelling fibrotic pathologies and seeking novel therapies.

1.5 Organoids

The gradual switch from animal to human cell sources in disease modelling is now frequently utilised, with modelling not only pertaining to the cellular content but to the microenvironment the biological material experiences (Kretzschmar and Clevers, 2016). It is important to recognise the importance of the 3D nature of same and different cell-cell interactions and how changes in the ECM composition can affect cellular behaviour (Kretzschmar and Clevers, 2016).

The shift towards 3D cell culture modelling started from the late 2000s in order to further analyse how one cell type interacts with other associated cells, subtypes and the matrix under *in vivo-like conditions* (Kretzschmar and Clevers, 2016). This interaction between cells can influence the behaviour and phenotype of cells, driving activation of certain pathways, switching off others and therefore should be considered when drawing conclusions based on earlier 2D models. The term ‘organoid’ is used to describe organotypic cultures from a cell source which still regains its pluripotent tendencies to self-organise into native 3D structures (Lancaster and Knoblich, 2014, Kretzschmar and Clevers, 2016). These structures would then crudely reflect the cellular compartmentalization seen *in vivo*, with differentiated cell types. ‘Crudely’ is used to stress that these structures cannot possibly be a true replicate of the organ under investigation (hence the suffix ‘oid’) but represent a simpler biomimicry of key functional subtypes in the correct spatial orientation as seen *in vivo*. The cellular sources are normally primary cells including pluripotent or adult stem cells. Epithelial cells are usually the main cell type used utilising their capacity to repair damaged surfaces but there are now many organoids or 3D cell culture that incorporates 2 cell types or even more, relevant towards development biology (Kretzschmar and Clevers, 2016).

The push in development of these structures originated from the discovery of stem cell niches in the gastrointestinal tract e.g., Lgr5⁺ stem cells (Kretzschmar and Clevers, 2016). The lifespan of organoid culture models is increased by the addition of necessary growth factors that also ensure the expected cell differentiation patterns to take place during the formation of the organoid from the polarised epithelium. Many of these are listed in **Table 1.4** and are taken from a review by Kretzschmar *et al* (Kretzschmar and Clevers, 2016). These include R-spondin1 which can activate the Wnt signalling pathway to maintain

the stem cell phenotype (Lugli et al., 2016, Urbischek et al., 2019), epidermal growth factor (EGF) which regulates cell proliferation (Boonstra et al., 1995), fibroblast growth factor 10 (FGF10) which is also a multifunctional mesenchymal-epithelial signalling growth factor with roles in encouraging cell proliferation and differentiation (Itoh, 2016). Organoids can be used to model a variety of processes; including but not limited to organogenesis, morphogenesis, cell plasticity and the activation of different signalling pathways. Co-culture systems can also be employed, for example being combined with other cell types such as endothelial cells, fibroblasts, immune cells as well as bacteria and viruses for infection assays (Kretzschmar and Clevers, 2016). Over the years, there has been many developments in organoid modelling of multiple organs in the human body that recapitulate specific functions. **Table 1.4** shows a representative list of publications whereby different organoids have been created from primary sources of pluripotent cells (e.g., tissue from biopsies), as well as the necessary presence of set growth factors required to encourage the lineage to a determined organ-specific path.

Regarding respiratory disease, the development of lung organoids has also progressed, creating organoids specific to different areas in the lung, as well as specific cell types present in the lung. For example, **Table 1.5** lists organoids composed of mesenchymal/fibroblast cells as well as spheroids imitating the alveolar structures in the distal lung parenchyma (Gotoh et al., 2014, Wilkinson et al., 2017). Models of cystic fibrosis, COPD and IPF have been created also listed in **Table 1.5**, which can be used not only to look at the cellular behaviour of epithelial cells but also to study the cell-to-cell interactions between complementary cell types including endothelial cells and fibroblasts. My PhD project is based on the model described by *Sachs* and *Hynds* as the *Tracheosphere* model that was published in 2016 (Butler et al., 2016, Hynds et al., 2019). From these protocols, bronchospheres will be cultured as the lung organoids in this *in-vitro* model. When fully matured, bronchospheres present pseudostratified epithelium whilst in a self-assembled 3D spheroid. Differentiated epithelial cell subtypes are formed from the single cell (progenitor basal epithelial cell) seeded, including goblet and ciliated cells recapitulating the airway epithelium with an enclosed lumen. This differentiation process is stimulated by the addition of growth factors and supplements in serum free medium for cell growth (Wu et al., 1997) e.g. insulin for glucose and amino acid utilisation (Morgan et al., 2022), transferrin for iron uptake (Sargent, 2012), epidermal growth factor (EGF) (Hebert et al., 2009) and retinoic acid

(RA) to promote cell proliferation and differentiation (Cardoso and Chen, 2015). Full list of the supplements added to the basal cell serum free media is listed in Chapter 2 (Methods).

Types of Organoids cultured

Organ	Cell source	Key growth/differentiation factors
Gallbladder	Adult stem cells	R-spondin 1, Noggin
Liver	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, Fgf10, A8301 and Notch inhibitor DAPT
Pancreas	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, RSPO1, Noggin, FGF10, Nicotinamide and/or PGE2
Stomach	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, R-spondin1, Noggin, FGF10, Wnt3A, Exendin 4, IGF, p38, GSK(3 β), PGE2
Fallopian Tube	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, RSPO1, Noggin, FGF10, Nicotinamide, TGF β R Kinase Inhibitor IV
Mammary Gland	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, R-spondin1, Wnt3A
Prostate	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, DHT, R-spondin 1, Noggin, A830, FGF10, FGF2, PGE2, SB202190 and Nicotinamide
Salivary Gland	Primary cells (minced tissue from biopsies, containing pluripotent cells)	EGF, FGF2, N2, Insulin and Dexamethasone
Taste Buds	Primary cells (minced tissue from biopsies, containing pluripotent cells)	R-spondin 1, Noggin, jagged-1, EGF, N2, B27, N-acetylcysteine

Table 1.4: A sample list of organoids that have been attempted by various research groups-ranging from organs like the gall bladder to the taste buds. Gallbladder (Lugli et al., 2016), Liver (Huch et al., 2013, Huch et al., 2015), Pancreas (Huch et al., 2013, Boj et al., 2015), Stomach (Barker et al., 2010, Stange et al., 2013, Bartfeld et al., 2015), Fallopian tube (Kessler et al., 2015), Mammary Gland (Linnemann et al., 2015, Zhang et al., 2017), Prostate (Chua et al., 2014, Karthaus et al., 2014), Salivary Gland (Maimets et al., 2016), Taste Buds (Ren et al., 2014). Data from (Kretschmar and Clevers, 2016).

Types of Lung Organoids cultured

Lung Organoids	Cell source	Mechanical feature	Key Features	Disease
Mesenchymal single cell-type	Human fetal lung fibroblasts and iPSC derived fibroblasts	Cell layer using alginate beads as scaffold to mimic distal lung alveolar sacs	Can be used to model cell to cell interactions if other cell types are introduced	IPF
Alveolar epithelial spheroids	Human ‘ventralised’ anterior foregut endoderm cells, co-culture with fetal human lung fibroblasts	Co-culture cell system, with substitute basement membrane (Matrigel)	Investigating enzymatic action on cellular behaviour and phenotype. Cell to cell interaction modelling	-
Human Lung Organoid	Induction of foregut endoderm	3D structure on Matrigel droplet	Alveolar structure and pseudostratified epithelium formed	-
‘Bronchospheres’	Primary human airway basal cells	25% Matrigel prelayer with 5% Matrigel medium	Pseudostratified epithelium formed. Inhibition of Notch2 signalling	Airway Diseases (Asthma, Cystic Fibrosis, COPD)
‘Tracheospheres’	Primary tracheal basal cells	1:1 Matrigel and culture media in Transwell inserts	Basal cell behaviour and phenotype study. Pseudostratified epithelium formed.	-
Airway Organoid	Human lung cell pellets	BME- cell suspension droplets	Pseudostratified epithelium formed. Viral infection and CFTR functional assays performed	Cystic Fibrosis
‘Tracheospheres’	Human Bronchial Epithelial Cells (with 3T3 co culture beforehand)	25% Matrigel prelayer with 5% Matrigel medium	Pseudostratified epithelium formed. Assessment of scalable cell culture	Possible clinical grafts suitability

Table 1.5: Summary of lung organoids protocols from research groups, focusing on different sections of the lung. Mesenchymal cell type (Wilkinson et al., 2017), Alveolar epithelial spheroids (Gotoh et al., 2014), Human lung organoid (Dye et al., 2015), ‘Bronchospheres’ (Danahay et al., 2015), ‘Tracheospheres’ (Rock et al., 2009), Airway Organoid (Sachs et al., 2019). The last row is used as a basis for the organoids created in this project (Butler et al., 2016).

1.6 Overarching Hypothesis

My project investigates the relationship between the matrix and the cells that reside within. Respiratory pathologies such as COPD and IPF have matrix stiffnesses which are greatly increased compared to that of the human healthy lung. It is proposed that bidirectional communication occurs at the cellular level with both having an influence on lung behaviour and phenotype. As fibrosis is one of the major key features found in both diseases and it progresses due to a positive feedback cycle, I hypothesise that the airway stiffness present in the matrix is a regulator in its own right, influencing the human primary bronchial epithelial cell function and impacting upon disease processes.

This thesis will explore the aims taken to prove the validity of this hypothesis, centring around the use of an *in-vitro* bronchosphere model and biosynthetic matrices with the specific aims to:

- **Develop photoinducible mechanically controlled 3D culture matrix**

The fabrication of the biosynthetic matrix will be studied, specifically looking at the methodology of photo-crosslinking, to mechanically define the 3D (lung organoid) culture matrix for this model. Many photoinitiator reagents will be tested, alongside the corresponding light wavelengths to allow for dynamic photocontrol of the matrix stiffness.

- **Examine the effect of altered gel stiffness on epithelial cell differentiation**

Bronchospheres created from normal human bronchial epithelial cells will be subjected to the proposed new matrix. These bronchospheres will be embedded and monitored under a time course assay and encapsulated under varying defined stiffness. RT-qPCR will be used to determine the effect of stiffness on the differentiated epithelial cells. Studies will investigate gene changes at the bench level and additionally at the single cell level using single cell (sc)RT-qPCR. Other techniques like immunocytochemistry will help visualise the pseudostratified layer encapsulated in the gel scaffold and in parallel controls. Studies will compare the responses between bronchospheres derived from healthy subjects and patients with COPD.

- **Study the differential effect on COPD epithelial cells/how COPD cells affect mechanoresponses**

Bronchospheres will be derived from both healthy subjects and patients with COPD. The effect of different matrix stiffnesses on the activation of mechano-sensitive pathways will be compared in bronchospheres derived from both groups.

- **Investigating the mechanisms of mechanotransduction**

Sections of the lung undergo stiffening during fibrosis, which is sensed and propagated by mechanosensitive proteins and signalling pathways. An example is the Hippo signalling pathway highlighting the transcriptional cofactors YAP/TAZ as well as the Wnt/ β -Catenin signalling pathway. Semi-quantitative methods including Western Blotting and genomic expression analysis using scRT-qPCR will be used to investigate the presence of such force sensing proteins together with confocal imaging after whole-mount bronchosphere antibody staining.

- **Determine the suitability of epithelial organoids as models of COVID-19**

The organoids created, can also be used in additional assays relating to the lung. As the differentiated cell types are present, exposure assays to bacteria and viruses like COVID-19 can be conducted. Investigating the entry points of viral attacks, on the cells can be done, beneficial for understanding the nature of the respiratory attack within 3D models instead of traditional 2D culture. This involves inverting/flipping the bronchospheres inside out so that the mucociliary cells are outward facing.

Chapter 2:

Methods

2.1 Cell culture

2.1.1 Bronchial epithelial cell culture

Primary human bronchial epithelial (HBE) cells were either purchased from Lonza (*Basel, Switzerland*), or thawed from the NHLI Airway Disease liquid nitrogen biobank. The bronchospheres were cultured, based on existing protocols to culture both tracheospheres and bronchospheres (Butler et al., 2016, Güney, 2018, Hynds et al., 2019). HBEs were thawed from liquid nitrogen by adding the vial to a 37°C water bath for 2 minutes- until a small crystal remained in the vial. Cells were added to the growth arrested 3T3 monolayer, pre-cultured in a T75 flask, with 10ml of complete co-culture medium (**Table 2.1**). Cell culture was washed with phosphate buffered saline (PBS) after 24 hours, to remove any remaining freezing reagent and media was changed every 2 days until colonies reach 80% confluence.

Reagent	Volume (ml)	Manufacturer
<i>Dulbecco's Modified Eagle Medium (DMEM)</i>	500	Sigma Aldrich/Merck, Dorset, UK
<i>Fetal Bovine Serum</i>	50	ThermoFisher, Paisley, UK
<i>L-glutamine</i>	5	Sigma Aldrich/Merck, Dorset, UK
<i>Penicillin-streptomycin</i>	5	Sigma Aldrich/Merck, Dorset, UK
DMEM mix*	373	-
Ham's F-12 Nutrient Mix	125	ThermoFisher, Paisley, UK
Hydrocortisone/EGF	0.5	Sigma Aldrich/Merck, Dorset, UK
Y-27632	0.5	LKT labs, Minnesota, USA
Cholera toxin	0.0043	Sigma Aldrich/Merck, Dorset, UK

*DMEM, FBS, L-glutamine and Pen-strep mix was made first, with 187ml removed before continuing to make the differentiation medium.

Table 2.1: List of reagents for co-culture medium. Table details from references (Butler et al., 2016, Hynds et al., 2016, Hynds et al., 2019).

2.1.2 Cell Stocks

Cell stocks (**Table 2.2**) were purchased from Lonza (*Lonza, Basel, Switzerland*) and Promocell (*Promocell, Heidelberg, Germany*) or from the liquid nitrogen cell bank housed at the NHLI (*Imperial College London, London, UK*). Normal Human Airway Smooth Muscle cells (NHASMs) were kindly gifted by Dr Charis Charalambous.

NHBE Donor	Lot	Tissue Acquisition Number	Age	Sex	Race
1	18TL215671	36311	47	F	H
2	18TL127524	35773	69	M	C
3	0000646466	33261	38	M	C
4	404Z008	29910	65	F	C
NHLF Donor	Lot	Tissue Acquisition Number	Age	Sex	Race
-	0042105.2	C-12360	65	F	C
CHBE Donor	Lot	Tissue Acquisition Number	Age	Sex	Race
1	18TL179343	36100	69	M	C
2	18TL204559	36221	54	F	B
3	0000440551	28154	63	M	B
4	0000407341	27425	76	F	C

Table 2.2: List of donors for normal human bronchial epithelial cells (NHBEs-healthy), COPD derived bronchial epithelial cells (CHBEs) and normal human lung fibroblasts (NHLFs) used. No cells from healthy smokers were included in the NHBE cohort. Legend for race: B-Black, C-Caucasian, H-Hispanic.

2.1.3 Feeder layer

NIH/3T3 fibroblast feeder layers are cultured and maintained alongside bronchial cell culture (Hynds et al., 2016). 3T3 fibroblasts cells were purchased from ATCC (*ATCC, Virginia, USA*) and stored in the NHLI Airway Disease liquid nitrogen biobank. Vials were thawed at 37°C in a water bath and seeded in a T75 flask, with feeder layer-dedicated media (**Table 2.3**). Cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Sigma Aldrich/Merck, Dorset, UK) after 24 hours and media was changed every 2 days until 80% confluent. Cells were passaged and expanded into T150s.

Reagent	Volume (ml)
Dulbecco's Modified Eagle Medium (DMEM)	500
Bovine Serum	50
L-glutamine	5
Penicillin-streptomycin	5

Table 2.3: *List of reagents for 3T3 medium. Table details from references (Butler et al., 2016, Hynds et al., 2016, Hynds et al., 2019).*

Feeder layers needed to be growth arrested before the addition of epithelial cells. Mitomycin C was used (final concentration at 4µg/ml) and added to a confluent layer of 3T3s for 2 hours at 37°C (Hynds et al., 2016). Once passaged, cells were reseeded at a density of 20,000 cells/cm².

2.2 *Passaging of cells*

2.2.1 3T3s

Cells were passaged using 0.25% Trypsin/EDTA (*Lonza, Basel, Switzerland*). After thaw, cells were passaged after reaching 70-80% confluence in monolayers. Cells were then incubated for 5 minutes at 37°C with Trypsin/EDTA, before neutralising with medium and centrifugation to pellet at 200xg for 5 minutes. Cells were passaged in T150s at 1:6 and at 1:10 thereafter.

2.2.2 Bronchial epithelial cells

Cells were passaged using 0.25% Trypsin/EDTA after reaching 80% confluency in colony formation. A double trypsinisation step was required to remove the 3T3 feeder layer first, before addressing the bronchial epithelial colonies. The first incubation step consisted of 2 minutes at room temperature (RT), placing the flask under a brightfield microscope to monitor the morphology of the feeder monolayer. As the incubation period ended, edges of the mouse fibroblasts 'rounded' up into the medium, forgoing their usual spindle and oblong-like structure. A couple of firm taps to the flask released most of the cells from the inner flask surface. The new suspension was aspirated, followed by a PBS wash. The remaining epithelial cells were resubmerged with 0.25% Trypsin/EDTA and incubated for 5 minutes at 37°C. Tapping the flask agitated the cells and created a suspension. Cells were neutralised with medium and centrifuged in a 50ml Falcon tube, at 200xg for 5 minutes. Reseeding was done in a T75 flask containing a growth arrested 3T3 monolayer, split according to the initial confluence of the previous epithelial colonies.

2.3 Bronchosphere culture

The bronchospheres used in this project were cultured, based on existing protocols to culture tracheospheres (Güney, 2018, Hynds et al., 2019). A suspension of 25% Matrigel® in *optimised* Airway Epithelial Cell Growth Medium, supplemented by the reagents listed in **Table 2.4** from the Growth medium supplement pack (*Promocell, Heidelberg, Germany*), was firstly coated onto the base of ultra-low attachment 96-well plates followed by incubation at 37°C, 5% CO₂ for 20 minutes (Chen et al., 2012, Barkauskas et al., 2017).

Reagent	Volume (ml)	Final concentration
Dulbecco's Modified Eagle Medium	500	-
L-glutamine	5	-
Airway epithelial basal medium	500	-
<i>Bovine Pituitary Extract</i>	2	0.004ml/ml
<i>Epidermal Growth Factor (recombinant human)</i>	0.5	10ng/ml
<i>Insulin (recombinant human)</i>	0.5	5µg/ml
<i>Hydrocortisone</i>	0.5	0.5µg/ml
<i>Epinephrine</i>	0.5	0.5µg.ml
<i>Transferrin (recombinant human)</i>	0.5	10µg/ml

Table 2.4: List of reagents for Bronchosphere culture medium. Table details from references (Butler et al., 2016, Hynds et al., 2016, Güney, 2018, Hynds et al., 2019).

Final medium was at **50:50** ratio of DMEM + L-glutamine and Airway Epithelial Cell Growth medium, completed with supplements listed above in **Table 2.4**. This media was augmented with Retinoic acid (final concentration at 1:5000, *Sigma Aldrich, Dorset, UK*) just before use (Chen et al., 2012, Danahay et al., 2015, Butler et al., 2016, Barkauskas et al., 2017).

Once epithelial cells had been passaged and counted, a suspension of 2500 cells/well (96-well plate) was centrifuged at 200xg for 5 minutes and resuspended in 5ml of bronchosphere medium, with Y-27632 (a ROCK inhibitor, final concentration 5µM, *Cambridge Biosciences, Cambridge, UK*) added when seeding only. 5% Matrigel® was added to the media when seeding and to every feed thereafter. 65µl of cell suspension was added to each well (the middle 24 wells of a 96 well plate, as seen in **Figure 2.1A**). Cells were fed on

day 3, 8 and 14, followed by once a week until day 30. By day 21, 3D lung bronchospheres with lumens and pseudostratified epithelium were formed, depicted in **Figure 2.1B**.

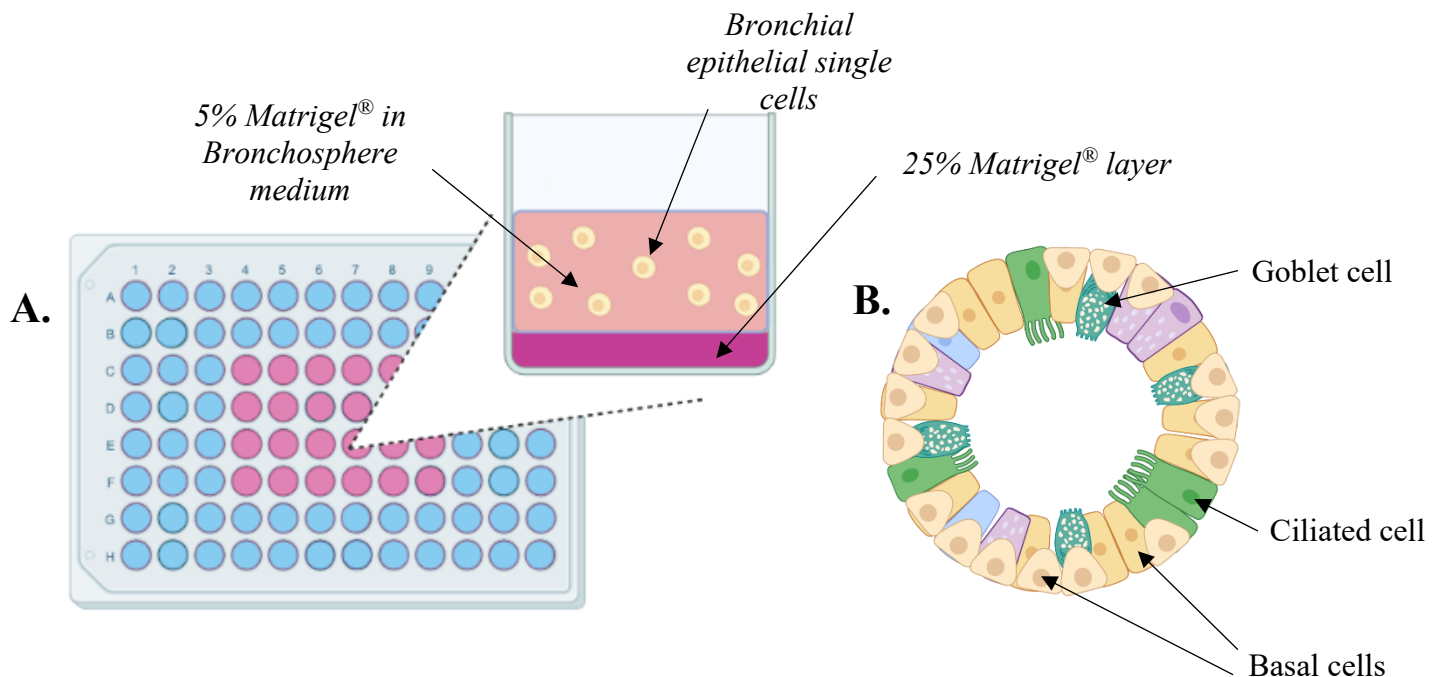


Figure 2.1: Schematic displaying (A) the bronchial epithelial single cell culture in Matrigel®. After 14+ days, single cells proliferated into (B) bronchosphere organoids, with differentiated cells including basal, ciliated and goblet (mucus-secreting) epithelial cells. Created with BioRender.com.

2.4 PMET (PEGDA-Matrigel®- Eosin Y and Triethanolamine) gel fabrication and encapsulation of bronchospheres

The fabrication of PMET gel extends the 25% Matrigel® system described in the previous bronchosphere culture section. The final volumes of the reagents used are listed below in **Table 2.5**, for a 150µl suspension, with the list of reagents followed as a sequence.

Reagent	Volume (µl)	Final percentage concentration	Manufacturer
Culture medium	52.5	35%	See section 2.3, pg.64
PEGDA (Mw 700Da)	30	20%	Sigma Aldrich/Merck, Dorset, UK
Co-initiator (Triethanolamine)	15	10%	Sigma Aldrich/Merck, Dorset, UK
Photoinitiator (Eosin Y)	15	10%	Sigma Aldrich/Merck, Dorset, UK
Matrigel®	37.5	25%	Corning Life Sciences B.V., Amsterdam, The Netherlands
Total	150	-	

Table 2.5: List of reagents for PMET gel suspension

Culture medium containing bronchospheres was gently pelleted (100xg for 2 minutes at RT) and resuspended in 150µl PMET gel suspension media in a 50ml Falcon tube. A pipette tip was then initially cut (under sterile conditions) and used to add PEGDA to the Falcon tube, ensuring reagent homogenisation by pipetting up and down within the culture medium.

After turning off the culture hood's light, Triethanolamine (0.5% v/v in sterile water) was added followed by Eosin Y (0.01% w/v in ethanol) (Fu et al., 2015). Matrigel® was the last reagent to be added to the suspension, to limit the solidification of Matrigel® as the room temperature will initiate the gelation process (Matrigel® must be stored cold and kept on ice just before use).

The 150µl suspension was transferred to a 24-well plate transwell insert. The transwell was moved to a 12-well plate, to ensure plastic-to-plastic contact with the bottom of the plate. The 12-well plate containing the uncured transwell was sealed with parafilm and placed on top of an 10W Pure Green light wavelength (525nm) LED array. Wearing orange lensed safety glasses, the power supply was switched on for 1 minute or 5 minutes at 10W

and at 1 ampere. The exposure of green light initiates the cross-linking reaction (**Figure 2.2A**) due to free radical propagation by the PEGDA monomers resulting in the formation of the PMET gel scaffold. Once completed, the plate was transferred to the hood, the parafilm was removed and the transwell was moved back into a 24-well plate. Bronchosphere medium was added to the transwell and the well, ensuring both sides were submerged in medium. **Figure 2.2B** shows schematically the resulting culture of the bronchosphere, embedded in PMET gel scaffold.

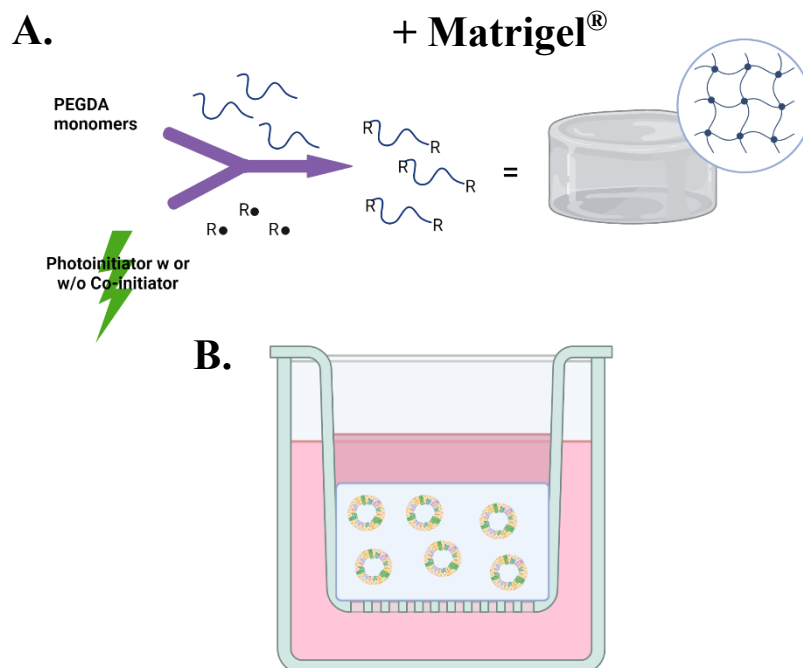


Figure 2.2: Schematic displaying (A) the photocuring mechanism of PMET gel, with free radicals reacting with the acrylate groups at the ends of the PEGDA monomers. (B) Bronchospheres in transwell insert encapsulated in PMET gel after photocuring. Created with BioRender.com.

2.5 RNA extraction

Standard bench RT-qPCR usually involves the use of dedicated pre-equipped kits, such as Qiagen RNeasy kits (*Qiagen, Manchester, UK*). With the presence of the PMET gel, typical protocols were optimised to allow for the manual mechanical homogenisation of matrix before RNA extraction.

An adapted protocol was therefore used instead (Gasparian et al., 2015):

2.5.1 Bronchosphere homogenisation

For the 72 hour RT-qPCR assay, each transwell -containing PMET gel was harvested every 24 hours alongside the parallel 25% Matrigel® cell culture.

2.5.1.1 *1 minute, 5 minutes and PMET gel uncured*

Gel was removed from the 24-well plate by scoring base of the transwell insert with a scalpel and was placed in a 50ml Falcon tube. The gel was then snap-frozen by placing the Falcon tube in liquid nitrogen until the gel was visibly frozen. Using a precooled spatula, the gel was quickly transferred to a pestle and mortar. Liquid nitrogen was added to the mortar and the gel was manually homogenised whilst kept on ice. 1ml of QIAzol (*Phenol*) (*Qiagen, Manchester, UK*) was added to the slurry and transferred to a 2ml glass dounce tissue grinder tube. 100 strokes of the large clearance pestle were initially performed, followed by 100 strokes of the smaller clearance pestle. The QIAzol-PMET gel slurry was incubated at RT for 5 minutes before being transferred to a pre-cooled 1.5 ml Eppendorf tube. The suspension was then snap frozen again and stored at -80°C until further processed.

2.5.1.2 *25% Matrigel® bronchosphere culture*

Bronchospheres were pooled into a 1.5ml Eppendorf tube and pulsed centrifuged to gently pellet the bronchospheres in media. The majority of the supernatant was removed, and the remaining bronchospheres were snap frozen in liquid nitrogen. 1ml of QIAzol was added to the frozen pellet and transferred to a 2ml glass dounce tissue grinder tube. 60-100 strokes of the large clearance pestle were initially performed, followed by 60-100 strokes of the smaller clearance pestle. The QIAzol-PMET gel slurry was incubated at RT for 5 minutes for nuclease inactivation before being transferred to a 1.5 ml Eppendorf tube, precooled in ice. The suspension was then snap frozen and stored at -80°C until further processing was performed.

2.5.2 Phenol-Chloroform extraction

Protocol adapted from adapted from (Toni et al., 2018).

200µl of Chloroform was added per 1ml of QIAzol-PMET gel/QIAzol-bronchosphere suspension in the respective Eppendorf tube and shaken vigorously for 15 seconds, to ensure homogenous mixing before incubating for 3 minutes at RT. Samples were then ultra-centrifuged at 12,000xg for 15 minutes at 4°C. After centrifuging, the colourless aqueous top layer was carefully removed by pipette to leave the phenol and sedimented bottom layer. An equal volume of ethanol (96-100%) was added to the sample in a clean, labelled Eppendorf tube and pipetted to mix thoroughly. The sample was then transferred into a Zymo-Spin™ IC Column (*Zymo Research, California, USA*) according to the Direct-zol™ RNA MicroPrep kit (Zymo Research) protocol. RNA was eluted and measured with a Nanodrop™ Lite spectrometer, with readings of the 260/280 ratio falling between 1.60-2.10 (fluctuations occurred due to traces of phenol). Due to the heavy mechanical homogenisation elements, the average RNA yield was lower than expected.

2.5.3 cDNA synthesis

cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (*Applied Biosystems, Warrington, UK*). Once the thermal cycler program had ended, cDNA was stored at 4°C until required.

2.5.4 Bench RT-qPCR

Replicates of each cDNA sample were probed with TaqMan primers (*ThermoFisher, Paisley, UK*) for the genes listed below (**Table 2.6**):

A.

Gene Symbol	Name	TaqMan Cat. No.
MUC5AC	Mucin 5AC, oligomeric mucus/gel-forming	Hs01365616_m1
MUC5B	Mucin 5B, oligomeric mucus/gel-forming	Hs00861595_m1
KI67	Marker of proliferation Ki-67	Hs04260396_g1
SOX2	SRY-Box transcription factor 2	Hs01053049_s1
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Hs00355782_m1
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Hs00923894_m1
TP63	Tumour protein 63	Hs00978340_m1
FOXJ1	Forkhead Box J1	Hs00230964_m1
TGFB1	Transforming growth factor beta 1	Hs00998133_m1

An average Ct value of 3 housekeeping genes was used:

B.

Gene Symbol	Name	TaqMan Cat. No.
18S	Eukaryotic 18S rRNA	Hs99999901_s1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02786624_g1
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	Hs00265885_g1

Table 2.6: (A) List of TaqMan probes used in RT-qPCR and (B) list of housekeeping genes used for normalisation.

Sample cDNA and TaqMan primer probes were formulated in TaqMan Fast Advanced Mastermix and ran under fast conditions, using the QuantStudio™ 3 Real-Time PCR System. Data analysed was performed using the $2^{-\Delta\Delta CT}$ method and normalised to the average of 18S, GAPDH and EEF1A1 expression.

2.7 *Single -cell genomics*

Two protocols were created for bronchosphere dissociation into single cells, depending on whether the bronchospheres had been embedded into PMET gel or not. Adaptations were from previously published single cell dissociation protocols (O’flanagan et al., 2019, Potter and Steven Potter, 2019).

2.6.1 **Harvesting of bronchospheres from 25% Matrigel® culture/uncured PMET gel**

Bronchospheres were transferred into Eppendorf tubes and gently pelleted at 200xg, 3 minutes at RT. The supernatant was removed and the bronchospheres microcentrifuged to pellet down after washing with PBS. The following reagents were used to create 1ml of protease solution (stored at 4°C) (**Table 2.7**):

Name	Final concentration	Manufacturer
DPBS (-Ca ²⁺ , -Mg ²⁺)	x1	Sigma Aldrich/Merck, Dorset, UK
Protease from <i>Bacillus Licheniformis</i> (Subtilisin A)	10mg/ml	Sigma Aldrich/Merck, Dorset, UK
DNase I	125U/ml	Sigma Aldrich/Merck, Dorset, UK
EDTA (0.5M)	5mM	Sigma Aldrich/Merck, Dorset, UK

Table 2.7: *Reagents list for single cell dissociation protease solution*

Once the protease solution was added, the samples were incubated for 7 minutes on a rotating shaker, at 4°C. After this incubation period, a needle (>21G) and syringe were used to physically aid sample suspension by enzymatic digestion. Another 10 minute incubation period at 4°C was performed followed by a second step of manual homogenisation unless the bronchospheres were originally harvested from uncured PMET gel.

10% FBS in PBS was used to neutralise the protease and bronchosphere suspension within a 15ml Falcon tube before being centrifuged at 600xg, 5 minutes at 4°C. Excess supernatant was removed, and the remaining suspension neutralised with 0.01% BSA in PBS. The eluted suspension was further dissociated by straining through a FACS tube with a cell strainer cap (40µm) and incubated on ice until being separated at the single cell level by cell sorting.

2.6.2 Harvesting of bronchospheres from PMET gel photocured for 1 minute and 5 minutes

Gels containing bronchospheres were removed from transwell inserts and roughly isolated with a scalpel, within a petri dish or a 6 well plate. Sections were added to Eppendorf tubes, along with cold protease solution (without EDTA) before incubation on a rotating shaker for 7 minutes, at 4°C. 5mM of MgCl₂ is also added to the suspension, to initiate the activation of DNase I in the protease solution (Guérault et al., 2010).

A cut P1000 tip was used to disrupt the suspension further physically before centrifuging at 600xg, for 2 minutes at RT. Excess protease solution was removed and substituted for 1ml of Accumax (RT, *Innovative Cell Technologies, California, USA*) per Eppendorf tube. The suspension was then added to a gentleMACS™ C Tube (*Miltenyi Biotec, Surrey, UK*) to further dissociate the gel, using the *brain_03* program on a gentleMACS™ Octo dissociator for 0.5-1 minute only (O'flanagan et al., 2019, Potter and Steven Potter, 2019). The suspension was neutralised with 10% FBS in PBS before pelleted at 600xg, 5 minutes for 4°C. Excess supernatant was removed and resuspended in 0.01% BSA in PBS. The suspension was passed through a 100µm Miltenyi filter before strained through the 40µm cap of a FACS tube and placed on ice.

2.6.3 Amplification of Single cell RNA and cDNA synthesis

As a single cell has minute amounts of RNA in comparison to typical cell pellets, RNA extraction would ideally need to be in unison with cell lysis, to try and preserve as much genetic information as possible. An amplification step was also included, boosting the synthesised cDNA. The protocols used were kindly provided by Dr Michela Nosedà (*Imperial College London, London, UK*) and her lab (Massaia et al., 2018).

A reverse transcription-specific target amplification was performed, using the following reagents to create a master mix, to add to the primer assay mix (**Table 2.8**):

Reagent name	Manufacturer
Cells Direct 2X Reaction mix	Sigma Aldrich/Merck, Dorset, UK
Ambion's SUPERase	Sigma Aldrich/Merck, Dorset, UK
TE buffer	Sigma Aldrich/Merck, Dorset, UK
SuperScript RT/Platinum Taq Mix	Sigma Aldrich/Merck, Dorset, UK

Table 2.8: *Reagents list for the master mix needed for single cell cDNA amplification*

The assay mix was prepared including the list of TaqMan genes (*Table 2.9*) to be investigated:

Gene	Name	TaqMan Cat. No.
KI67	Marker of Proliferation Ki-67	Hs04260396_g1
SOX2	SRY-Box Transcription Factor 2	Hs01053049_s1
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	Hs00355782_m1
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	Hs00923894_m1
TGFβ1	Transforming Growth Factor Beta 1	Hs00998133_m1
MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming	Hs01365616_m1
MUC5B	Mucin 5B, Oligomeric Mucus/Gel-Forming	Hs00861595_m1
TP63	Tumor Protein P63	Hs00978340_m1
FOXJ1	Forkhead Box J1	Hs00230964_m1
EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1	Hs00265885_g1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Hs02786624_g1
HK18S	RNA, 18S Ribosomal	Hs99999901_s1
ACTB	Beta Actin	Hs01060665_g1
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Hs00420895_gH
TBP	TATA-Box Binding Protein	Hs00427620_m1
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Hs02800695_m1
RPL13A	Ribosomal Protein L13a	Hs04194366_g1
YAP1	Yes1 Associated Transcriptional Regulator	Hs00902712_g1
ACE2	Angiotensin Converting Enzyme 2	Hs01085333_m1
MUC2	Mucin 2, Oligomeric Mucus/Gel-Forming	Hs00159374_m1
CEACAM5	CEA Cell Adhesion Molecule 5	Hs00944025_m1
CHGA	Chromogranin A	Hs00900370_m1
KRT4	Keratin 4	Hs00361611_m1
SNTN	Sentan	Hs00419632_m1
CSF3	Colony Stimulating Factor 3	Hs99999083_m1
CXCL10	C-X-C Motif Chemokine Ligand 10	Hs00171042_m1
CTNNB1	Catenin Beta 1	Hs00355045_m1
CRIM1	Cysteine Rich Transmembrane BMP Regulator 1	Hs01070663_m1
DOCK5	Dedicator Of Cytokinesis 5	Hs00227848_m1
VCL	Vinculin	Hs00419715_m1

Table 2.9: TaqMan primer list for the 0.2X assay list needed for single cell cDNA amplification.

The single cell suspension was sorted into individual wells of a 96 well plate via the Aria Fusion Cell Sorter, kindly operated by the FACS sorting facility based at Hammersmith Campus (www.imperial.ac.uk/lms-nihr-flow-cytometry). Once sorted, the seeded cells were immediately amplified via a 22-cycle pre-amplification protocol before being diluted (1:4 dilution factor) and stored at -20°C until further use.

2.6.4 Single cell (sc)RT-qPCR

scRT-qPCR was ran using Fluidigm® Dynamic Array™ IFC chips, with a 48.48 format. The chips when loaded were automated on the Biomark™ HD system (*Fluidigm®*, *California, USA*) and subsequent data analysed on Biomark HD Real time PCR analysis and the Singular Analysis Toolset software (*Fluidigm®*, *California, USA*).

2.7 Bronchosphere cell viability

Due to the green light wavelength (~525nm) causing adverse effects to components of the PMET gel when imaging (e.g., PEGDA and photoinitiators Eosin Y and Triethanolamine), standard live/dead assays using fluorophores in this wavelength could not be used as the autofluorescence of the PMET matrix was also quite prominent. Fluorophores that use this wavelength (e.g., Alexa Fluor 488 or FITC) or cell-permeant dyes (Calcein AM) were therefore not used as a viability marker for the 3D models. Furthermore, the spherical shape of the bronchosphere had an adverse effect on the rate of diffusion for antibodies and cell markers into the middle of the bronchosphere. Instead, a 2D and a 3D cell viability study were performed, looking at reagent toxicity and the maintenance of morphological integrity of the cells as a marker of viability, respectively. The protocols are as follows:

2.7.1 2D cell viability assay

Bronchial epithelial cells, Normal Human Airway Smooth Muscle cells and Normal Human Lung Fibroblasts were seeded into wells of 48 well plates and left to attach for 4 hours. Photoinitiator Eosin Y and co-initiator Triethanolamine were added to the culture media (15ul of each reagent, the same volume required in the precursor PMET solution, see pages 66-67), exposed to green light for 1 minute and monitored over 3 days. The same assay was also repeated and ran in parallel; without the addition of photoinitiators and light (control) and with the addition of green light exposure only. By day 3, the following stains (*Table 2.10*) were used as per manufacturer instructions:

Stain	Viability of cell	Manufacturer
Calcein AM	Live	Tocris, Abingdon, UK
Hoechst 3342	Live and dead	ThermoFisher, Paisley, UK
Propidium Iodide	Dead	Sigma Aldrich/Merck, Dorset, UK

Table 2.10: Cell stains used for live/dead viability assay

2.7.2 3D cell structural integrity assay

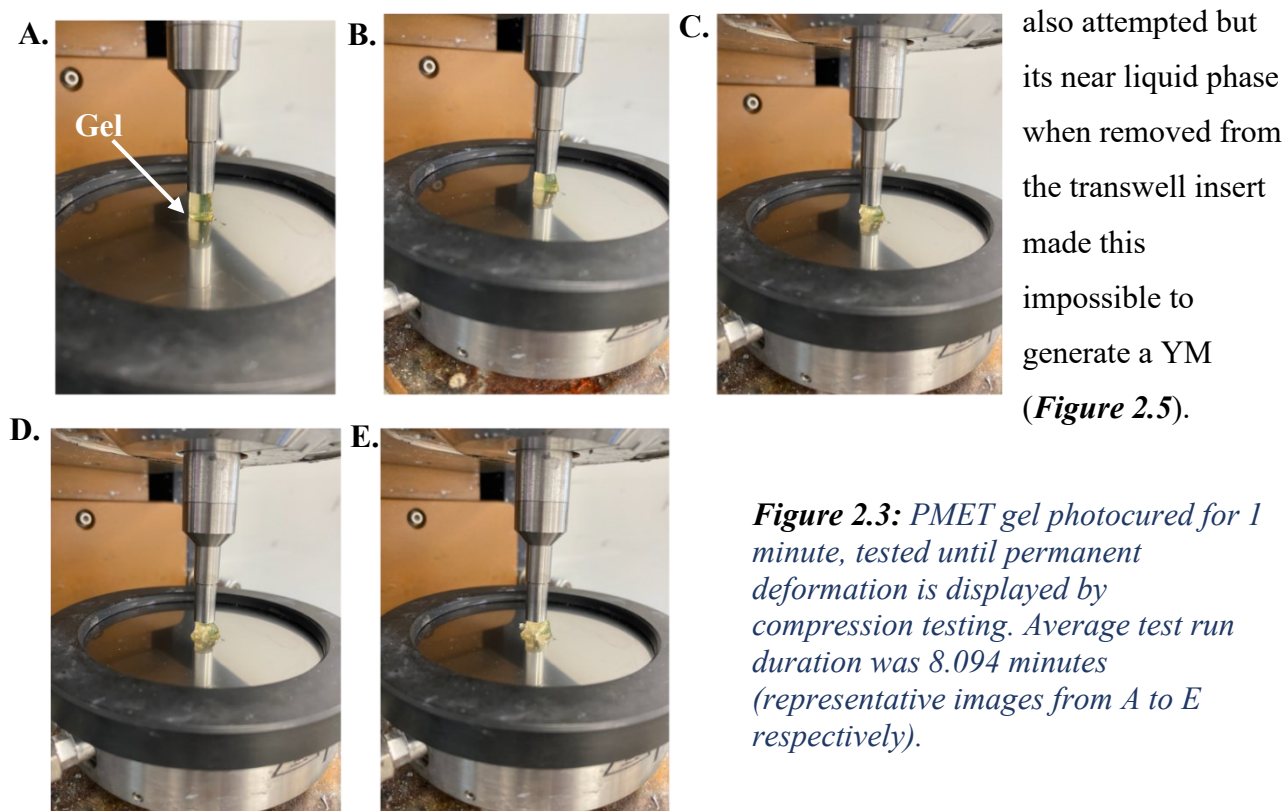
Structural morphology and integrity were used to assess the bronchospheres when encapsulated in PMET gel. Evaluating prior positive (dead) controls, bronchospheres cannot maintain their spheroid shape as the cellular death takes place. The end conformation results in the organoid dissociating, compromising the structure to collapse inwards or rupture into its environment. For this assay, bronchospheres were cultured and embedded into photocured PMET gel (1 minute exposure to green light) as per protocols on pages 66-67.

Bronchospheres were then cultured for 3 days in PMET gel, harvested per day, and fixed with 4% paraformaldehyde (PFA) (*ThermoFisher, Paisley, UK*). DAPI (*ThermoFisher, Paisley, UK*) was used to stain the nuclei of each bronchosphere before imaged via confocal microscopy.

2.8 Gel characterisation

2.8.1 Compression testing for Young's Moduli values

PMET gel was characterised through means of compression testing via rheometer parallel plates. Cylindrical moulded gels were placed under the Discovery Hybrid 10 Rheometer testing probe and linearly compressed at a rate of $8.3\mu\text{m}$ per second until the gel fractured (permanent deformation was seen). PMET gel photocured for 1 minute and 5 minutes were tested, with $N=5$ (graphs of resulting data seen in chapter 3, page 122). **Figure 2.3A-E** shows representative time-lapse images of a compression test run where loading force was increased until the PMET gel photocured for 1 minute collapsed, average test run duration was 8.094 minutes before fracture. Although enhanced cross-linking is presumed with the 5 minute photocured gel, the time to gel collapse was shorter with these gels (**Figure 2.4**) with an average of 5.158 minutes before fracture. Measurements of axial force and ratio between displacement of gel's height to original height during testing were used to calculate stress (calibrated to the gel's geometry, using its cross-sectional area) and strain values. The Young's Modulus (stiffness or YM) for each photocured condition, was then calculated, and the linear relationship between stress versus strain plotted. Testing of 25% Matrigel® was



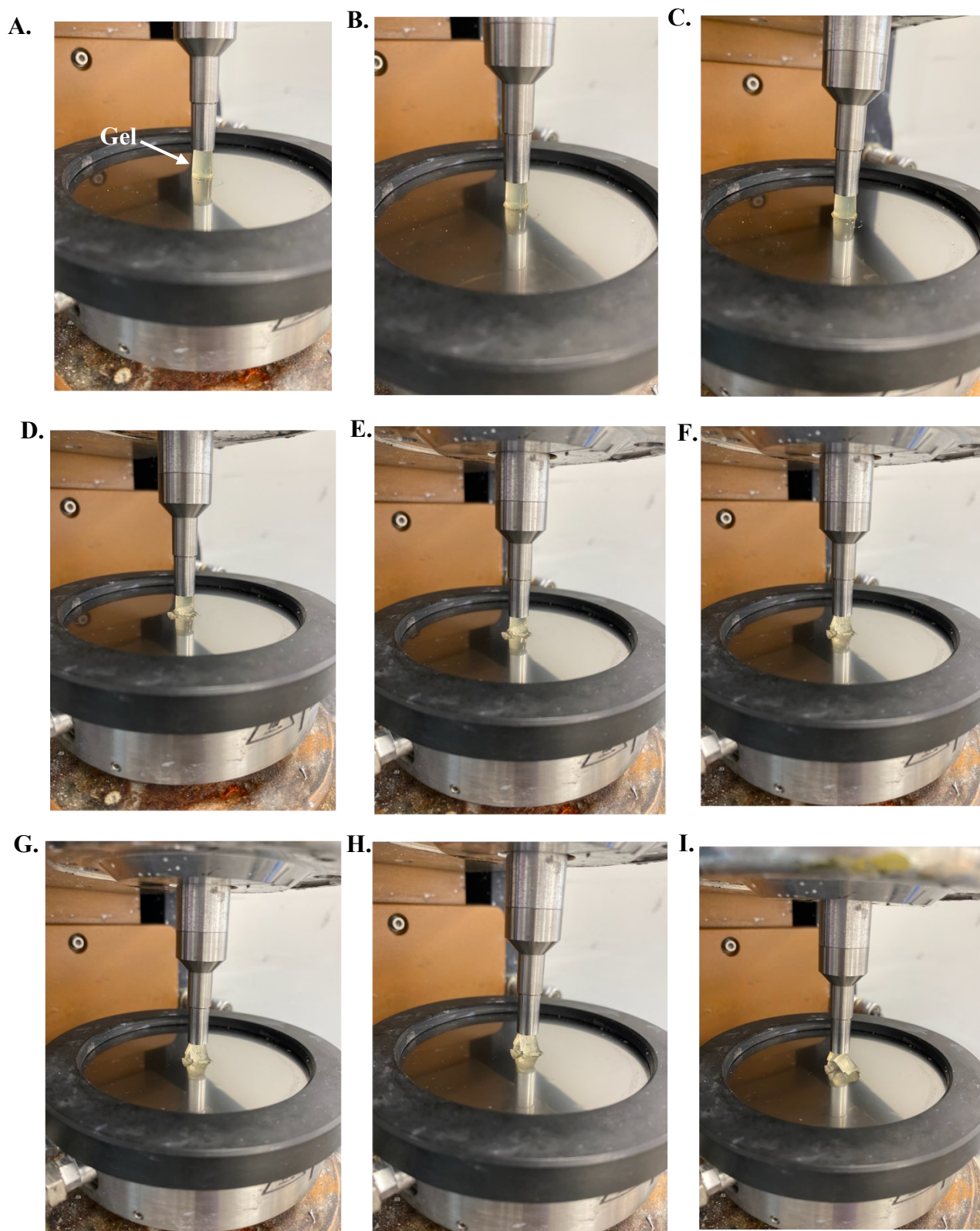


Figure 2.4: PMET gel photocured for 5 minutes, tested until permanent deformation by compression testing over time. Average test run duration was 5.158 minutes (representative images from A to I respectively).

A.



B.

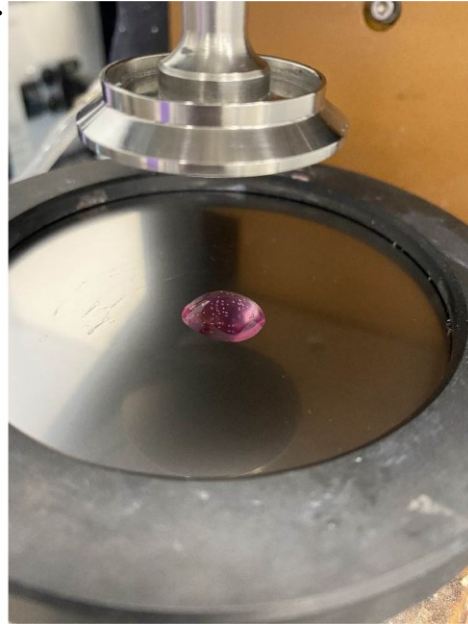


Figure 2.5: (A) 25% Matrigel[®] in transwell insert. Without the plasticware, 25% Matrigel[®] cannot hold form (B).

2.9 Immunocytochemistry (ICC) and imaging of bronchospheres

Typical sample processing for ICC staining and were explored (Abcam, Biologicals) but the presence of the biohybrid matrix created the need for optimisation as the chemistry of the gels were incompatible with the standard paraffin embedding preservation process. Cryo-sectioning was also considered and worked to some extent, however sample slices were blindly processed and stained and did not always provide good cross-sectional images. The following protocol was used and was adapted from recently published protocols involving organoids (Nikolić et al., 2017, Dekkers et al., 2019), following advice from the Facility for Imaging by Light Microscopy (FILM) institute at Imperial College London (www.imperial.ac.uk/medicine/facility-for-imaging-by-light-microscopy).

2.9.1 Whole mount immunofluorescence staining

2.9.1.1 Bronchospheres harvested from 25% Matrigel[®] culture/uncured PMET gel

Bronchospheres were harvested from the culture media, pipetted into an empty 50ml Falcon tube and topped up with cold DPBS. The suspended bronchospheres were pelleted at 200xg for 3 minutes. Excess supernatant was carefully removed and 4% paraformaldehyde (*ThermoFisher, Paisley, UK*) was added to fix the bronchospheres for 30 minutes at RT. Bronchospheres were washed with PBS once more and then stored at 4°C in fresh PBS. The protocol follows the same steps below from this point on as for the bronchospheres harvested from PMET gel.

2.9.1.2 Bronchospheres harvested from PMET gel photocured for 1 minute and 5 minutes

Gels containing bronchospheres were removed from the transwell insert and fixed in 4% PFA for 1 hour, at RT. After washing with PBS, gels were transferred to a 6 well plate, with enough PBS to cover the surface. Under a dissecting microscope, needles (30G) were used to finely dissect the matrix and remove as much gel as possible from the surface of the bronchospheres without damaging the bronchospheres themselves. A Pasteur pipette was used to transfer the bronchospheres to a V bottom 96 well plate and the plate was spun at 1000xg for 1 minute, to pellet the bronchospheres so the supernatant can be easily removed (to allow for subsequent buffer exchange).

Bronchospheres were permeabilised for 1 hour in 0.5% (v/v) Triton-X and washed in washing solution (0.5% (w/v) bovine serum albumin (BSA) (*ThermoFisher, Paisley, UK*), 0.2% Triton-X in PBS). Blocking was done at RT for 1 hour in 1% BSA, 5% normal goat serum (NGS), 0.2% Triton-X in PBS.

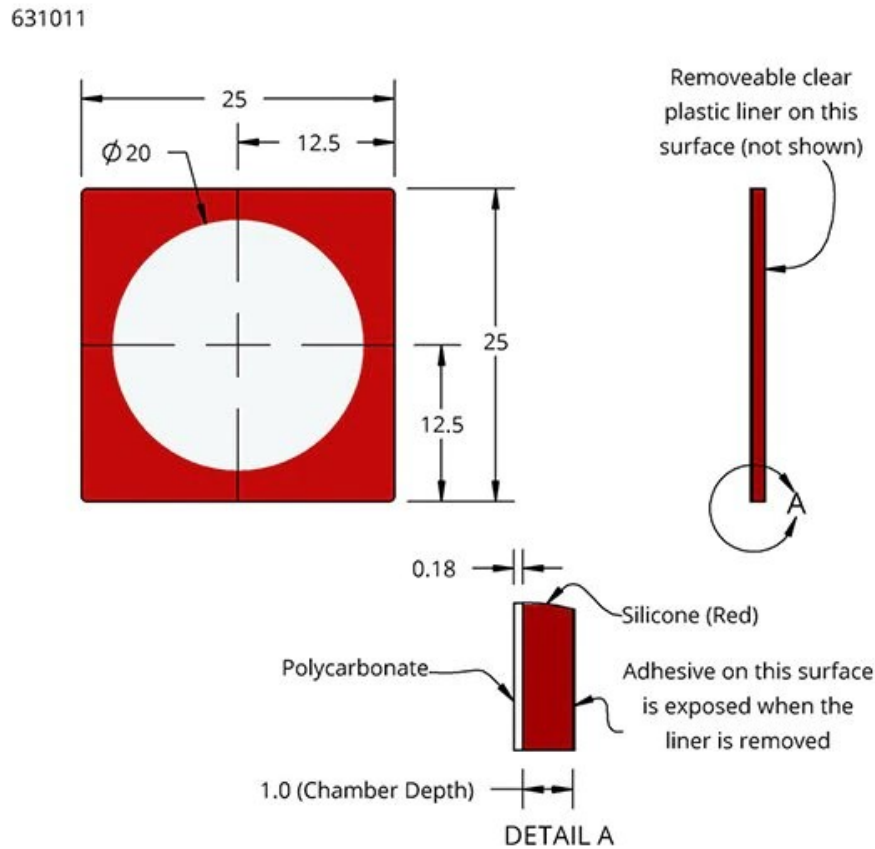
Table 2.11 lists the primary antibodies used for incubation at 4°C overnight in blocking solution. Following 3 x 5 minute washes at 4°C on a rocker with 1000xg spins to pellet the bronchospheres, and secondary antibodies including DAPI (**Table 2.11**) were incubated at 4°C overnight, in 5% NGS, 0.2% Triton-X in PBS. Washings were repeated before the bronchospheres were cleared and mounted with 2'-2'-thio-diethanol (TDE) (*Sigma Aldrich/Merck, Dorset, UK*). 1 hour incubations were initially performed with 10%, 25% and 50% TDE followed by 97% (v/v) TDE in PBS overnight at 4°C on a rocker.

Antibody name	Final Concentration	Manufacturer	Cat. No.
Primary			
Muc5ac	1:100	ThermoFisher, Paisley, UK	MA5-12178
Muc5b	1:200	Elabscience, Texas, USA	EAB-15988-ELA
TP63	1:200	Abcam, Cambridge, UK	ab124762
Acetylated α-tubulin	1:200	Sigma Aldrich/Merck, Dorset, UK	T7451
E-cadherin	1:200	Abcam, Cambridge, UK	ab1416
YAP1	1:100	Abcam, Cambridge, UK	ab52771
Secondary			
Goat anti-mouse AF488	1:500	ThermoFisher, Paisley, UK	A21121
Goat anti-mouse AF568	1:500	ThermoFisher, Paisley, UK	A21144
Goat anti-rabbit AF568	1:500	ThermoFisher, Paisley, UK	A11036
Goat anti-rabbit AF647	1:500	ThermoFisher, Paisley, UK	A27040
DAPI (4',6-diamidino-2-phenylindole)	1:1000	ThermoFisher, Paisley, UK	D1306

Table 2.11: ICC primary and secondary antibodies.

Bronchospheres harvested from 25% Matrigel® culture/uncured PMET gel were mounted in 65µl of 97% TDE onto a glass slide with a Grace Bio-Labs SecureSeal™ imaging spacer (*Sigma Aldrich/Merck, Dorset, UK*) attached and coverslipped. Bronchospheres harvested from PMET gel photocured for 1 minute and 5 minutes were mounted within 180-200µl of 97% TDE, using CoverWell™ Incubation Chamber Gaskets (*ThermoFisher, Paisley, UK*) with the adhesive side bound to a glass slide (**Figure 2.6**). The

optically thin clear side of the gasket allows for detailed images to be captured via confocal microscopy (Leica SP8).



Dimensions are in millimeters and are for reference only.

Figure 2.6: Schematic for Coverwell™ incubation chamber gaskets, used for encapsulated bronchospheres after antibody labelling (Diagram from ThermoFisher, Paisley, UK (Invitrogen, 2022)).

2.9.2 Time-lapsed live imaging of bronchospheres (from 25% Matrigel® culture)

Bronchospheres were transferred to a glass bottom dish, with enough media to keep them submerged. A Zeiss Axio Observer inverted widefield microscope was used to acquire time lapsed videos of inverted and non-inverted bronchospheres at a frame speed of 500/0.01 seconds.

2.10 Zymography

Matrix metalloproteases were also investigated, especially MMP 2 and MMP 9 due to their roles in unregulated tissue remodelling and associations in COPD. Supernatant from the apical side of the bronchosphere gel /transwell culture and the basal side were harvested separately over a 72 hour assay and stored at -20°C until required. Samples were first concentrated with Amicon Ultra-0.5 Centrifugal Filter Units (*Merck/Millipore, Dorset, UK*) before being ran on Novex™ 10% Zymogram Plus (Gelatin) gels (*ThermoFisher, Paisley, UK*), alongside MMP2 and MMP9 protein standards (*R & D Systems, Oxford, UK*). Densitometry was analysed using Image Studio Lite software (*Li-Cor Biosciences, Cambridge, UK*).

2.11 Western Blot proteomics

Protein characterisation was performed using the Pierce 660nm Protein Assay Kit Substrate (*ThermoFisher, Paisley, UK*). 24-27 day old bronchospheres were mechanically disrupted using a dounce tissue grinder set as described previously before being lysed with RIPA lysis buffer (*Sigma Aldrich/Merck, Dorset, UK*). Samples were reduced before gel electrophoresis and analysed with SuperSignal™ West Femto Maximum Sensitivity Substrate (*ThermoFisher, Paisley, UK*) on Image Studio Lite software (*LI-COR Biosciences, Cambridge, UK*).

2.11.1 Total protein normalisation

As the housekeeping genes CT values were seen to be unstable during RT-qPCR, total protein normalisation was used as an alternative normalisation method for the results derived from the Western Blot data. VersaBlot™ CF®770 Total Protein Normalisation kits (*Biotium, San Francisco, USA*) were used, applied to the cell lysate samples before processing. The 800nm channel was used to detect the staining, on the LI-COR® Odyssey® Fc system.

2.12 Inverted bronchospheres

Bronchospheres were harvested from 25% Matrigel[®] cell culture and gently pelleted by centrifuging at 200xg, 3 minutes at RT. Following steps from the protocol (Co et al., 2019), bronchospheres were incubated in 5mM EDTA in PBS for 1 hour, 4°C on a rotating platform. Bronchospheres were then re-pelleted by centrifugation and resuspended in bronchosphere medium but without any Matrigel[®] present. Low attachment tissue culture plastic was used to prevent any adherence of the bronchospheres and incubated at 37°C, 5% CO₂. Inversion, with cilia facing on the outside of the bronchospheres structure was observed by 72 hours. Mucus secretion into the media was also observed at this time (**Figure 2.7**).

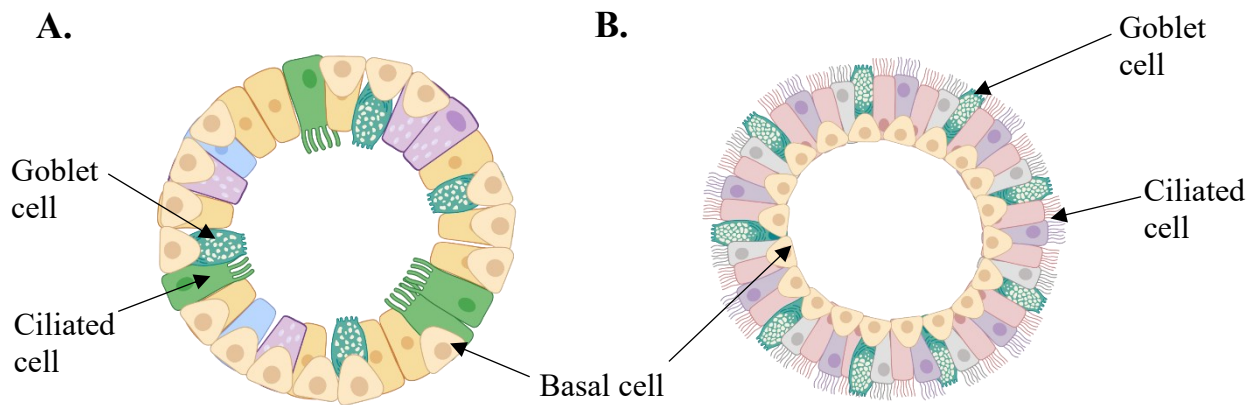


Figure 2.7: *Bronchospheres schematic displaying (A) the apical ‘in’-basal ‘out’ orientation of the bronchosphere when cultured in 25% Matrigel[®]. (B) Inverted bronchosphere, with orientation showing apical side facing out into the media and basal side facing inwards. Created with BioRender.com.*

Chapter 3:

Development of
photocured culture
matrix gel as a model
for varying matrix
stiffness

3.1 *Introduction*

The use of gels has been widely used in many biological applications, in particular hydrogels which are often employed to introduce a scaffold for complex cell culture (Choi et al., 2014, Chang et al., 2016, Greene et al., 2017). They normally consist of hydrophilic polymers or biomolecules that have been crosslinked, providing an architectural 3D support for cells to be embedded within and cultured further.

Different methods and materials/reagents can be used to create hydrogels. For cell culture, an important factor is biocompatibility. The cells embedded or encapsulated must be able to thrive in this new environment without significant impact on cell viability. The pore-size of the network induced by crosslinking must not be too restrictive, so that diffusion can occur. This enables media, or any other reagent, to readily permeate through and waste products can be easily excreted (Alaribe et al., 2016, Nikolova and Chavali, 2019).

Much emerging multidisciplinary research uses a mix of both biological and synthetic reagents to develop a biomimicry of the microenvironment found within the body. Mechanical and chemical properties are also taken in account. The hydrogel's stiffness should be able to be controlled; malleable and compliant to allow long term cell culture if needed, but stable to remove the risk of structures collapsing unless degradable linkers have been added by design (Blondel and Lutolf, 2019).

Moving from 'sol'ution precursor phase to gel phase can be done via different mechanisms, usually categorised under thermal, ionic and photo crosslinking (Gasperini et al., 2014). Thermal crosslinking is aided by either increasing or decreasing the temperature to create a hydrophobic effect. An upper critical solution temperature (UCST) forces the polymers to interact with themselves, when the conditions are below this thermal level (Gasperini et al., 2014). A lower critical solution temperature (LCST), however, has the opposite effect whereby polymers need to be heated or warmed to form a gel (Gasperini et al., 2014). Both attributes have a threshold whereby the polymers no longer interact with water in solution, displaying a change in entropy (LCST), creating a more uniformed structure of the repeated monomer. A change in enthalpy is seen with UCST instead, as thermal energy is lost (Gasperini et al., 2014). Many natural or biological proteins gel using this method e.g., agarose, collagen, gelatin and elastin (Gasperini et al., 2014). Hydrogels created by ionic crosslinking bring opposite charges in close proximity. The polymers used hold a net charge (polyelectrolytes) and crosslink with reagents that are oppositely charged to form an insoluble product e.g., a gel. Polymer-water interactions become secondary as a

result. Examples of such gelation occur with polysaccharides like alginate and chitosan (Gasperini et al., 2014).

Amongst the 3 mechanisms mentioned, thermal and ionic crosslinking are reversible whereas photocrosslinking is not. In photocrosslinking, a photoinitiator is used. When excited by a specific wavelength of light, the photoinitiator molecule is excited and produces free radicals in solution (*initiation*), which in turn reacts with functional groups found on the polymer chain to allow for intermolecular crosslinking (*propagation*), until most, or all, free radical groups have been depleted (*termination*) (Ferreira, 2011). Photoinitiators can be divided into two groups, based on how the reaction proceeds between the solution and gel formation; Norrish type I photoinitiators are able to self-fragment, creating free radicals in order to react with the functional ends of the polymer chain (Ferreira, 2011, Chatani et al., 2014). Norrish type II photoinitiators cannot do this- instead, they require another accessory co-initiator (Chatani et al., 2014). Such co-initiators facilitate the excitation by the donation of hydrogen, forming free radicals for polymerisation to occur. **Figure 3.1** depicts the mechanism that occurs with excitation of electrons when light is absorbed. The schematic of the Jablonski process shows how free radical generation can occur via alternate transitions back to ground state from an excited triplet state (Soofi et al., 2009).

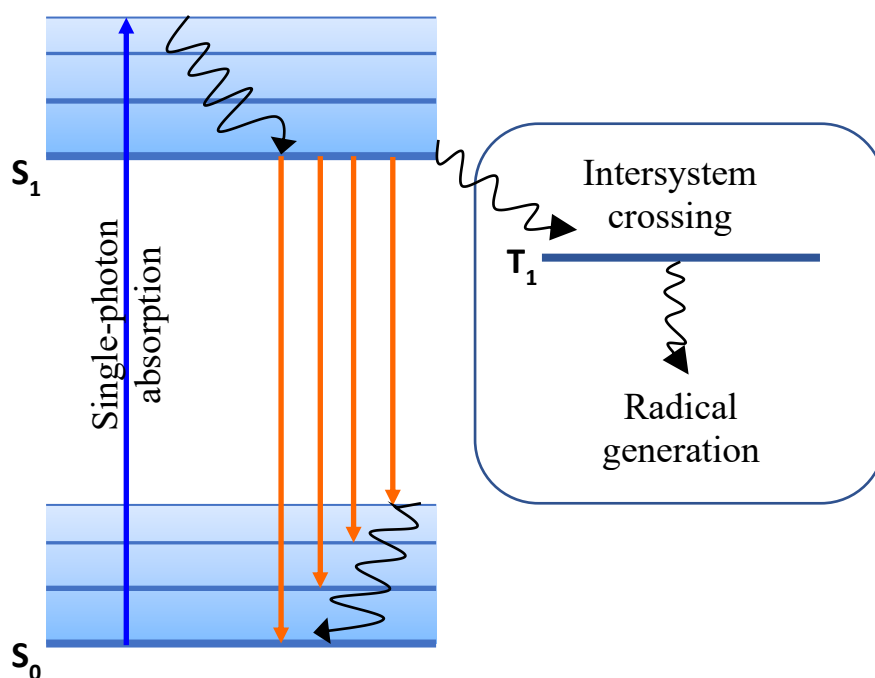


Figure 3.1: Jablonski diagram, demonstrating the photochemical process occurring when light is absorbed. Photons from the selected wavelength excite electrons from S_0 (ground state) to excited singlet state (S_1). Intersystem crossing (ISC) to triplet state (T_1) is an alternative pathway back to ground state, whereby radical generation can occur, forming an intermediary exciplex species instead of traditional heat dissipation/fluorescence back to S_0 . Image reproduced from (Scott et al., 2011, Chatani et al., 2014, Kim et al., 2021).

3.2 Hypothesis

For this project, the hydrogel matrix to be created, should biomimic the human fibrotic lung ECM. From this, properties of the gel would then be comparable to stiffnesses found in pathologies such as chronic obstructive pulmonary disease (COPD). The stiffness should also be able to be fine-tuned to realistically, replicating the more severe fibrotic ECM microenvironment found in idiopathic pulmonary fibrosis (IPF). I hypothesise that by manipulating the hydrogel's stiffness to simulate a fibrotic setting, the effects of this newly remodelled microenvironment in which cells are embedded can identify disease mechanisms.

3.3 Aims

To achieve this objective, the following aims were completed:

- Investigating different reagents to induce photocrosslinking when fabricating the hydrogel; particularly pertaining to the use of photoinitiators and applicable polymers in order to achieve gelation
- Demonstrating mechanical tuneability of gel created, switching mechanical properties at will regarding stiffness
- Characterisation of the proposed hydrogel; measurements of stiffness generated from photocuring method via gel compression (rheometry)
- Biocompatibility of hydrogel; viability of cell culture once encapsulation has taken place through brightfield and confocal imaging (immunocytochemistry)

As this chapter describes technology development, the methods used will be discussed not as a separate section but merged with the results and discussed alongside the analysis. Therefore, the rationale for each step in the methods will also be addressed. Finally, I describe how the final outcome of the protocol was decided and employed to create the hydrogel used within the remainder of this project.

3.4 Methods

3.4.1 Structural components

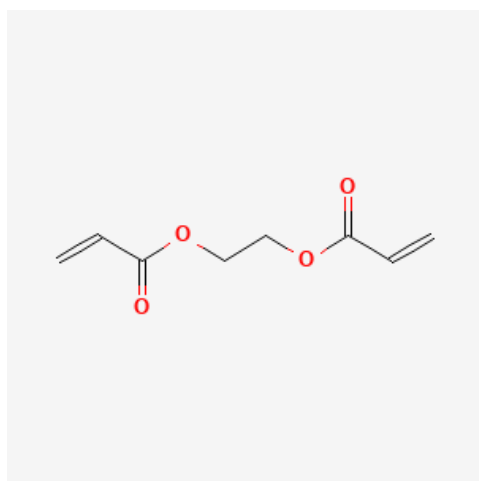


Figure 3.2: 2D structure of Polyethylene glycol diacrylate (Kim et al., 2021)

Polyethylene glycol diacrylate (PEGDA, MW 700Da) will be used within this hydrogel, utilising the acrylate groups found on both ends of the monomer (**Figure 3.2**). It is these groups that are attacked with the free radicals supplied by the photoinitiator (and co-initiator if present), to propagate with other monomer segments and form a polymer (and gel). PEG-based hydrogels are also often used in synthesis of synthetic and biosynthetic hydrogel scaffolds, largely due to its bioinert properties and malleability to further functional modification. The risk of causing an additional inflammatory response is then reduced (Burdick and Anseth, 2002, Roberts and Bryant, 2013,

Aisenbrey and Murphy, 2020).

The final hydrogel complex also involves the presence of Matrigel[®]. Matrigel[®] is a commercially available basement membrane from Corning[™], popularly characterised and used in many 3D cell cultures due to multiple ECM proteins within its composition (Aisenbrey and Murphy, 2020, Corning, 2021). Adding 25% Matrigel[®] to the hydrogel will alter the new matrix properties and hence, has been tested in parallel to investigate whether these induced changes from its addition are significant enough to merit further optimisation (Güney et al., 2021).

3.4.2 Photoinitiator selection

I explored a range of photoinitiators to use within the hydrogel's synthesis, as displayed in **Table 3.1**. Irgacure® 2959 (2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1propanone) is popularly used in biomedical applications (Ferreira, 2011), however the desire to move away from photoinitiators using the UV wavelength is preferred, limiting any potential DNA, membrane and protein damage from exposure (Larsen et al., 2016).

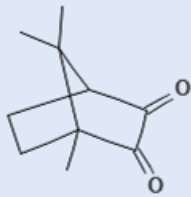
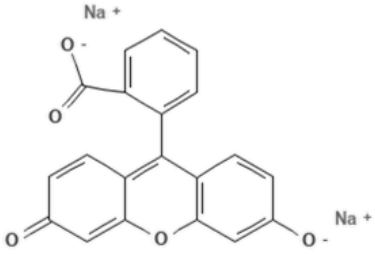
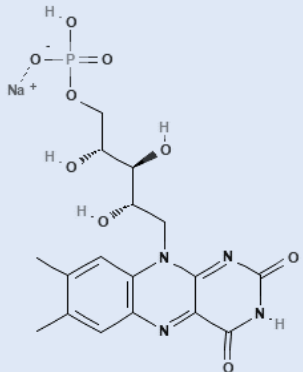
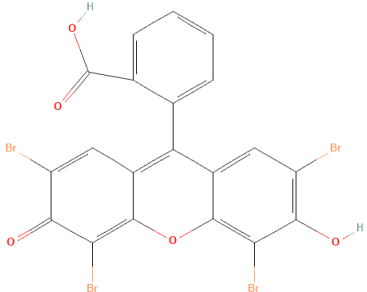
Abbreviation	Photoinitiator		2D Structure
CQ	Camphorquinone	Diketone within structure absorbs in the blue light sector of the visible light spectrum (~450nm) (Hu et al., 2012, Larsen et al., 2016, Kim et al., 2021)	
FR	Fluorescein (disodium salt)	Xanthene dye, requiring the presence of triethanolamine (TEAO) to use as a co-initiator. Exposure of green light from the visible light spectrum (max. absorption at ~494nm) (Hu et al., 2012, Kim et al., 2021)	
RF	Riboflavin (5'-monophosphate sodium salt)	Water soluble flavin (Vitamin B2). Requires the presence of TEAO with blue light from the visible light spectrum (max. absorption at ~460nm) (Hu et al., 2012, Kim et al., 2021)	
EY	Eosin Y	Derivative of Fluorescein. Requires the presence of TEAO with green light from the visible light spectrum (max. absorption at ~524nm) (Fu et al., 2015, Kim et al., 2021)	

Table 3.1: Table showing photoinitiators investigated for hydrogel fabrication. Information and diagrams derived from PubChem (Kim et al., 2021).

Camphorquinone (2,3-bornanedione, CQ)(Kim et al., 2021) is a photoinitiator, commonly used in dentistry for its rapid photocuring in situ. Although CQ is part of the Type II photoinitiator cohort, CQ's structure is an aliphatic α -diketone with two carbonyl groups, both becoming reduced during exposure to blue visible light $\sim$ 450nm (Hu et al., 2012, Kamoun et al., 2016). The use of visible light is advantageous as it can penetrate more deeply than UV. It is also less thermogenic and delivers less cytotoxicity to cells (Fu et al., 2015, Darvell, 2018).

Fluorescein (FR) and riboflavin (RF) are also type II photoinitiators that are frequently used within medicinal applications e.g., vitamin supplements and diagnostic tracers. The organic xanthene dye FR absorbs around 495nm (Hu et al., 2012, Kim et al., 2021), which is shifted more towards the green segment of visible light. Riboflavin also known as vitamin B2, is a ribitol phosphate (Kim et al., 2021). Found naturally in tissues, it absorbs around 330nm as well as 460nm (Fawzy et al., 2012, Hu et al., 2012, Tirella et al., 2012) within the blue segment of visible light.

Eosin Y (EY) is a red crystalline powder, a common dye used in microscopy for haematoxylin-eosin (H&E) staining process. As a derivative of FR and a type II photoinitiator due to its photosensitizing properties, its absorbance sits within the green light wavelength of the visible light spectrum ($\sim$ 524nm) (Fu et al., 2015, Sigmaaldrich.Com, 2022).

To identify the optimal photoinitiator, the 3 candidates (CQ, FR and RF) were titrated in different percentages of PEGDA (molecular weight: 700 Da) in deionised water. 10W LED arrays of the required wavelengths were used at a current of 1 ampere. FR and RF, but not CQ, required a co-initiator to facilitate the reaction. Triethanolamine (TEAO) was chosen based on previously published work (Fu et al., 2015). The tertiary amino group provides the necessary electrons as a photoreductant to the photoinitiator-polymer solution, starting off the polymerisation process by further donating a proton (Stansbury, 2000) and allowing the gelation process to occur (**Figure 3.3**). Eosin Y was also considered and added to the assay at a later timepoint.

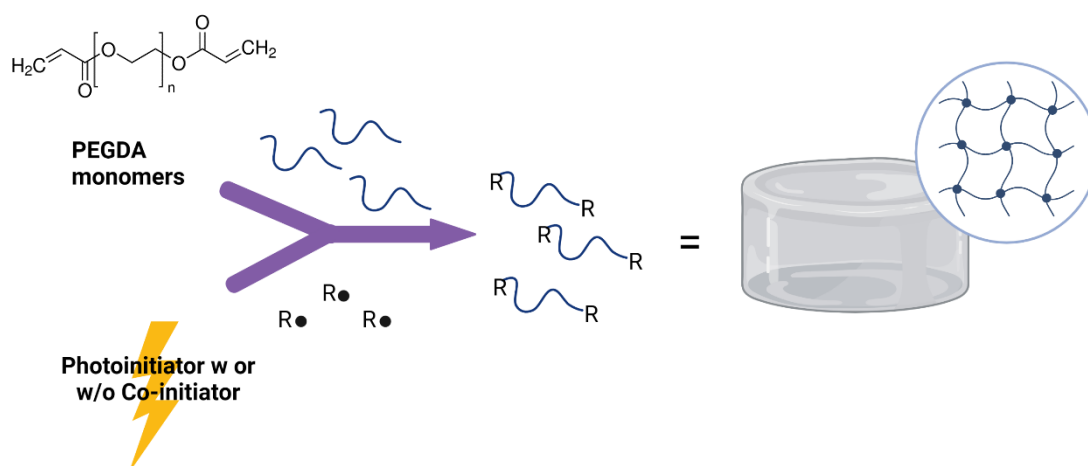


Figure 3.3: Schematic displaying photochemical reaction when light of the correct wavelength is exposed to PEGDA monomers and photoinitiator (with or without co-initiator) in solution. Free radicals (**R**) produced from tertiary amines in situ interact with acrylate end groups on PEGDA monomers and initiate the polymerisation process, forming a crosslinked hydrogel. Created on Biorender.com

3.5 Results

3.5.1 Photocuring times: Camphorquinone (CQ), Riboflavin (RF) and Fluorescein (FR)

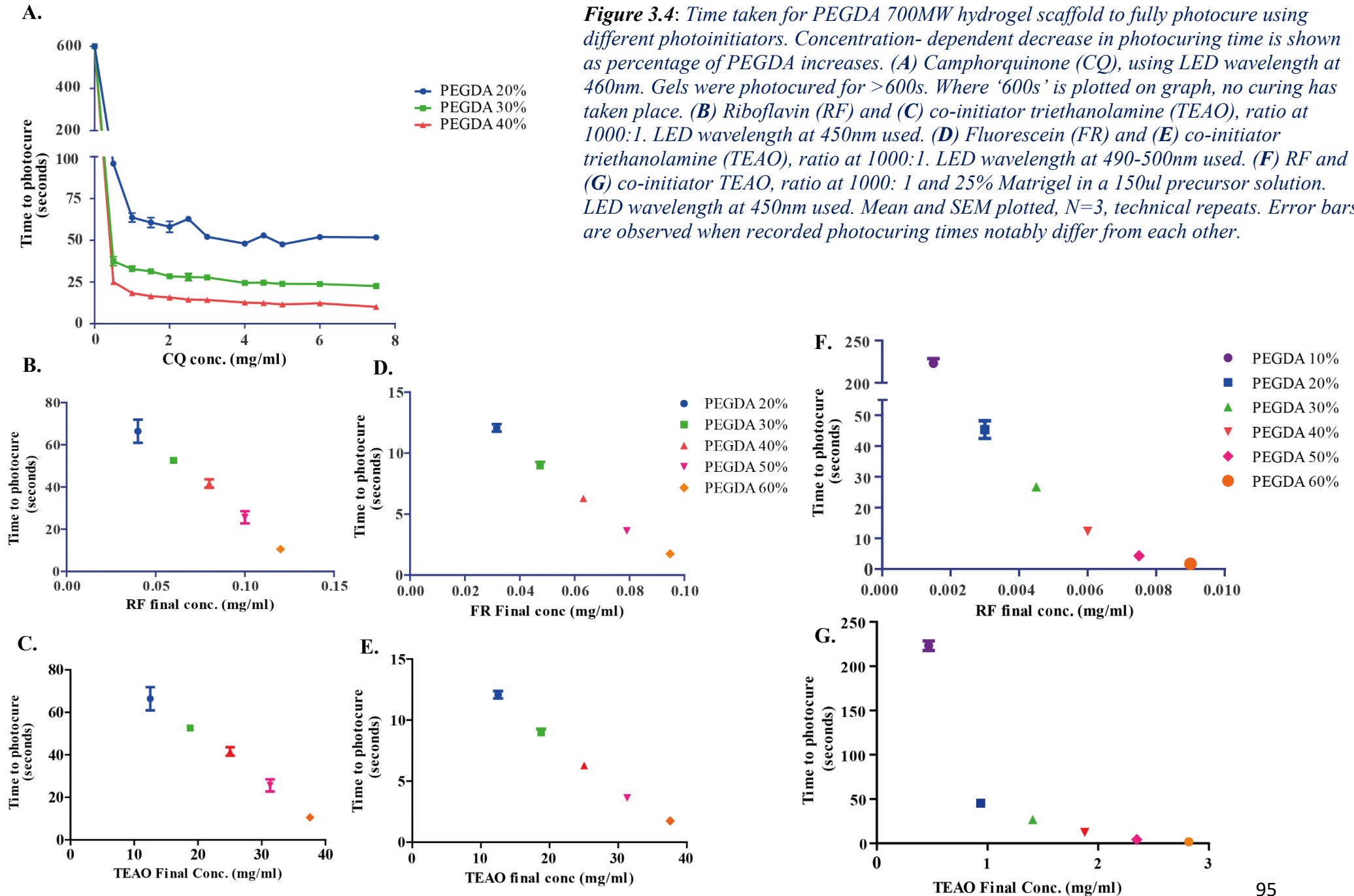
Out of the 3 photoinitiators tested, CQ was the slowest photoinitiator for gelation to occur. The fastest times to photocure was at (average) 10.08 seconds (CQ concentration at 7.5mg/ml) with 40% PEGDA, shown in **Figure 3.4A**. The slowest time to cure was on average 95.84 seconds, with 20% PEGDA and CQ concentration at 0.5mg/ml. Although PEGDA was initially selected for being bioinert, it is preferred to reduce its percentage within the gel's composition as much as possible without requiring excessive photoinitiators. Therefore 20% was used as the chosen limit when testing.

In comparison, RF (**Figure 3.4B and C**) and FR (**Figure 3.4D and E**) photocure gelation timings were much faster. When a co-initiator is present, 50µl of photoinitiator complex is used per 1ml of polymer (Fu et al., 2015). Paired with TEAO, the fastest time for FR to cure was on average 1.75 seconds (FR concentration at 0.095mg/ml and TEAO concentration at 37.6mg/ml, 60% PEGDA). The slowest photocure time was on average 12.09 seconds (FR concentration at 0.0316mg/ml and TEAO concentration at 12.532 mg/ml, 20% PEGDA). The reaction of the FR photoinitiator solution may be too fast to control when processing many gel scaffolds and therefore may not be as consistent every time the gel was made.

The RF and TEAO combinations were initially more favourable, with the slowest photocuring time at an average of 66.46 seconds (RF concentration at 0.04mg/ml and TEAO concentration at 12.53mg/ml, 20% PEGDA) and an average of 10.58 seconds as the fastest photocure (RF concentration at 0.12mg/ml and TEAO concentration at 37.6 mg/ml, 60% PEGDA). The 20% PEGDA combination was long enough to control the gelation process but still relatively short if preparing multiple scaffolds.

Matrigel[®] was also added to the precursor composition and tested when photocuring 150ul of the precursor solution, with cell culture media used instead of water, to reflect the real culture conditions (**Figure 3.4F and G**). As a result, the photocure timings altered slightly to be under 2.30 minutes. RF and TEAO were therefore initially chosen as the photoinitiator (and co-initiator) to use for hydrogel synthesis.

Figure 3.4: Time taken for PEGDA 700MW hydrogel scaffold to fully photocure using different photoinitiators. Concentration- dependent decrease in photocuring time is shown as percentage of PEGDA increases. (A) Camphorquinone (CQ), using LED wavelength at 460nm. Gels were photocured for >600s. Where ‘600s’ is plotted on graph, no curing has taken place. (B) Riboflavin (RF) and (C) co-initiator triethanolamine (TEAO), ratio at 1000:1. LED wavelength at 450nm used. (D) Fluorescein (FR) and (E) co-initiator triethanolamine (TEAO), ratio at 1000:1. LED wavelength at 490-500nm used. (F) RF and (G) co-initiator TEAO, ratio at 1000: 1 and 25% Matrigel in a 150ul precursor solution. LED wavelength at 450nm used. Mean and SEM plotted, N=3, technical repeats. Error bars are observed when recorded photocuring times notably differ from each other.



3.5.2 Photocuring times: Eosin Y (EY) and Triethanolamine (TEAO)

The use of EY and TEAO allows for low phototoxicity towards cells with the use of green light, in comparison to blue or UV light (Fu et al., 2015). The combination is already used in FDA-approved medical adhesives (Fu et al., 2015) and therefore further investigated for suitability with the photocuring system.

Concentrations of EY and TEAO were tested, to find their optimal concentrations (**Figure 3.5**). The fastest curing time was on average 12.06 seconds, at 40% PEGDA, EY conc. at 0.0125mg/ml and TEAO conc. at 0.7025mg/ml. The slowest curing time was on average 18.15 seconds (with 20% PEGDA, EY conc. at 0.0125mg/ml and TEAO conc. at 0.7025mg/ml). This still appears to be rapid, however when including Matrigel® and the bronchosphere medium, the photocuring slows down considerably to an average of 45.36 seconds, which is less time than required to gel than with the RF/TEAO combination. The photocuring time was increased to 1 minute to ensure enough photocrosslinking has taken place.

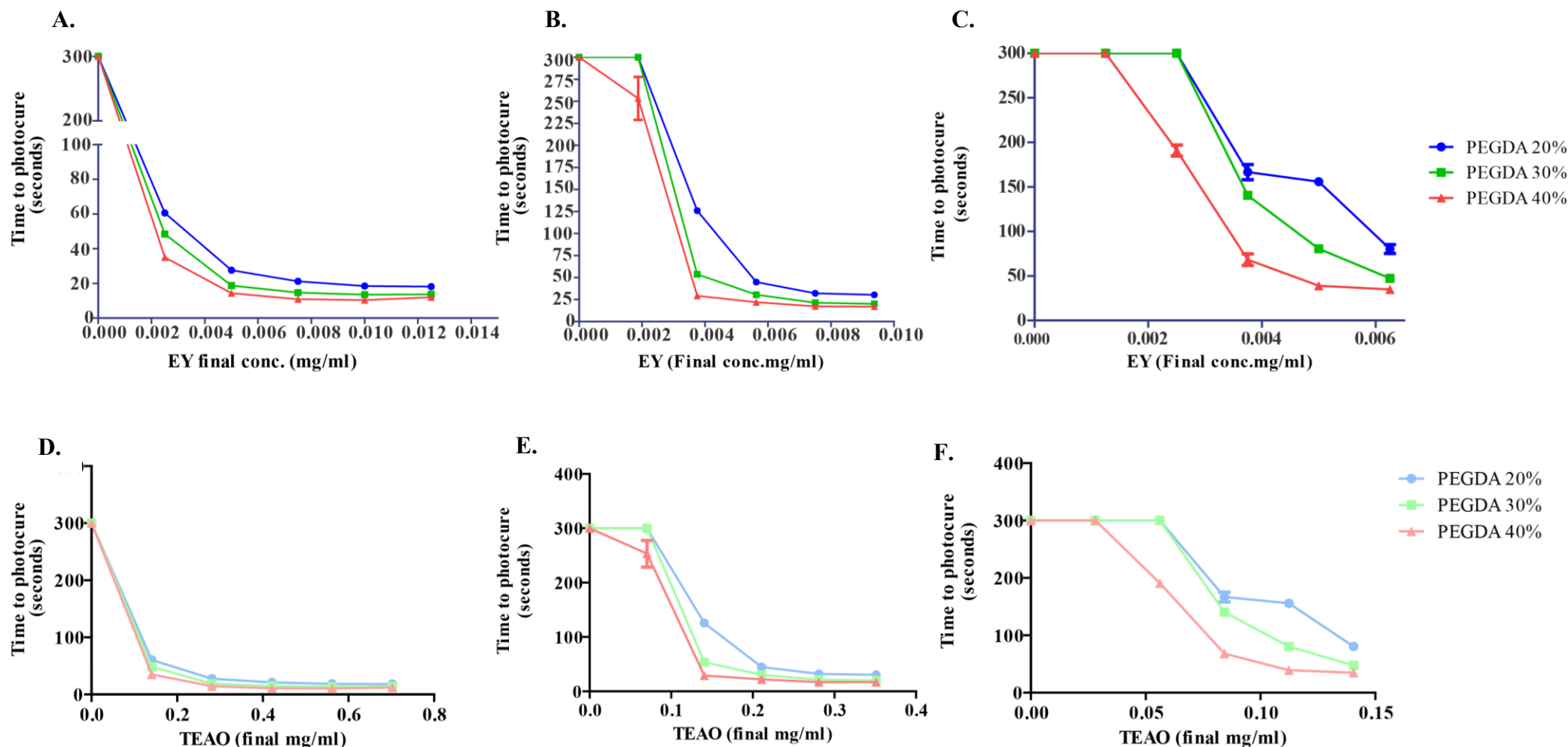


Figure 3.5: Time taken for Polyethylene Glycol Diacrylate (PEGDA) 700MW hydrogel scaffold to fully photocure using photoinitiator eosin Y (EY) and co-initiator triethanolamine (TEAO), LED wavelength at 525nm used. Concentration- dependent decrease in photocuring time is shown as percentage of PEGDA increases. Gels were photocured for >300s. Where '300s' is plotted on graph, no curing has taken place. (**A and D**) EY: TEAO, at the ratio of 1:5.62. (**B and E**) EY: TEAO, at the ratio of 1:37.47. (**C and F**) EY: TEAO, at the ratio of 1:22.48 (Mean and SEM plotted, $n=3$, technical repeats). Error bars are observed when recorded photocuring times notably differ from each other.

3.5.3 2D cell viability assay

To test out any potential phototoxicity, a 2D cell culture system was first utilised. Cells were seeded into wells 4 hours prior to 1 minute exposure to green light alone or in combination with the photoinitiator and co-initiator solution and monitored over 3 days. Results were compared to cells seeded at the same time. This assay is comparable to a wound healing assay and used to determine whether any cell attachment, cell death and/or recovery occurred after exposure. A summary of the results is provided immediately below with more details for each experiment following. Full method can be found on page 75 in Chapter 2. It should be noted that any free radicals produced would normally be mopped up by the PEGDA monomers in order to begin the crosslinking reaction.

Bronchial epithelial cells (NHBEs) showed no adverse reaction over 72 hours to green light (525nm) following initial exposure on day 0 when compared to the control sample - unexposed cells, no stimulus (**Figure 3.6A-D (control)** and **3.7A-E**). EY and TEAO show initial phototoxicity as determined by morphological changes, when added to the media together with light exposure (**Figure 3.8A-B**). This is presumed to be due to the number of free radicals produced in the cell culture. However, within 72 hours, a full cell monolayer was formed (**Figure 3.8E**). The Calcein AM green (live cell marker), and Hoechst 33342 stain overlay show good cell viability within the monolayer formed, with Propidium iodide (red) used to indicate the very few dead cells at day 3. Hoechst 3342 stains for all cells (blue).

As NHBEs are just one of the many types of cells present in the lung parenchyma, normal human lung fibroblasts (NHLFs) were also grown and exposed to green light alone as well as in combination with the photoinitiator and co-initiator complex. This cell type displayed the fastest recovery time, with the monolayer layer formed within 1 day in the presence of green light ($\lambda=525\text{nm}$) and EY and TEAO (**Figure 3.9, control**), **Figure 3.10 (green light)** and **Figure 3.11 (green light and photoinitiator)**. These results match the results seen in control (no stimuli).

Normal human airway smooth muscle cells (NHASMs) were also exposed to both stimuli. These cells had a better recovery from the generation of free radicals than NHBEs, exhibiting a faster recovery (by day 2) after exposure to green light, photoinitiators and co-initiators in combination and when exposed to just green light (day 1) in comparison to the results seen in control untreated cells (**Figure 3.13, control**), **Figure 3.14 (green light)** and **Figure 3.15 (green light and photoinitiator)**.

3.5.3.1 2D cell viability assay: Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic (Figure 3.6)

The cells were seeded onto standard tissue culture (TC) plastic and in controls, cells in suspension were left to attached to TC plastic on day 0 and monitored over 72 hours (**Figure 3.6A-D**). At the end of the assay, a live/dead viability stain was performed, with *Calcein AM green* (live cells stained green) and *Hoechst 33342* (all cells stained blue) (**Figure 3.6E**). *Propidium iodide* (dead cells stained red) was also used in conjunction with *Hoechst 33342* as an additional visual for viability (**Figure 3.6F**).

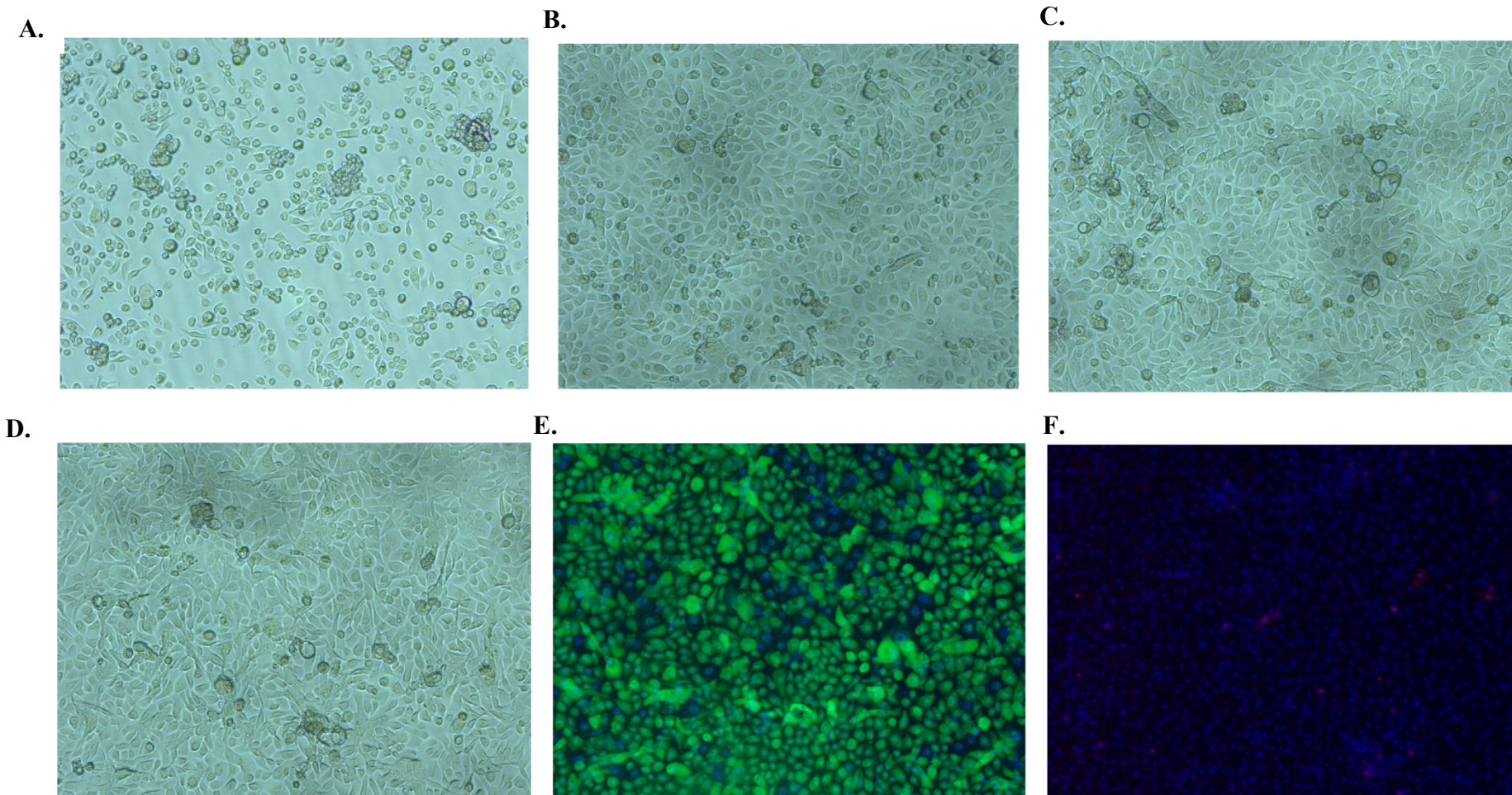


Figure 3.6: 2D cell viability assay. Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic (control). Timecourse over 72 hours, once cells have been seeded and left for 4 hours to attach: (A) Day 0, (B) Day 1, (C) Day 2, (D) Day 3. (E and F) NHBE cells stained with LIVE/DEAD stain. **Calcein AM green:** live cells/green, **Hoechst 33342:** all cells/blue, **Propidium iodide:** dead cells/red. Images are representative of 2 independent experiments.

3.5.3.2 2D cell viability assay: Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic with photo-exposure to green light wavelength 525nm (**Figure 3.7**)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic before exposure to green light (525nm wavelength). After light exposure for 1 minute, the cells were observed over 72 hours (**Figure 3.7B-E**). A live/dead viability stain was then performed with *Calcein AM green* (live cells stained green) and *Hoechst 33342* (all cells stained blue) (**Figure 3.7F**). *Propidium iodide* (dead cells stained red) was also used in conjunction with *Hoechst 33342* as an additional visual for viability (**Figure 3.7G**).

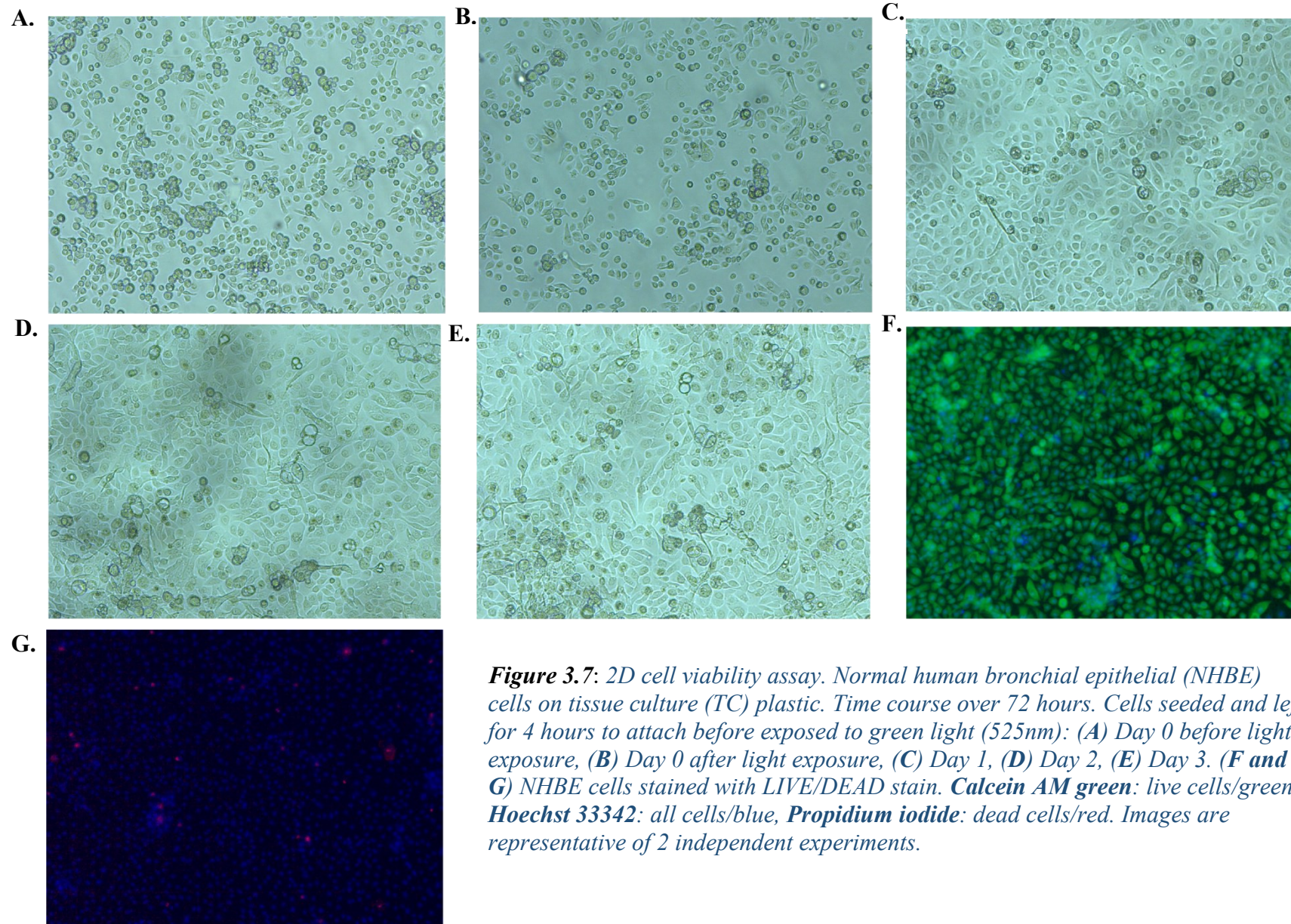


Figure 3.7: 2D cell viability assay. Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm): (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1, (D) Day 2, (E) Day 3. (F and G) NHBE cells stained with LIVE/DEAD stain. **Calcein AM green:** live cells/green, **Hoechst 33342:** all cells/blue, **Propidium iodide:** dead cells/red. Images are representative of 2 independent experiments.

3.5.3.3 2D cell viability assay. Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic, with photoexposure (green light wavelength 525nm) and exposure to photoinitiator/co-initiator Eosin Y and triethanolamine (TEAO) (Figure 3.8)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic before exposure to green light (525nm wavelength) together with the photoinitiator EY and the co-initiator TEAO. After light exposure for 1 minute and the same volumes of the photosensitizing reagents required for hydrogel synthesis, the cells were observed over 72 hours (**Figure 3.8B-E**). A live/dead viability stain was then performed with *Calcein AM green* (live cells stained green) and *Hoechst 33342* (all cells stained blue) (**Figure 3.8F**). *Propidium iodide* (dead cells stained red) was also used in conjunction with *Hoechst 33342* as an additional visual for viability (**Figure 3.8G**).

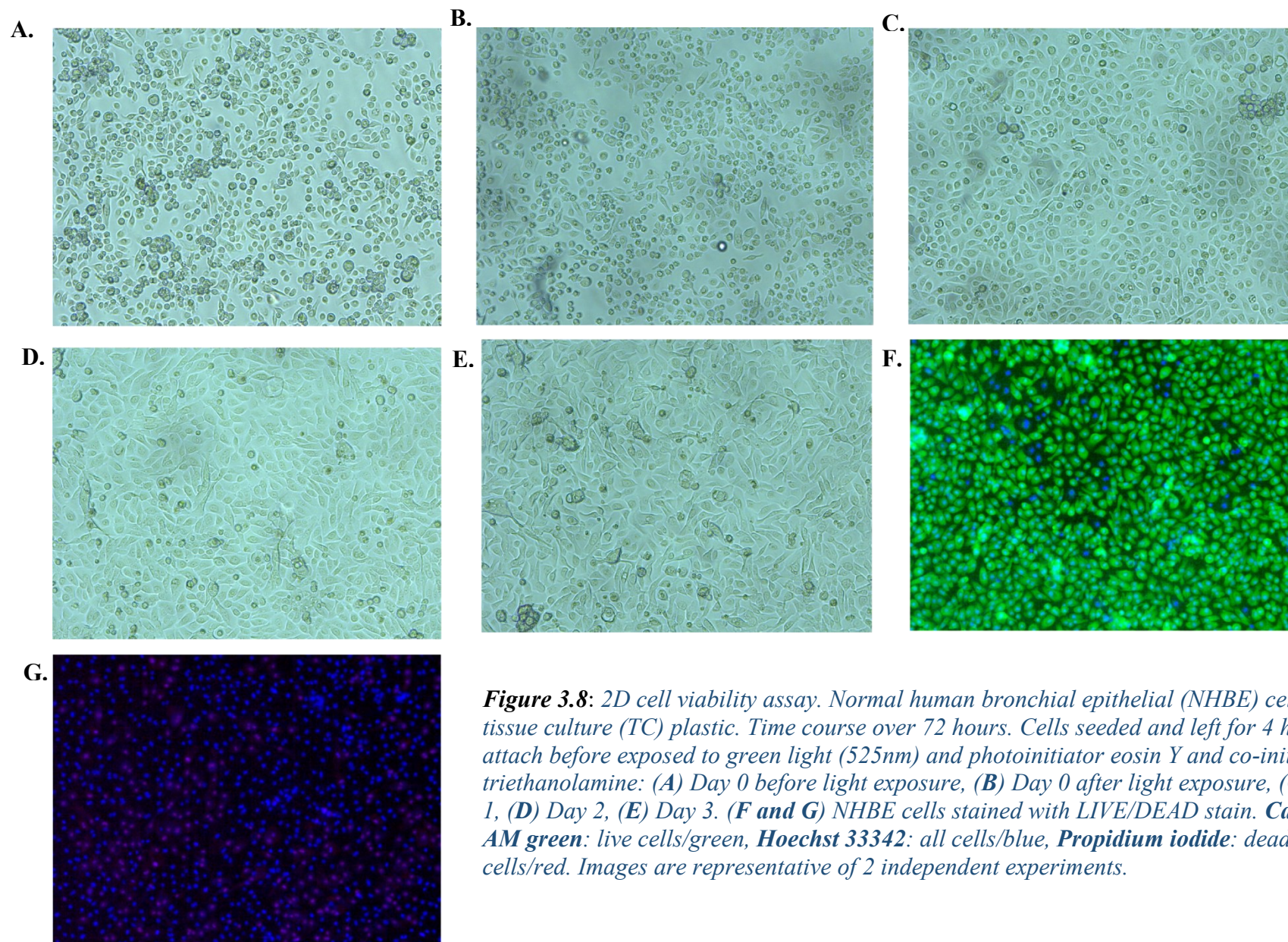


Figure 3.8: 2D cell viability assay. Normal human bronchial epithelial (NHBE) cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm) and photoinitiator eosin Y and co-initiator triethanolamine: (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1, (D) Day 2, (E) Day 3. (F and G) NHBE cells stained with LIVE/DEAD stain. **Calcein AM green:** live cells/green, **Hoechst 33342:** all cells/blue, **Propidium iodide:** dead cells/red. Images are representative of 2 independent experiments.

3.5.3.4 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic- control (**Figure 3.9**)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic as the control to be used for comparative analysis in the experiments conducted where stimuli were also introduced (**Figure 3.9A**). A time course of 72 hours was allocated for monolayer development but by day 1, a monolayer had formed (**Figure 3.9B**).

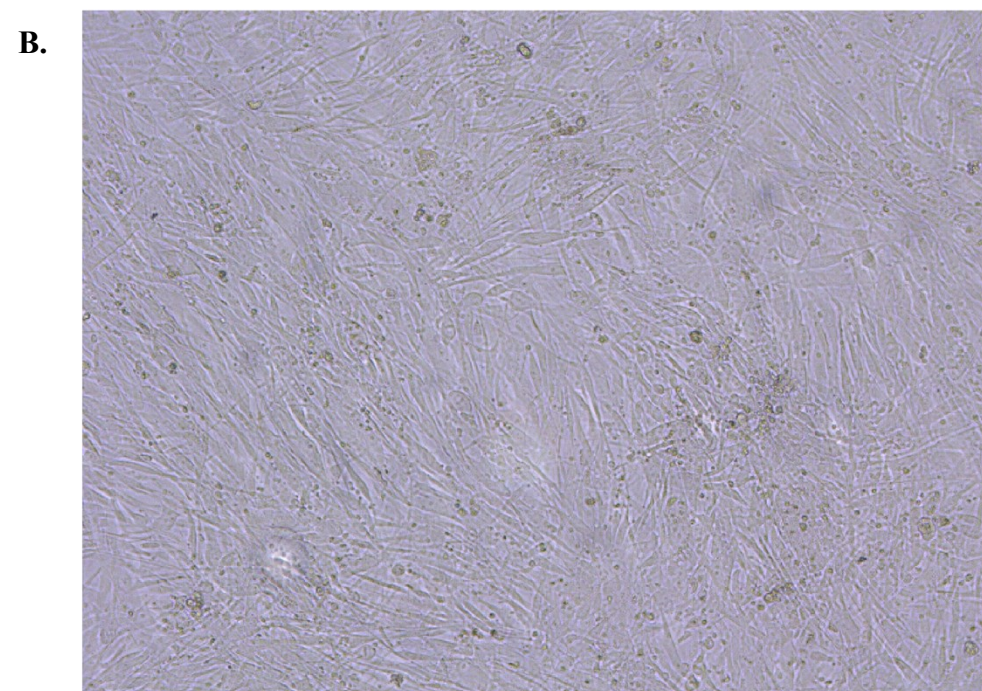
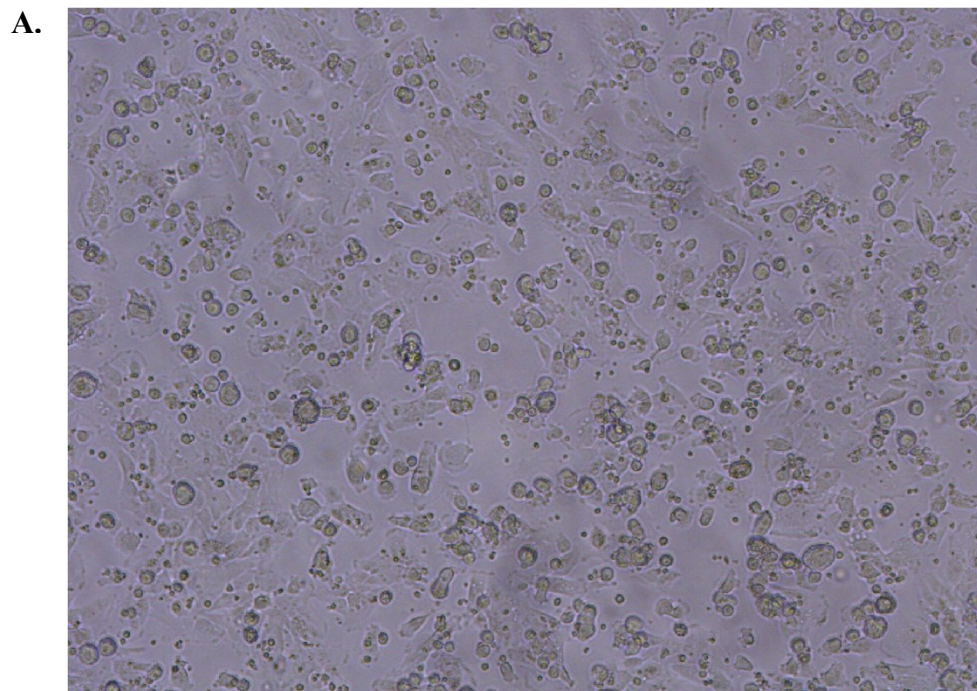


Figure 3.9: 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic (control). Time course over 72 hours, once cells have been seeded and left for 4 hours to attach: (A) Day 0, (B) Day 1. Images are representative of 2 technical replicates.

3.5.3.5 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic with photo-exposure to green light wavelength 525nm (Figure 3.10)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic (**Figure 3.10A**) before exposure to green light (525nm wavelength). After light exposure for 1 minute, the cells were observed over 72 hours from day 0 (**Figure 3.10B**) for monolayer formation. However, by day 1, a monolayer had already formed (**Figure 3.10C**).

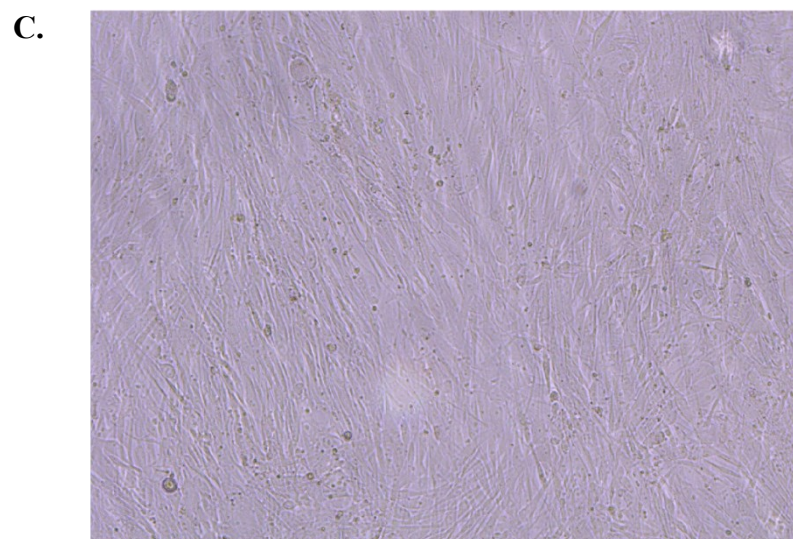
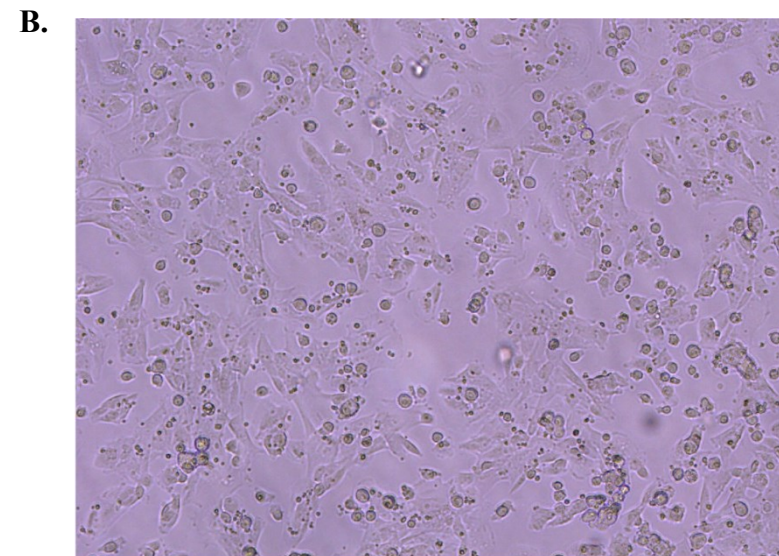
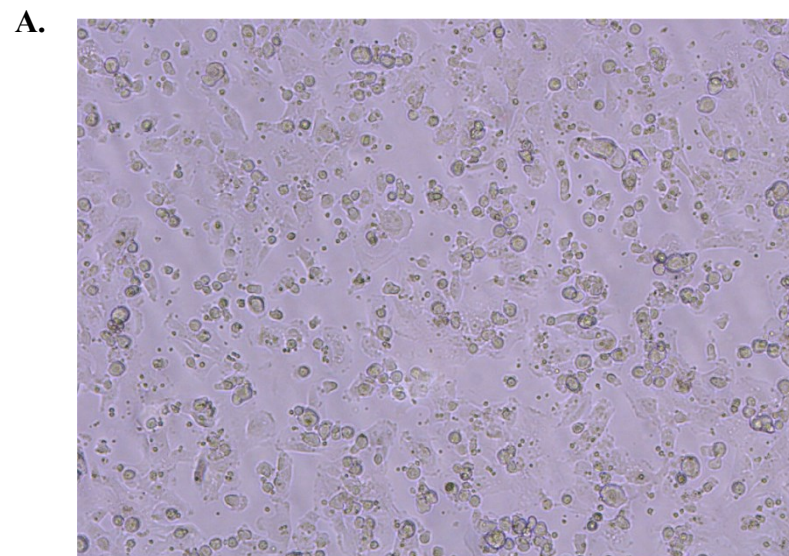
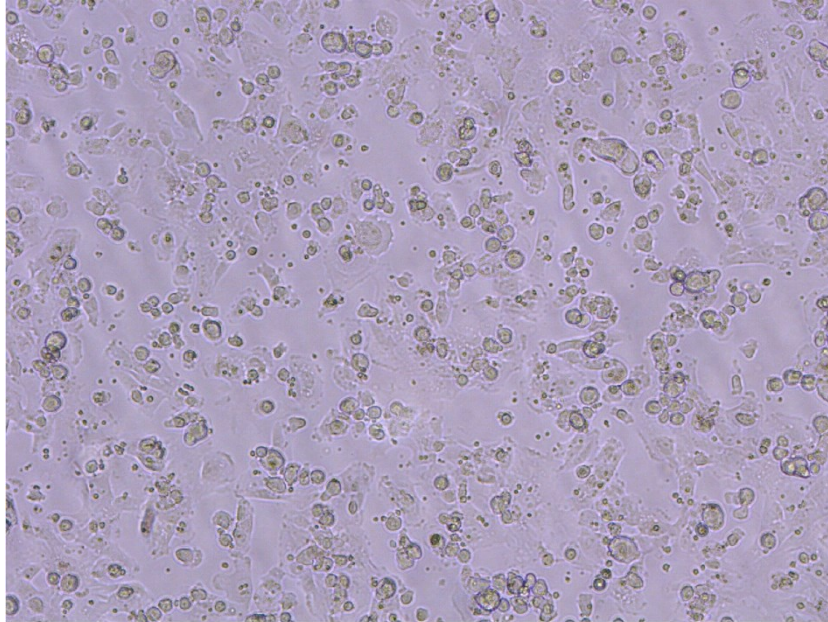


Figure 3.10: 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm): (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1. Images are representative of 2 technical replicates.

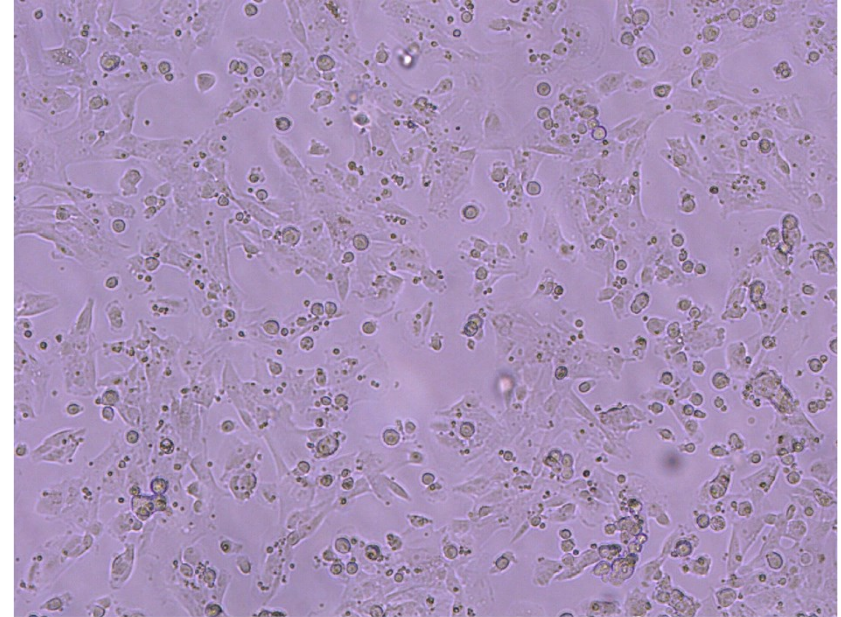
3.5.3.6 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic, with photoexposure (green light wavelength 525nm) and exposure to photoinitiator/co-initiator Eosin Y and triethanolamine (TEAO) (Figure 3.11)

Cells were seeded on day 0 and left to attach to the TC plastic (**Figure 3.11A**) before exposure to green light (525nm wavelength) and the photoinitiator/co-initiator combination of EY and TEAO. After photoexposure for 1 minute and the introduction to photosensitizing reagents, the cells were observed over 72 hours from day 0 (**Figure 3.11B**) for monolayer formation. However, by day 1, a monolayer had already formed (**Figure 3.11C**).

A.



B.



C.

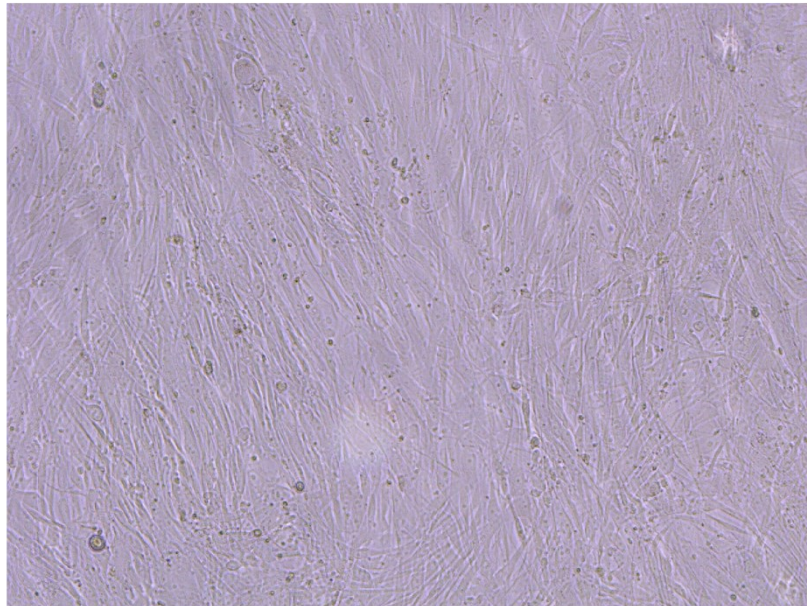


Figure 3.11: 2D cell viability assay. Normal human lung fibroblast cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm) and photoinitiator eosin Y and co-initiator triethanolamine: (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1. Images are representative of 2 technical replicates.

3.5.3.7 2D cell viability assay. Normal human airway smooth muscle (NHASM) cells on tissue culture (TC) plastic-control (**Figure 3.12**)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic as the control to be used for comparative analysis in the experiments conducted where stimuli were also introduced (**Figure 3.12A**). Cells were monitored over 72 hours (**Figure 3.12B-E**). At the end of the assay, a live/dead viability stain was performed with *Hoechst 33342* (all cells stained blue) and *Propidium iodide* (dead cells stained red) (**Figure 3.12F**).

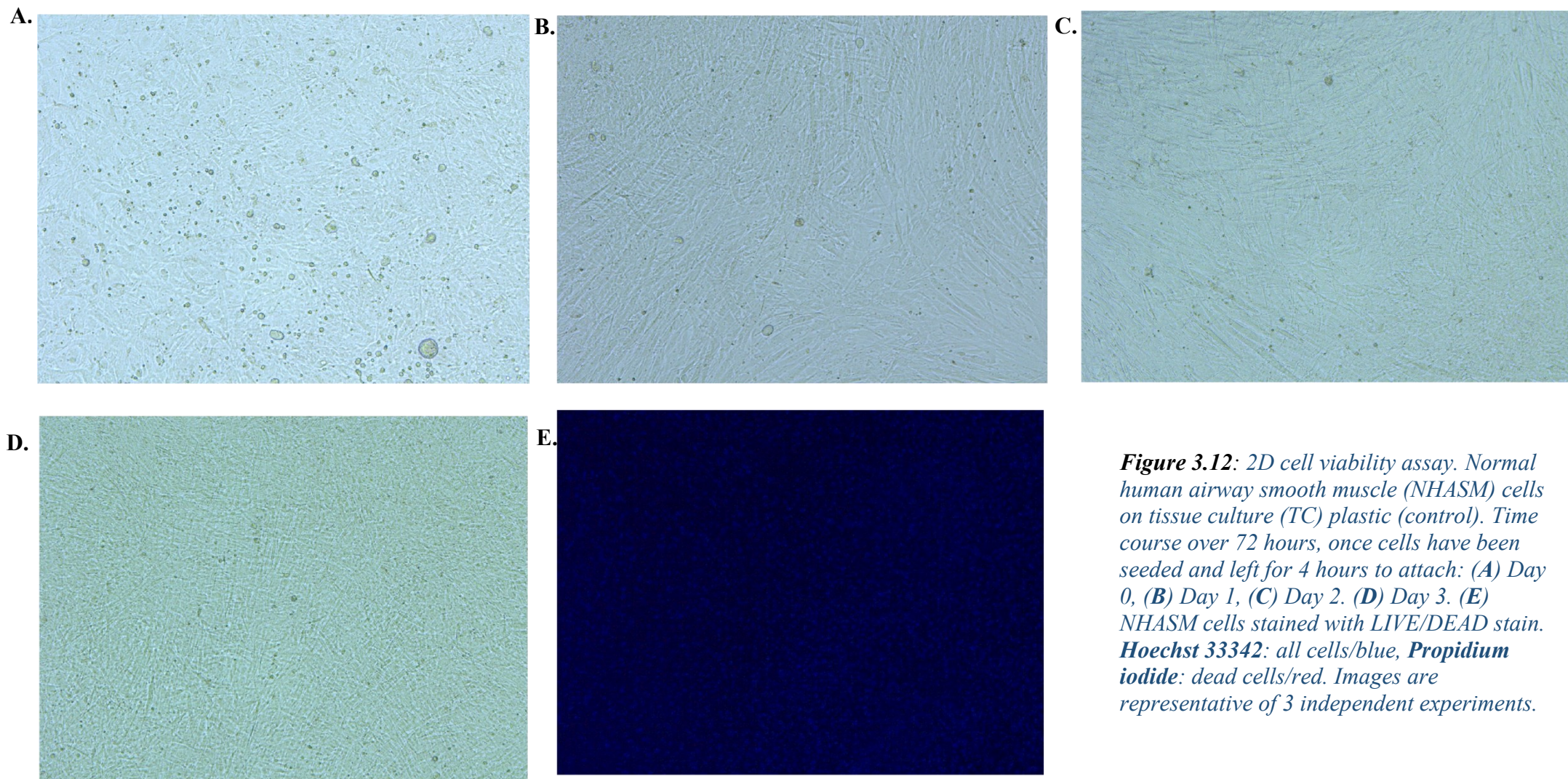


Figure 3.12: 2D cell viability assay. Normal human airway smooth muscle (NHASM) cells on tissue culture (TC) plastic (control). Time course over 72 hours, once cells have been seeded and left for 4 hours to attach: (A) Day 0, (B) Day 1, (C) Day 2. (D) Day 3. (E) NHASM cells stained with LIVE/DEAD stain. **Hoechst 33342:** all cells/blue, **Propidium iodide:** dead cells/red. Images are representative of 3 independent experiments.

3.5.3.8 2D cell viability assay. Normal human airway smooth muscle (NHASM) cells on tissue culture (TC) plastic with photo-exposure to green light wavelength 525nm (Figure 3.13)

Cells were seeded on day 0 and left to attach to the tissue culture (TC) plastic before exposure to green light (525nm wavelength) (**Figure 3.13A**). After light exposure for 1 minute, cells were monitored over 72 hours (**Figure 3.13B-E**). At the end of the assay, a live/dead viability stain was performed with *Hoechst 33342* (all cells stained blue) and *Propidium iodide* (dead cells stained red) (**Figure 3.13F**).

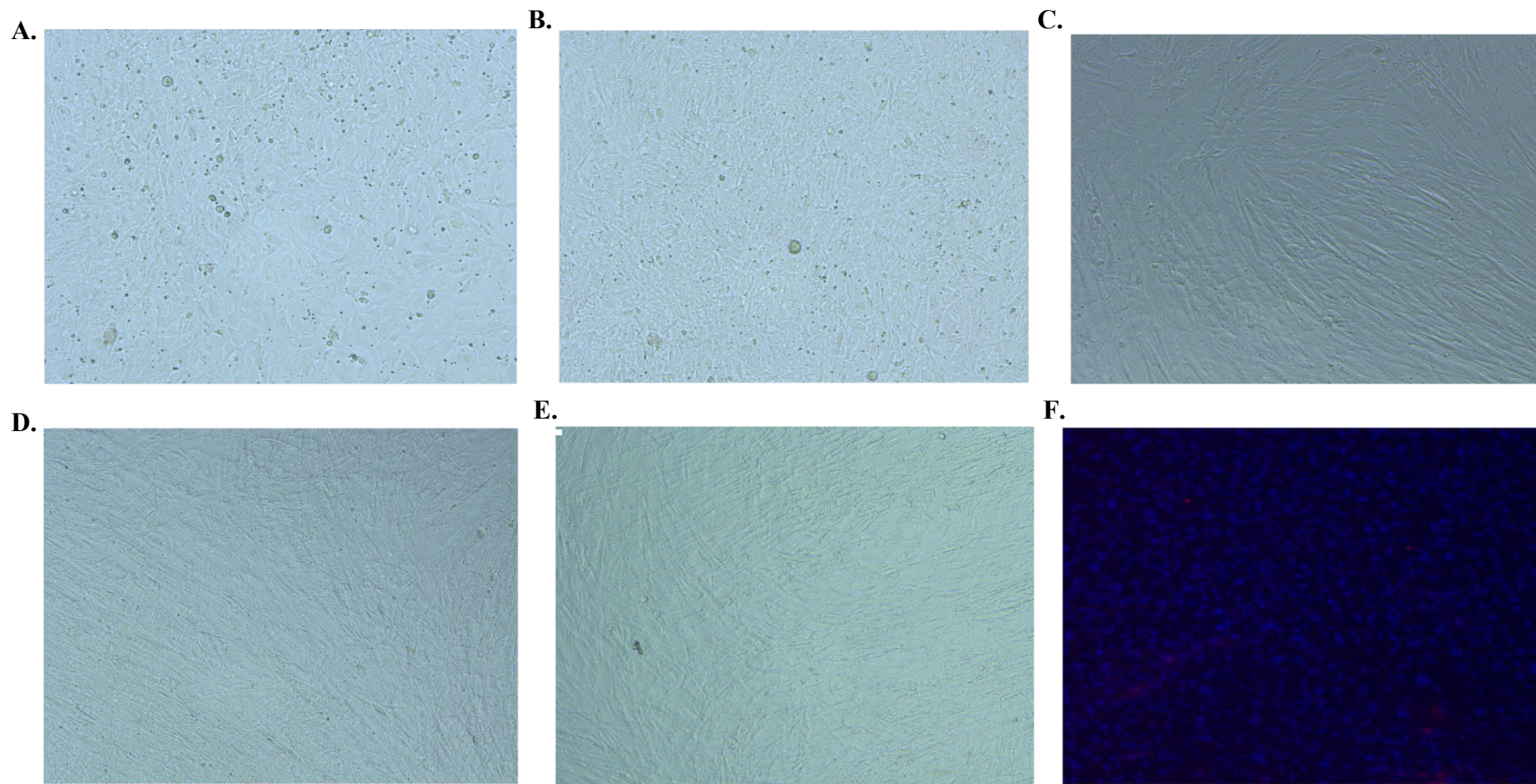


Figure 3.13: 2D cell viability assay. Normal human airway smooth muscle (NHASM) cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm): (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1, (D) Day 2, (E) Day 3. (F) NASM cells stained with LIVE/DEAD stain. **Hoechst 33342**: all cells/blue, **Propidium iodide**: dead cells/red. Images are representative of 3 independent experiments.

3.5.3.9 2D cell viability assay. Normal human airway smooth muscle (NHASM) cells on tissue culture (TC) plastic, with photoexposure (green light wavelength 525nm) and exposure to photoinitiator/co-initiator Eosin Y (EY) and triethanolamine (TEAO) (Figure 3.14)

Cells were seeded on day 0 and left to attach to the TC plastic (**Figure 3.14A**) before exposing to green light (525nm wavelength) and the combination of photoinitiator/co-initiator EY and TEAO. After photoexposure for 1 minute and the introduction to photosensitizing reagents, the cells were observed over 72 hours from day 0 (**Figure 3.14B**) for monolayer formation. At the end of the assay, a live/dead viability stain was performed with *Hoechst 33342* (all cells stained blue) and *Propidium iodide* (dead cells stained red) (**Figure 3.14F**).

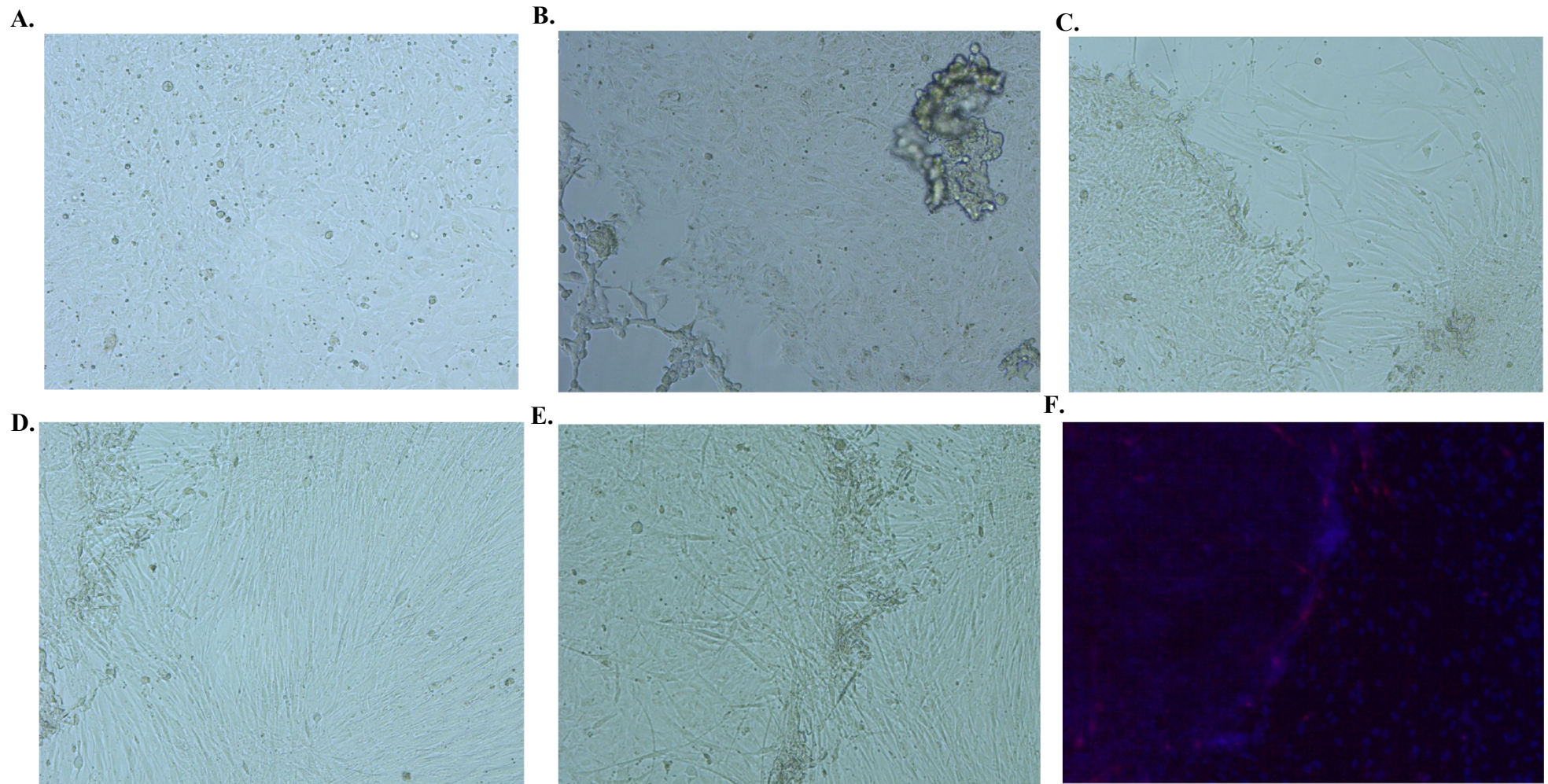


Figure 3.14: 2D cell viability assay. Normal human airway smooth muscle (NASM) cells on tissue culture (TC) plastic. Time course over 72 hours. Cells seeded and left for 4 hours to attach before exposed to green light (525nm) and photoinitiator eosin Y and co-initiator triethanolamine: (A) Day 0 before light exposure, (B) Day 0 after light exposure, (C) Day 1, (D) Day 2, (E) Day 3. (F) NHASM cells stained with LIVE/DEAD stain. **Hoechst 33342:** all cells/blue, **Propidium Iodide:** dead cells/red. Images are representative of 3 independent experiments.

3.5.4 3D cell structural integrity assay

To test the viability of the bronchospheres in response to cross-linking conditions, traditional methods were adapted. With bronchospheres, their spherical shape make common live/dead dyes and fluorophores difficult to diffuse through and penetrate down to all the cells rather than just the topmost layers. In addition, using Calcein AM green as a live cell marker was problematic since the PMET gel begins to autofluoresce at the same wavelength. This may be due to EY and TEAO being stimulated at the same time as using the FITC/495-530nm emission wavelength when imaging via confocal or LEDs, as well as general autofluorescence from the non-cellular reagents and material.

As an alternative, the morphology of the bronchospheres was investigated. DAPI was used to stain to cells due to its ability to enter the bronchospheres once embedded and fixed with paraformaldehyde. The rationale being that the bronchosphere structure will not be sustained if there was significant cellular death with either the bronchosphere collapsing inwards or rupturing outwards if the bronchosphere was located at edge of the PMET gel scaffold. Bronchospheres grown in ultra-low attachment tissue culture (TC) plastic wells would exhibit these indicators of cell death if conditions became unsuitable for maintenance once matured.

The 3D cell viability assay uses the same 3 day time course and 1 minute exposure format as the 2D cell viability assay. After each 24 hours, the PMET gels containing embedded bronchospheres were washed with PBS and fixed in PFA for an hour. Each gel was then stained with DAPI for 1 hour and submerged in PBS until imaged via the 405 laser with a Leica confocal SP8. A control cell culture group of bronchospheres were also fixed and stained in parallel. In this instance, the bronchospheres were embedded in the PMET gel but not exposed to green light so no photocuring occurred. Full method on page 76, in Chapter 2.

Figure 3.15 demonstrates that the morphology of the bronchospheres remained intact as the time course progresses following photocrosslinking (**Figure A-D**) with the results comparable to bronchospheres placed in non- photocured gel (**Figure E-H**). The circumference of the bronchospheres identified by the stained nuclei, are still well defined in the stiffer gel compared to the uncured environment. If the organoid's viability were compromised, this distinction would be lost entirely as the structure would degrade and

collapse. There were no observed differences in bronchosphere viability over the 72 hours of the experiment in the PMET gel compared to control non-photocured gels.

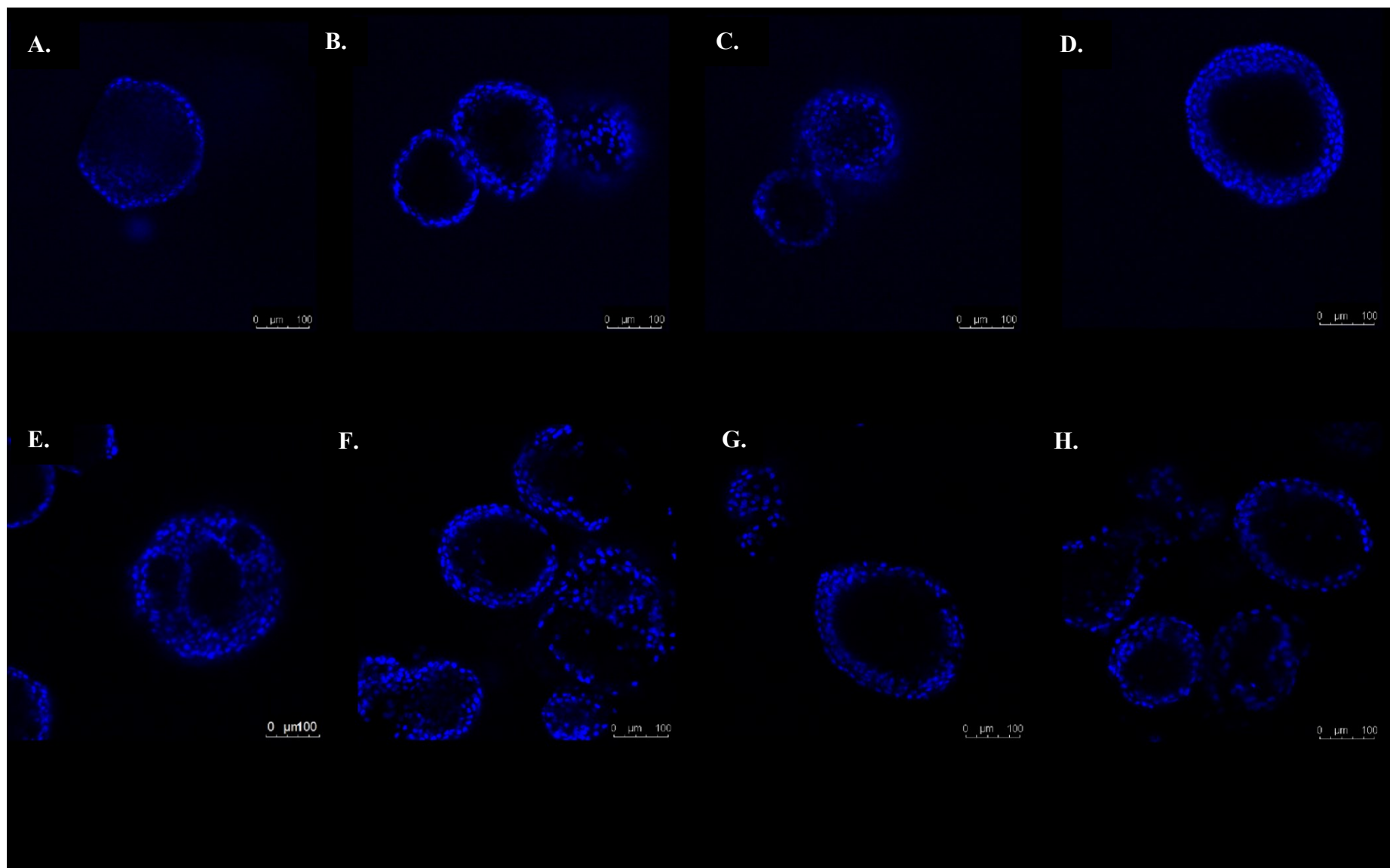


Figure 3.17: Confocal images of bronchospheres over a 72 hour time course, fixed with DAPI staining in 1 minute photocured PMET (PEGDA, Matrigel, Eosin Y and Triethanolamine) gel matrix (**A-D**) and in PMET gel without photocuring conditions (**E-H**). **A, E**: 0hrs, **B, F**: 24hrs, **C, G**: 48hrs and **D, H**: 72hrs. Images are representative of 4 independent experiments.

3.5.5 PMET gel characteristics

The resulting gel (PEGDA, Matrigel, EY and TBAO, referred to as *PMET* gel henceforth) can be further manipulated by fine-tuning the stiffness. The stiffness of the human lung can range from 1-7.5 kPa* (*approximation) (Liu et al., 2010b, Booth et al., 2012, Hinz, 2012, White, 2015) depending on the cell type encountered and measured as the YM ($\Delta\text{Stress}/\Delta\text{Strain}$) (Booth et al., 2012, White, 2015) as well as the technique used (Polio et al., 2018). With fibrotic respiratory pathologies like COPD and IPF, the stiffness can be at least nearly 3 times more than the maximum measurement in normal lung, in the range of 20-100 kPa with some cases reporting even higher (Goffin et al., 2006, Hinz, 2009, Hinz, 2010, Booth et al., 2012, Hinz, 2012).

Figure 3.16 highlights the new range that can be effectively reproduced, when exposing the PMET gel to green light (525nm) for longer. Under compression testing (for methods, please refer to pages 77-79 in Chapter 2), PMET gel after 1 minute photocure generated an average stiffness of $37 \pm 4 \text{ kPa}$, whilst after 5 minutes, the gel's stiffness increased to $138 \pm 23 \text{ kPa}$ - calculating from the initial linear region of the stress/strain curves; 0-5% for 1 minute photocure and 0-2% for the 5 minutes photocure (**Table 3.2**). In comparison, an attempt to measure Matrigel[®]'s stiffness was also performed. According to published data, Young's Modulus (YM) of Matrigel[®] is less than 1kPa, with many values stated from $160 \pm 93 \text{ Pa}$ to a higher value measured at $443 \pm 285 \text{ Pa}$ at 37°C using atomic force microscopy (Reed et al., 2009, Soofi et al., 2009, Kiss et al., 2011). Nonetheless, in many organoid-generating protocols, 25% Matrigel is also used to culture cells (Chen et al., 2012, Danahay et al., 2015, Butler et al., 2016)- whilst it can facilitate cellular growth, it is too soft to imitate actual lung ECM.

Using the PMET gel photocured at 1 minute and 5 minutes as pseudo-ECM for bronchospheres allows for the *in-vitro* model to effectively biomimic fibrosis in the lung at different scales with ~37 kPa at the lower end of the range, with ~ 138 kPa seen with severe IPF ECM (Booth et al., 2012). Having access to these different stiffnesses eliminates the disconnect often seen in lab *in-vitro* models where tissue culture (TC) plastic is often used, where the YM for plasticware is in the GPa cohort, at 2-4 GPa (White, 2015). Moreover, the bronchospheres can additionally be encapsulated by the PMET gel whilst a standard TC model cannot.

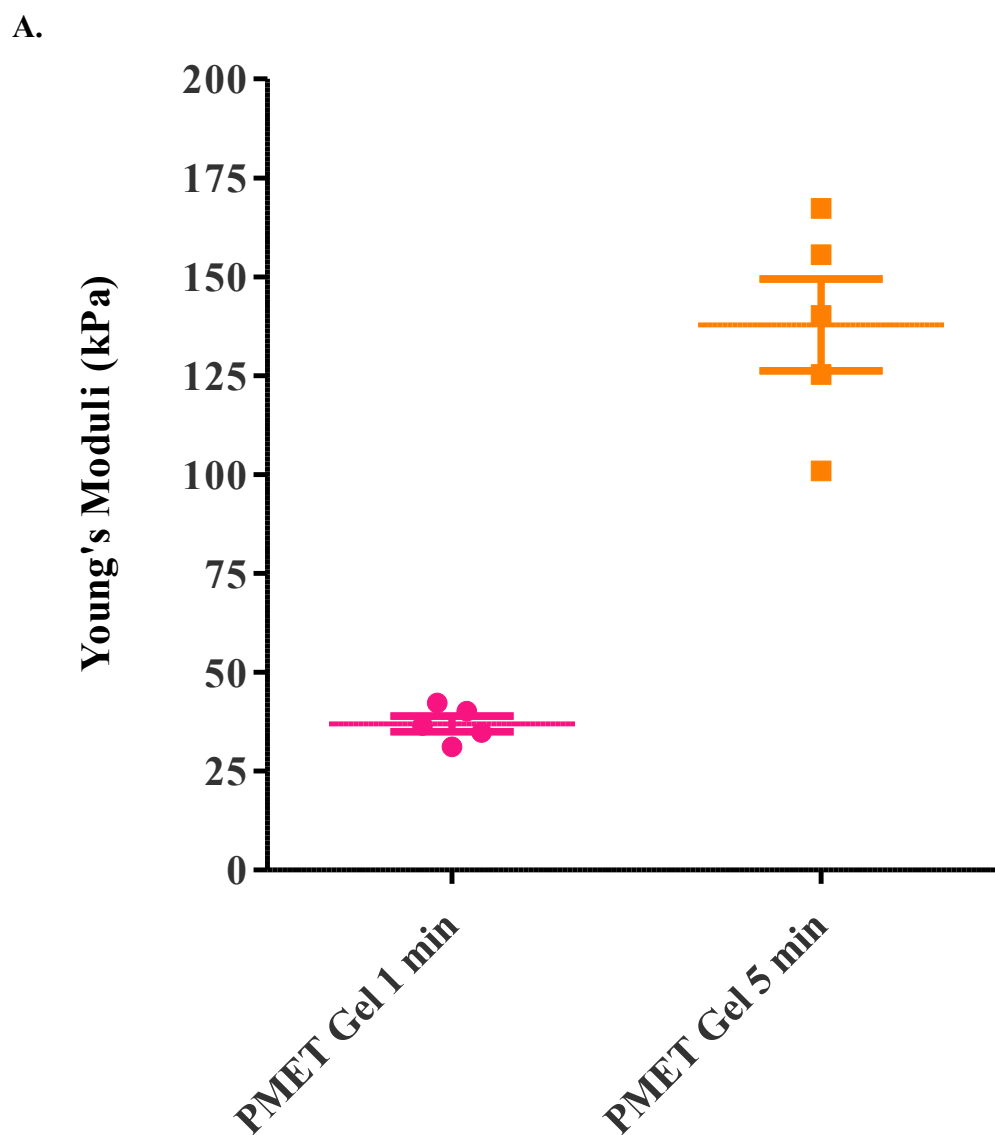
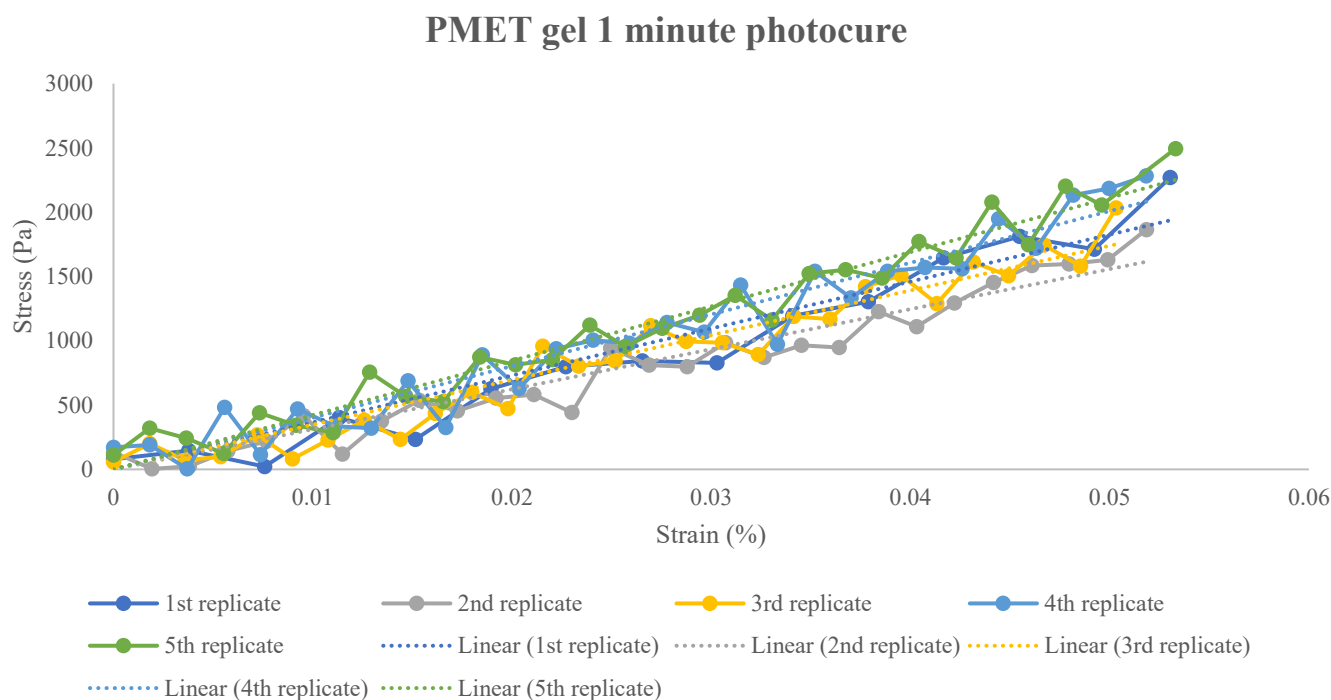


Figure 3.16: Scatter dot plot (mean and SEM) of PMET gel photocured at different times under green light: 1 minute and 5 minutes.

Photocuring Time	Young's Moduli (kPa)
1 min	37 ± 4
5 min	138 ± 23

Table 3.2: Each timepoint produced a different stiffness, depicted as Young's Modulus (kPa). Increasing time of light exposure, increases YM. N=5 (technical repeats). In contrast, YM of 100% Matrigel has been reported values of 160 Pa and 443 Pa when at 37°C (Soofi et al., 2009, Kiss et al., 2011).

A.



B.

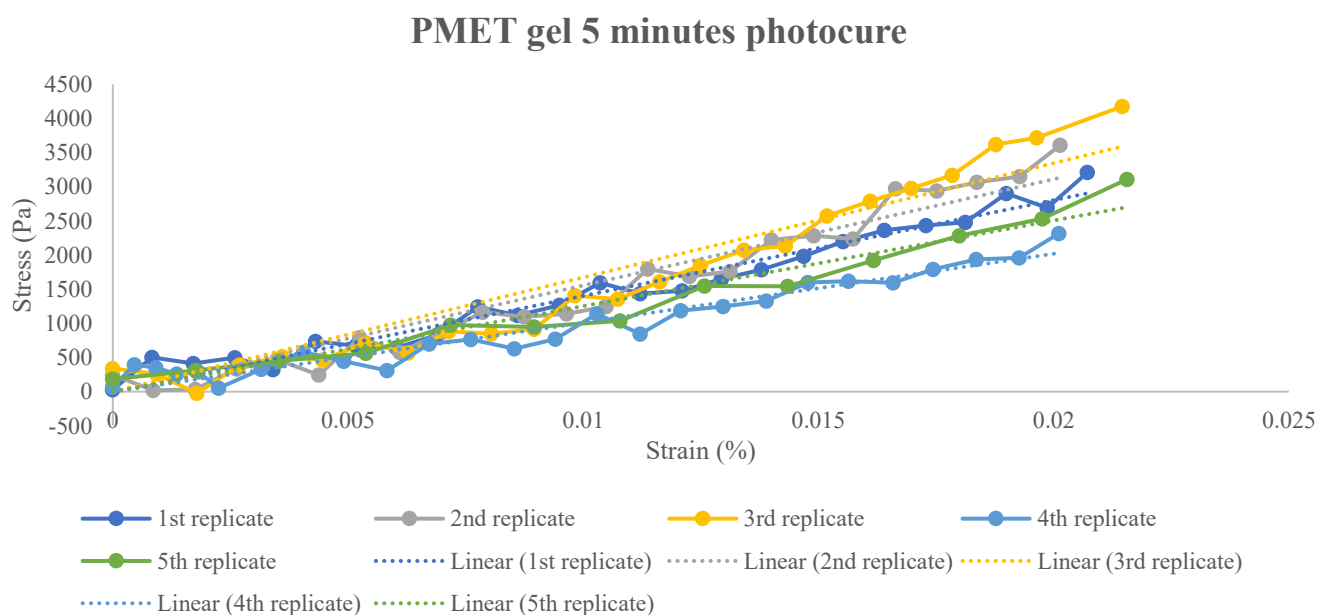


Figure 3.17: Plotted data points of rheometer compression tests of (A) 1 minute photocured PMET gel and (B) 5 minute photocured PMET gel. Produced data points are just before significant deformation is observed in gel as stress is applied. Young's Moduli values are derived from slope gradient of each technical replicate ($N=5$), with strain at 5% for the 1 minute photocured gels and 2% for the 5 minute photocured gels.

3.6 Summary

From the results presented, the newly designed PMET gel offers a tuneable mechanism for stiffness within the HBEC *in-vitro* model. The use of photoinitiators such as EY and TEAO allow for minimal toxicity and provide a relatively quick photochemical reaction, which is reproducible and scalable compared to the other options explored. The cultured bronchospheres can be encapsulated into pseudo-fibrotic ECM, with a stiffness akin to that seen within the lung of patients with fibrosis-associated diseases such as COPD and IPF. The ability to achieve a range of YM starting from ~37 kPa and scaling towards YM values seen within the stiffer /severe fibrotic ECM-like conditions such as IPF with a 5 minute photocure (~138 kPa) makes this a useful model.

The exploration of encapsulating organoids for disease modelling (in gels of controlled stiffness) is also a novel concept, in comparison to most published literature at the time of thesis submission. Of these, studies include cells cultured on top of synthesised matrices or individual cells embedded within the designed hydrogel (Fu et al., 2015, Chang et al., 2016, Greene et al., 2017, Nizamoglu et al., 2022). Using differentiated spherical bronchospheres in this model allows the determination of the effect of the matrix on cells when in native conformation, which includes the relationship between cell-matrix as well as the consideration of cell-cell interactions. The ability to adapt or tune the matrix at a specific point in time during culture is also a relatively new technique in scientific literature and has been since adopted in research and commercially (as very soft ECM mimics are commonly used e.g. Matrigel®), allowing the researcher to investigate the effect of stiffness from onset (Shih and Lin, 2016, Hu et al., 2017, Fu et al., 2019, Yan et al., 2022).

Although the process of PMET gel synthesis is relatively simple, limitations are still apparent. Optimisation is required to allow for the initial cell culture to be undertaken in the uncured PMET precursor solution, allowing the maturation of the bronchospheres to occur before photocuring. The development of a healthy lung ECM biomimic would also be beneficial as a stiffness reference point before matrix manipulation. This was initially attempted in the early stages of my research, using higher molecular weights of PEGDA (therefore a looser ‘mesh network’ is made when crosslinked), however the polymer created was not fully crosslinked and subsequently swelled within the transwell once media was added, altering the stiffness independently from the photoinitiator effects. In addition,

different techniques for hydrogel synthesis could be implemented, such as electrospinning as well as the potential for optical fabrication/3D printing—allowing the user to have orderly control over spatial geometry design as well as stiffness (Cristovão et al., 2019, Nikolova and Chavali, 2019, Zhu et al., 2020). Regarding the multiple cell types tested for viability, NHASMs and NHLFs appear to react similarly and recover quickly, which is expected as they are more resilient in cell culture, in comparison to NHBEs (Alberts, 2002). There may also be some differences in interaction between these two cell types and the photoinitiator components as the NHLFs recover the fastest—however, this would need to be further studied as only one donor was available to test. The preliminary 2D cell viability assays were performed with the intention of carrying on this potential tri-cell culture, especially when concerning organoid work. However, due to time constraints and extensive optimisation required for the bronchospheres, I streamlined the 3D cell culture to be only epithelial cell-based for the future assays conducted in the PhD project.

In summary, this hydrogel matrix model is a useful addition to the area that allows the impact of stiffness to be assessed in a time-dependent manner. The effects of photogenerated stiffness on bronchosphere mRNA will be investigated in the following chapter.

Chapter 4:

Matrix stiffness impacts
epithelial cell differentiation
markers in encapsulated
bronchospheres

4.1 *Introduction*

The natural range of stiffnesses seen in the healthy lung can vary, interacting and influencing cellular behaviour. This range increases exponentially in fibrotic diseases due to the onset and continuous remodelling of the ECM; the loss of elastic fibres and increased presence of ECM proteins e.g. tenascin C seen in COPD (Annoni et al., 2012) and the enhanced crosslinking of collagen and elastin in IPF (Burgstaller et al., 2017, Giménez et al., 2017, Plantier et al., 2018). Both disease states, are also accompanied by hallmark changes to the parenchyma such as increased alveolar airspace (COPD) and collapsed alveoli with subsequent presence of fibroblastic foci from fibroblasts and myofibroblasts in IPF (Burgstaller et al., 2017).

Within the airways, there are several groups of mucins present, distributed according to the different segments of the lung. Division is also found between gel-forming secretory mucins e.g., MUC2, MUC5AC, MUC5B and MUC6 which trap inhaled particles and those which are membrane-bound e.g., MUC1, MUC3, MUC4, MUC12 and MUC13 (Caramori et al., 2004, Whitsett, 2018). Amongst these, MUC5AC and MUC5B are the most dominant (Caramori et al., 2004, Boucherat et al., 2013). MUC5B is mostly expressed by submucosal glands and by the superficial epithelia of the distal airways whilst MUC5AC is found in the superficial epithelium of the cartilaginous and proximal section of the airways (Caramori et al., 2004, Whitsett, 2018, Okuda et al., 2019). Aggravators, such as cigarette smoke and dust, can become ‘stuck’ in the airway lining fluid at bifurcations causing a positive feedback loop of mucosal deposition and increase in mucous-secreting (goblet) cells (Okuda et al., 2019, Walentek, 2021).

It can be hypothesised that the differing stiffened matrix itself acts as a driver of cellular differentiation and function, favouring pathological features such as those stated above- associated with those seen in human airway diseases with distinct ECM characteristics.

4.2 *Methods*

In order to determine the influence the microenvironment has on lung organoids, bronchospheres can be embedded into PMET gel of varying stiffness, to investigate epithelial cell function and differentiation over time. At each timepoint, the expression of 9 genes were examined via RT-qPCR and immunohistochemical analysis of mRNA and protein expression, respectively. The used markers ranged from those for mucus production to specific cell markers of proliferation, inhibition, and cell types. They were selected to determine whether a stimulus consisting of a different stiffness of the surrounding matrix alone would demonstrate specific changes in such 3D cell culture, which are akin to those observed in fibrotic diseases in the respiratory system (such as mucus hypersecretion and unregulated tissue remodelling/proliferation). A list of the genes studied, and their major functions is provided in *Table 4.1*.

Gene Symbol	Name	Function
MUC5AC	Mucin 5AC	One of the major gel-forming glycoprotein mucins in the airways, secreted to protect the epithelium from inhaled particles from infection via muco-ciliary clearance. Marker of goblet cell
MUC5B	Mucin 5B	Another member of the major gel-forming glycoprotein mucins in the airways, secreted to protect the epithelium from inhaled particles from infection via muco-ciliary clearance. Marker of goblet cell
KI67	Ki-67 (Marker of proliferation)	Nuclear protein present during cellular proliferation
SOX2	SRY-Box transcription factor 2	Part of the transcription factor complex involved in proliferation and embryonic development
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Inhibitor of cell cycle progression of G1 and S phases via cyclin dependent kinases
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Regulates cell cycle progression via G1 and G2 phases and regulatory proteins e.g., CDK4 and p53
TP63	Tumour protein 63	DNA transcriptional regulator. Basal cell marker (epithelial development)
FOXJ1	Forkhead Box J1	Part of the transcription family involved in the production of proteins for motile cilia. Marker of ciliated cell
TGFB1	Transforming growth factor beta 1	Part of the TGFβ protein family for cell regulation

Table 4.1: List of genes to be observed. Definitions sourced from the GeneCard database (Stelzer et al., 2016, Safran M, 2022).

RT-qPCR was performed via a modified phenol-chloroform RNA extraction method. Bronchospheres were embedded in PMET gel (1 minute, 5 minute and no photocure) or cultured in 25% Matrigel before being removed from cell culture matrix and flash frozen with liquid nitrogen. This was swiftly followed by manually grinding the now frozen gel/organoid

pellet via pestle and mortar, keeping the gel/pellet cold with liquid nitrogen throughout (Gasparian et al., 2015).

The ground gel/pellet was then transferred to a 2ml dounce tissue grinder set with 1 ml of QIAzol™ Lysis Reagent (Qiagen, Venlo, The Netherlands) with Yeast tRNA at 5µg/ml and subjected to 100 strokes using both the larger pestle (A) and the smaller pestle (B) to create the homogenate. The homogenate was transferred to a 2ml Eppendorf and at this point, the homogenate can be stored at -80°C until continuing with the extraction.

Chloroform was used to separate the aqueous layer from the phenol. A Direct-zol RNA kit (Zymo Research, Irvine, California) was used to complete the extraction and RNA quantified using Nanodrop™ Lite spectrometer. 260/280 ratio ranged between 1.60-2.1. cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, Massachusetts) and stored at 4°C thereafter.

Contrary to standard normalisation methods, no singular housekeeping (HK) gene was used to normalise the gene mRNA detected as the Ct values obtained for all 8 housekeeping genes tested, changed over the time course of the experiment once the matrix surrounding the organoids was altered. Out of the 8 screened, the best 3 housekeeping genes were averaged to calculate a normalised Ct value. All data were then normalised to bronchospheres cultured with 'no light' stimulus, to eliminate any influence from the photoinitiators and PEGDA polymer used. No statistical tests were performed as the biological samples are discrete. Instead, each donor data set for NHBE and CHBE cells used to generate bronchospheres have been plotted to reflect the great variation seen and the results are provided as a narrative based on the trends observed during the analysis. Details regarding the donor details, culturing of these cells and the generation of bronchospheres in provided in Chapter 2 (pages 60-67).

4.3 Results

4.3.1 NHBE Mucins

The effect of time after photocuring on the expression of MUC5AC mRNA in NHBE (**Figure 4.1A**) was not as clear as that for MUC5B expression (**Figure 4.1B**). Overall, there was upregulation of the inflammatory mucin paralog MUC5AC seen by 48 hours, with the exception of donor 4 at 5 minute photocure showing peak MUC5AC mRNA expression at 24 hours (**Figure 4.1A**).

The high baseline expression of MUC5AC seen with donor 3 at 0 and 72 hours suggest that this may also be an outlier. The peak in MUC5AC mRNA expression was especially pronounced with the 5-minute samples. In contrast, there was a clear peak of MUC5B mRNA expression at 48 hours, except with donor 2 which showed no elevation of MUC5B expression over time, across photocured conditions. Upregulation was greater with the 5 minute photocure condition but fell back towards baseline levels by 72 hours.

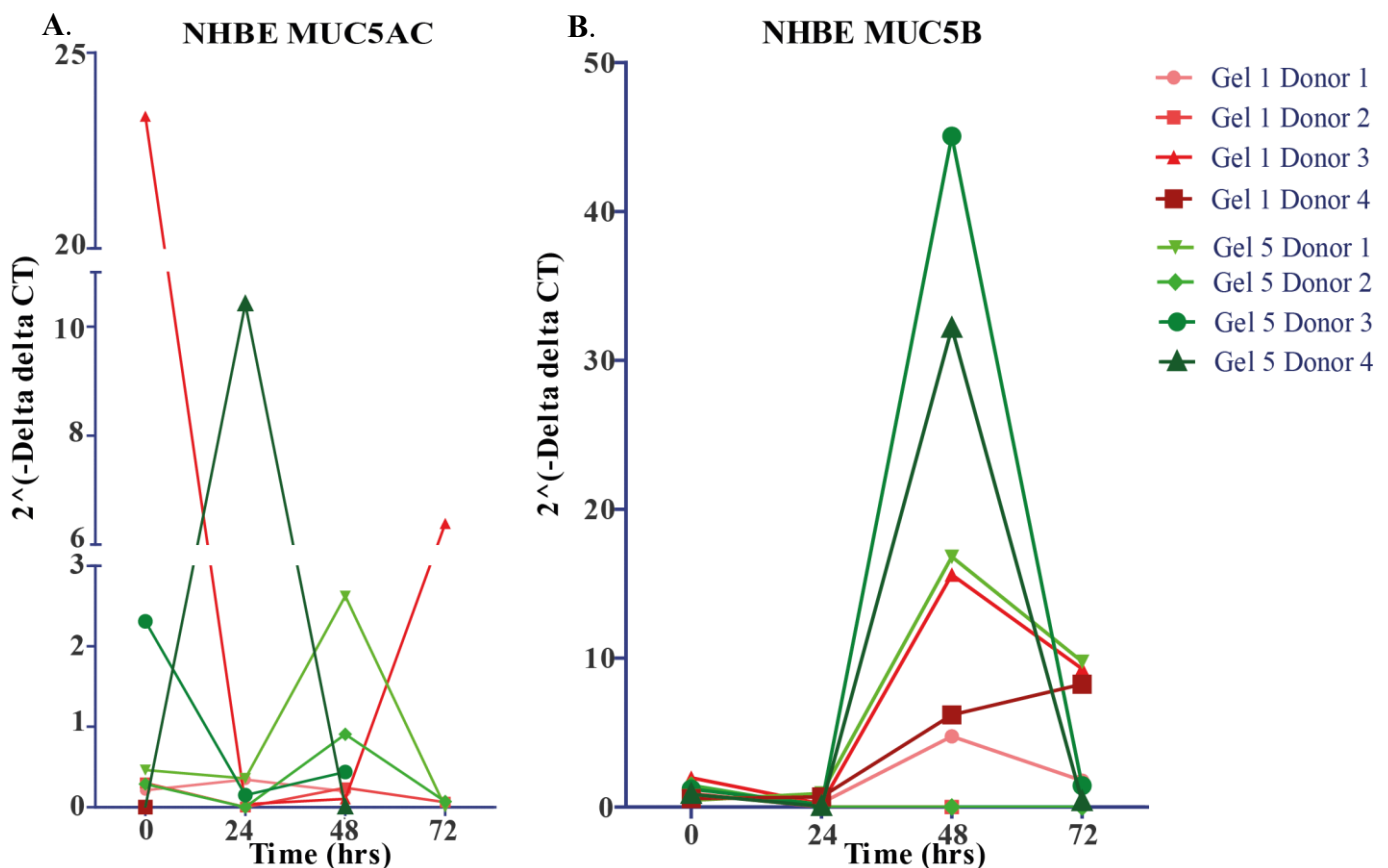


Figure 4.1: Mucin expression in normal human bronchial epithelial (NHBE) cells. (A) Mucin 5AC- MUC5AC (B) Mucin 5B- MUC5B. RT-qPCR gene expression **normalised to PMET gel stimuli on bronchospheres without photocuring (light)**. 0-72 hour time series performed following from encapsulation of normal human bronchial epithelial cell (NHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, $n=4$.

4.3.1.1 Immunocytochemistry (ICC) images of NHBE bronchospheres

Confocal ICC was used to examine the expression of MUC5AC and MUC5B in NHBE bronchospheres over 72 hours after photocuring for 1 minute (**Figure 4.2**) or 5 minutes (**Figure 4.3**).

In **Figure 4.2**, bronchospheres cultured in the control condition (25% Matrigel®) exhibited low expression and secretion of MUC5AC and MUC5B at 0 hours (**Figure 4.2A**) and after 72 hours (**Figure 4.2B**). The low levels of MUC5AC and MUC5B expression were also seen in bronchospheres encapsulated in PMET gel photocured for 1 minute immediately after transfer (**Figure 4.2C**) and after 72 hours (**Figure 4.2D**). The supplementary 3D rendering of **Figure 4.2D** (created with Volocity® software- please see supplementary folder, **Figure 4.2E**) displayed the colocalization of the secreted mucins within the bronchosphere after 72 hours.

Similar results were seen when exposing bronchospheres to 5 minutes photocuring with bronchospheres cultured in the control condition (25% Matrigel®) without cross-linking agents. These bronchospheres have low expression and secretion of MUC5AC and MUC5B at 0 hours (**Figure 4.3A**) and after 72 hours (**Figure 4.3B**). However, with bronchospheres cultured in PMET gel, photocured for 5 minutes displayed more MUC5B expression after 72 hours, particularly within the luminal space (**Figure 4.3C** and **4.3D** respectively), in comparison to the control (**Figure 4.3A** and **4.3B**). In contrast, there was no consistent increase in MUC5AC production over time in these experiments, reflecting the results seen with mRNA expression (**Figure 4.1**).

Comparable results of mucin secretion (emphasis of MUC5B than MUC5AC) were also observed when bronchospheres were examined 48 and 72 hours after photocuring.

Time after
photocure

0hrs:

48-
72hrs:

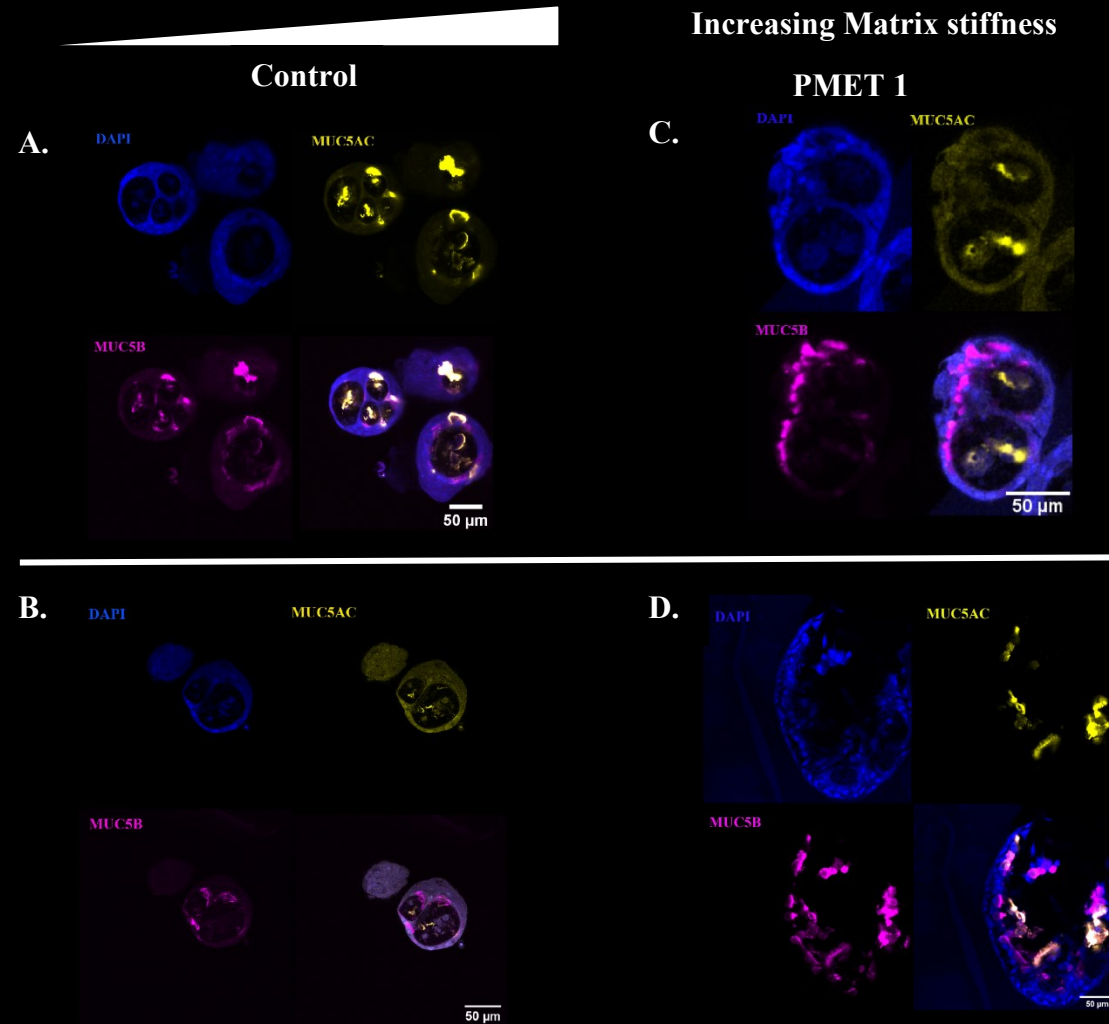


Figure 4.2: Representative confocal images of bronchospheres derived from normal human bronchial epithelial cells. ‘Control’ bronchospheres imaged at 0hrs (A) and 72hrs (B), cultured in 25% Matrigel. Bronchospheres imaged at 0hrs (C) and (D) 72hrs after embedding in PMET gel and exposed to light for 1 minute. Bronchospheres were stained for MUC5AC (yellow), MUC5B (magenta). Nuclei stained with DAPI (blue). Images are representative of results from 4 independent experiments.

Time
after
photocure

0hrs:

48-
72hrs:

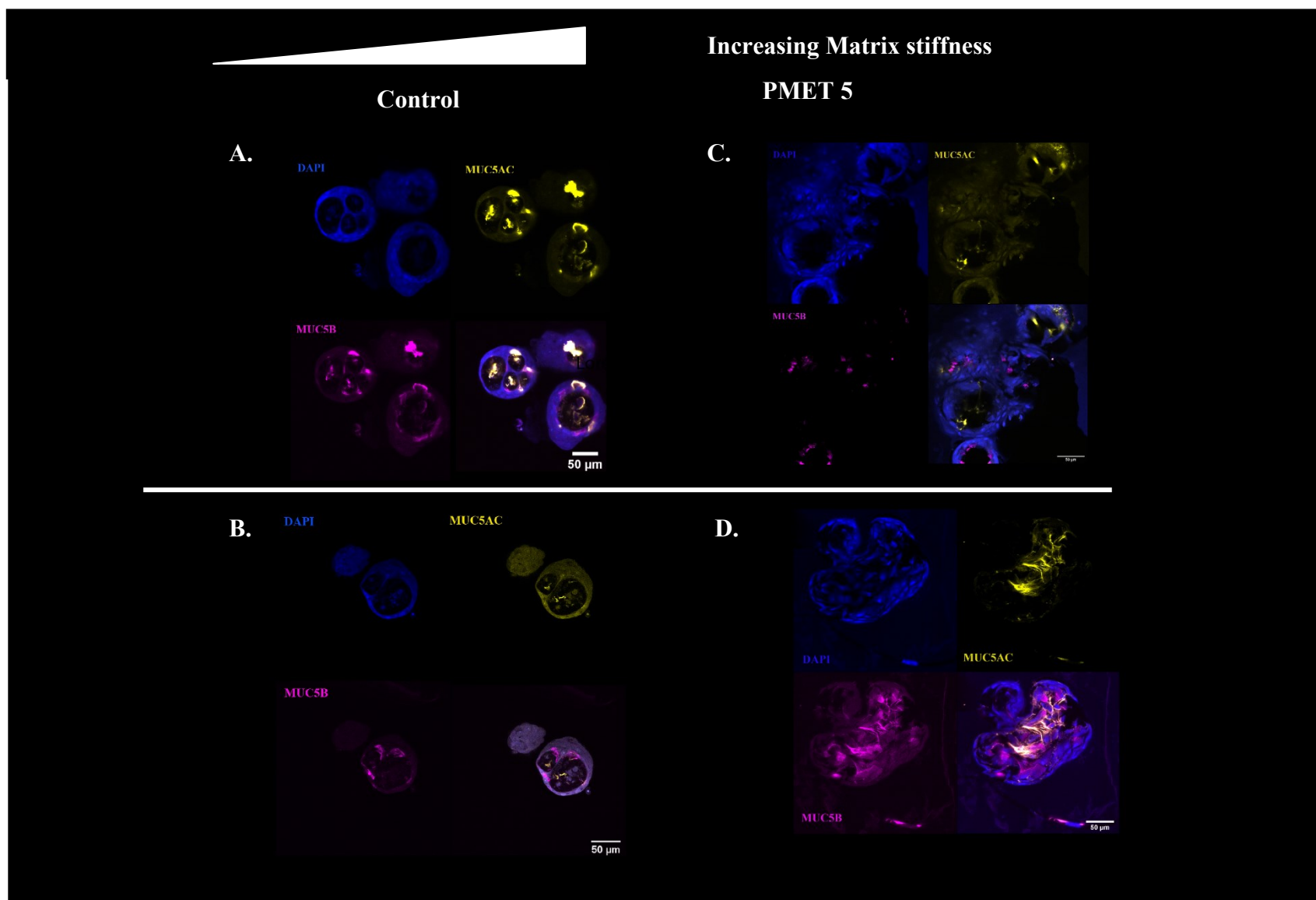


Figure 4.3: Representative confocal images of bronchospheres derived from normal human bronchial epithelial cells. ‘Control’ bronchospheres imaged at 0hrs (**A**) and 72hrs (**B**), cultured in 25% Matrigel. Bronchospheres imaged at 0hrs (**C**) and (**D**) 72hrs after embedding in PMET gel and exposed to light for 5 minutes. Bronchospheres were stained for MUC5AC (yellow), MUC5B (magenta). Nuclei stained with DAPI (blue). Images are representative of results from 4 independent experiments.

4.3.2 CHBE Mucins

CHBE MUC5AC mRNA expression (**Figure 4.4A**) was the greatest at 48 hours over the 72 hour time series, with 5 minutes photocuring generally eliciting stronger expression. MUC5B mRNA peaked at 24 hours instead, in all conditions (**Figure 4.4B**), following from initial photocuring. Expression waned by the 72-hour mark (bar outlier donor 4, which began with a high baseline in comparison) as the impact from the stiffened stimuli seemed to decrease over time from day one, whilst MUC5AC mRNA declined in expression between 48 and 72 hours (**Figure 4.4A**).

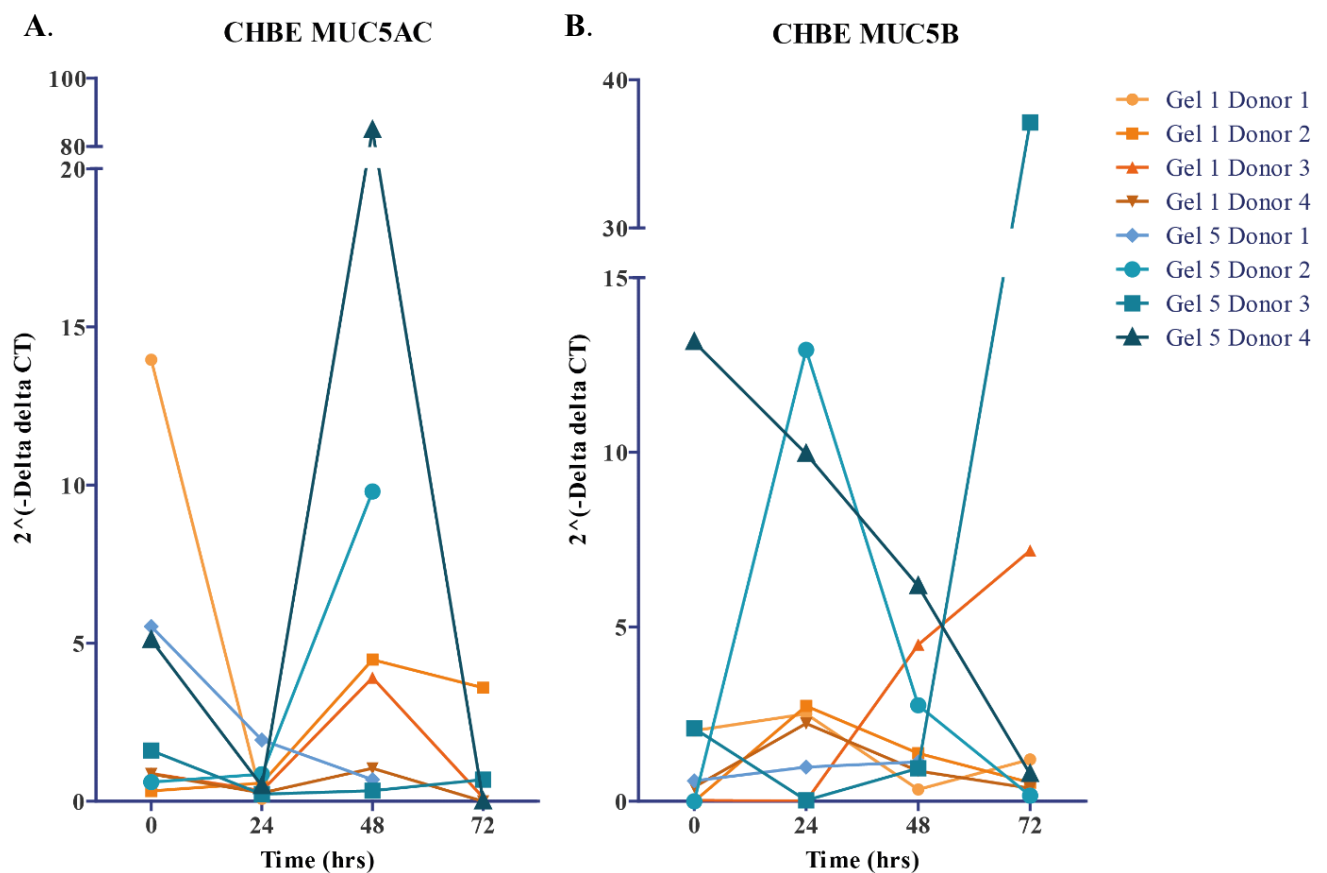


Figure 4.4: Mucin expression in COPD derived Human Bronchial Epithelial cells (CHBEs). (A) Mucin 5AC- MUC5AC (B) Mucin 5B- MUC5B. RT-qPCR gene expression normalised to PMET gel stimuli on bronchospheres without photocuring (light). 0-72 hour time series performed following from encapsulation of COPD human bronchial epithelial cell (CHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, n=4.

4.3.2.1 ICC images of CHBE bronchospheres

Confocal ICC was used to examine the expression of MUC5AC and MUC5B in CHBE bronchospheres over 48 hours after photocuring for 1 minute (**Figure 4.5**) or 5 minutes (**Figure 4.6**).

In **Figure 4.5**, bronchospheres cultured in the control condition (25% Matrigel[®]) exhibited low expression of both mucus paralogs (5AC and 5B) secretion, at 0 hours (**Figure 4.5A**) and after 48 hours of embedding (**Figure 4.5B**). Under the 1 minute generated stiffness, bronchospheres displayed some expression of MUC5AC after 48 hours, in comparison to the control (**Figure 4.5C at 0 hours, Figure 4.5D at 48 hours**). When encapsulated within the 5 minute photocured stiffness, the bronchospheres displayed increased levels of MUC5AC within the lumen at 48 hours, compared to the switch of MUC5B seen in the NHBE counterpart (**Figure 4.6C at 0 hours, Figure 4.6D at 48 hours**).

As with the NHBEs, the CHBEs displayed similar results of mucin secretion (MUC5AC favoured over MUC5B) at 48 and 72 hours- both timepoints used as endpoints of the assay.

Time after
photocure

0hrs:

48-
72hrs:

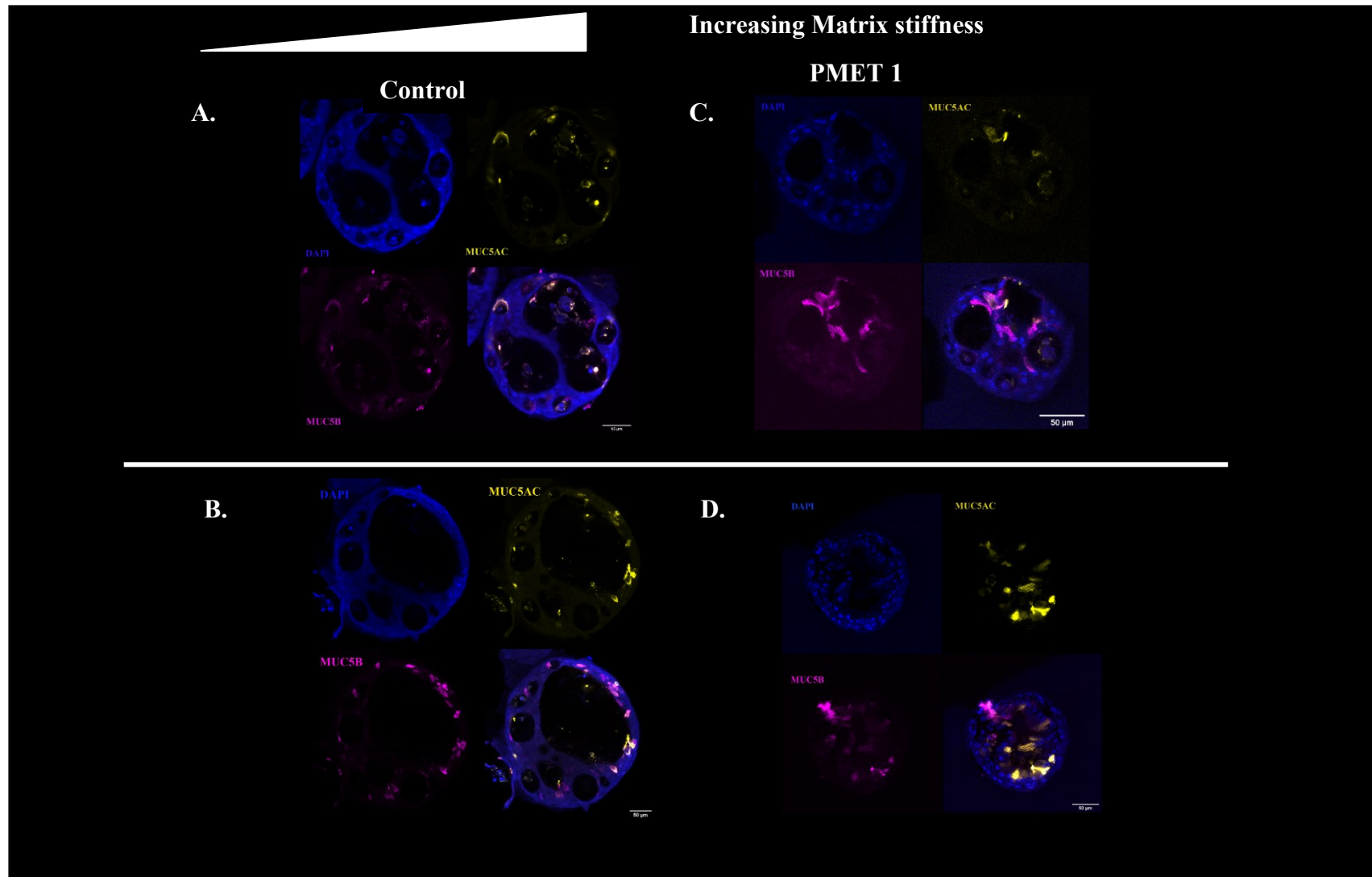


Figure 4.5: Representative confocal images of bronchospheres from COPD derived human bronchial epithelial cells (CHBEs). ‘Control’ bronchospheres imaged at 0hrs (**A**) and 48hrs (**B**), cultured in 25% Matrigel. Bronchospheres imaged at 0hrs (**C**) and (**D**) 48hrs after embedding in PMET gel and exposed to light for 1 minute. Bronchospheres were stained for MUC5AC (yellow), MUC5B (magenta). Nuclei stained with DAPI (blue). Images are representative of results from 4 independent experiments.

Time after
photocure

0hrs:

48-
72hrs:

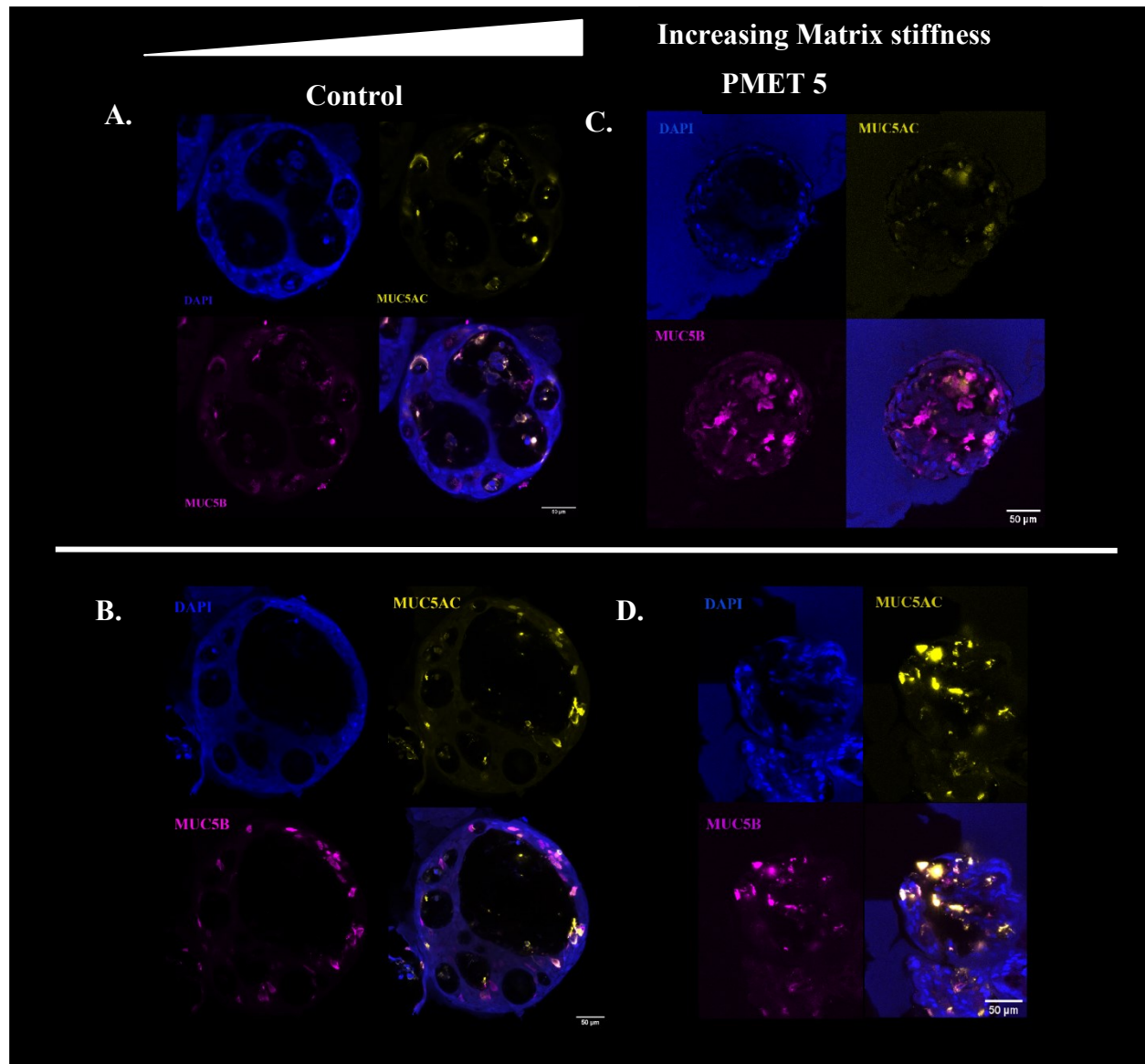


Figure 4.6: Representative confocal images of bronchospheres from COPD derived human bronchial epithelial cells (CHBEs). ‘Control’ bronchospheres imaged at 0hrs (A) and 48hrs (B), cultured in 25% Matrigel. Bronchospheres imaged at 0hrs (C) and (D) 48hrs after embedding in PMET gel and exposed to light for 1 minute. Bronchospheres were stained for MUC5AC (yellow), MUC5B (magenta). Nuclei stained with DAPI (blue). Images are representative of results from 4 independent experiments.

4.3.3 NHBE cell cycle promoters and inhibitors

No true trend was seen with proliferative marker Ki67 mRNA expression amongst the donors sampled (**Figure 4.7A**). Some upregulation was noted at 24 to 72 hours but missing data points within donor sets made analysis hard to complete.

With SOX2 mRNA, mixed levels of upregulation were seen across both donor groups (**Figure 4.7B**). However, a noticeable peak was seen at 24 hours, followed by a smaller peak at 48 hours. The increase in SOX2 mRNA expression was also split between the data produced by the donor sets, although 1 min/5 min counterparts did not always follow the related photocured condition (e.g., Donor 2 and 3).

The main mRNA peak of the cell cycle arrest marker CDKN1A (p21) was seen at the 48 hour time point, especially after a 5 minute photocure (**Figure 4.7C**). Donor 2 data set may be identified as potential outliers for cell cycle arrest marker CDKN2A (p16) due to the large increase seen in the first 48 hours compared to the rest of the data sets. Overall downregulation was seen amongst both photocure conditions towards the 72 hour timepoint (**Figure 4.7D**).

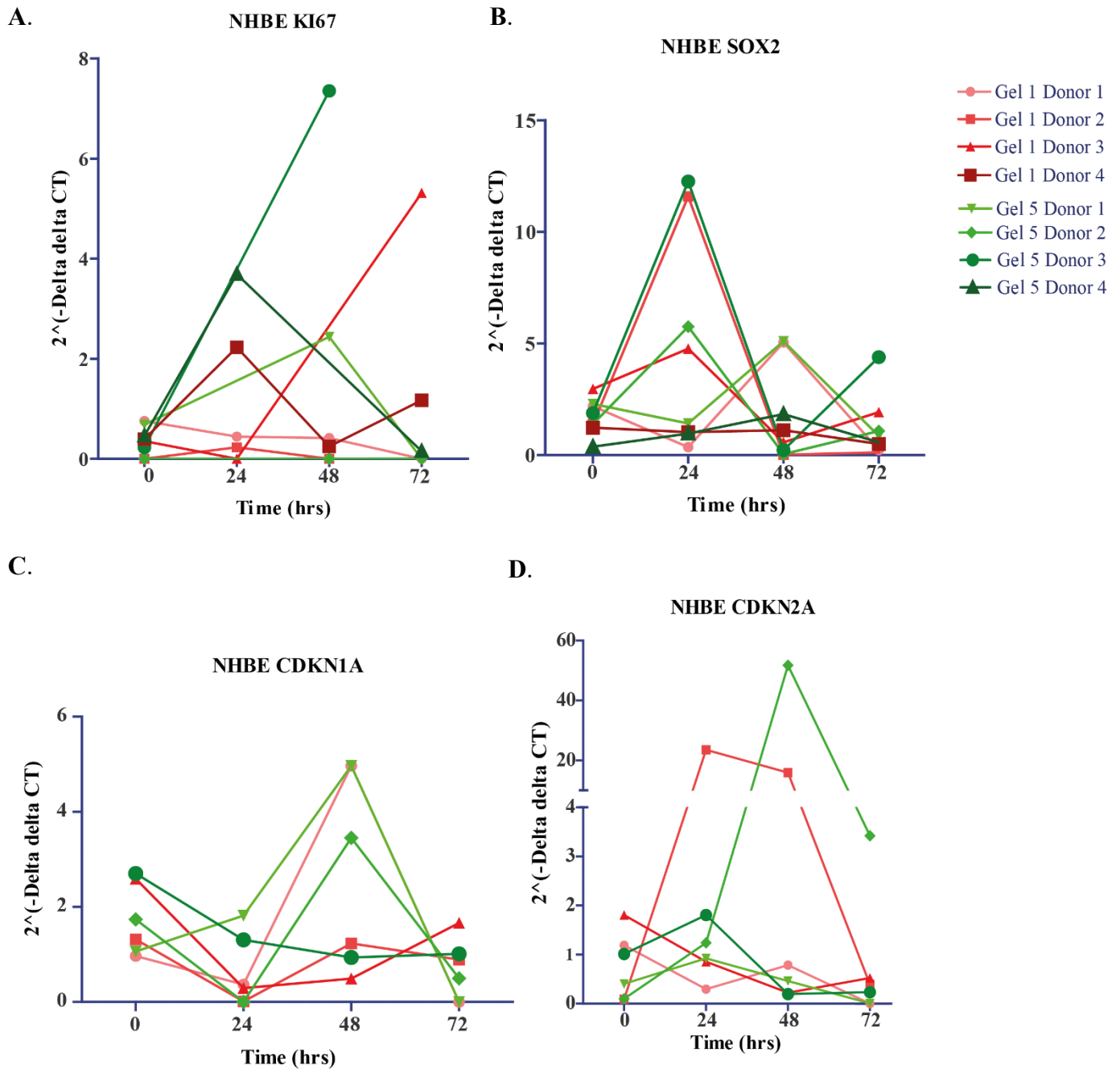


Figure 4.7: Expression levels of the proliferation marker Ki-67- KI67(A), SRY-Box transcription factor 2- SOX2 (B), Cyclin-dependent kinase inhibitor 1A- CDKN1A (p21, C) and cyclin-dependent kinase inhibitor 2A- CDKN2A (p16, D). RT-qPCR gene expression **normalised to PMET gel stimuli on bronchospheres without photocuring (light)**. 0-72 hour time series performed following from encapsulation of normal human bronchial epithelial cell (NHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, n=4 (A-B) and n=3 (C-D) respectively.

4.3.4 CHBE cell cycle promoters and inhibitors

With the COPD bronchospheres, Ki67 mRNA expression was also variable. A general dip was seen at 24 hours, but irregular/no Ct value was detected at some timepoints, making it hard to decipher what trend was occurring (**Figure 4.8A**).

SOX2 mRNA expression mimicked Ki67 data with the dip at 24 hours but with an increase seen at 48 hours (**Figure 4.8B**). Most data plateaued between 48-72 hours, except for that of donor 1 with both photocuring conditions and donor 4 after 5 minutes photocuring.

CDKN1A and CDKN2A mRNAs, however, displayed similar patterns with an initial decrease in expression and then a rise at 48-72 hours. Partial upregulation of CDKN1A was displayed at 24 hours by differing donors - donor 3 at 1 minute photocure and donor 1 at 5 minutes photocure (**Figure 4.8C**). In contrast, CDKN2A mRNA expression was reduced at 24 hours in each donor, with the same trend seen with PMET gels photocured for either 1 minute or 5 minutes. There was generally a greater fold change induction of p16 mRNA with bronchospheres embedded in the PMET gel given 5 minutes photocuring (**Figure 4.8D**).

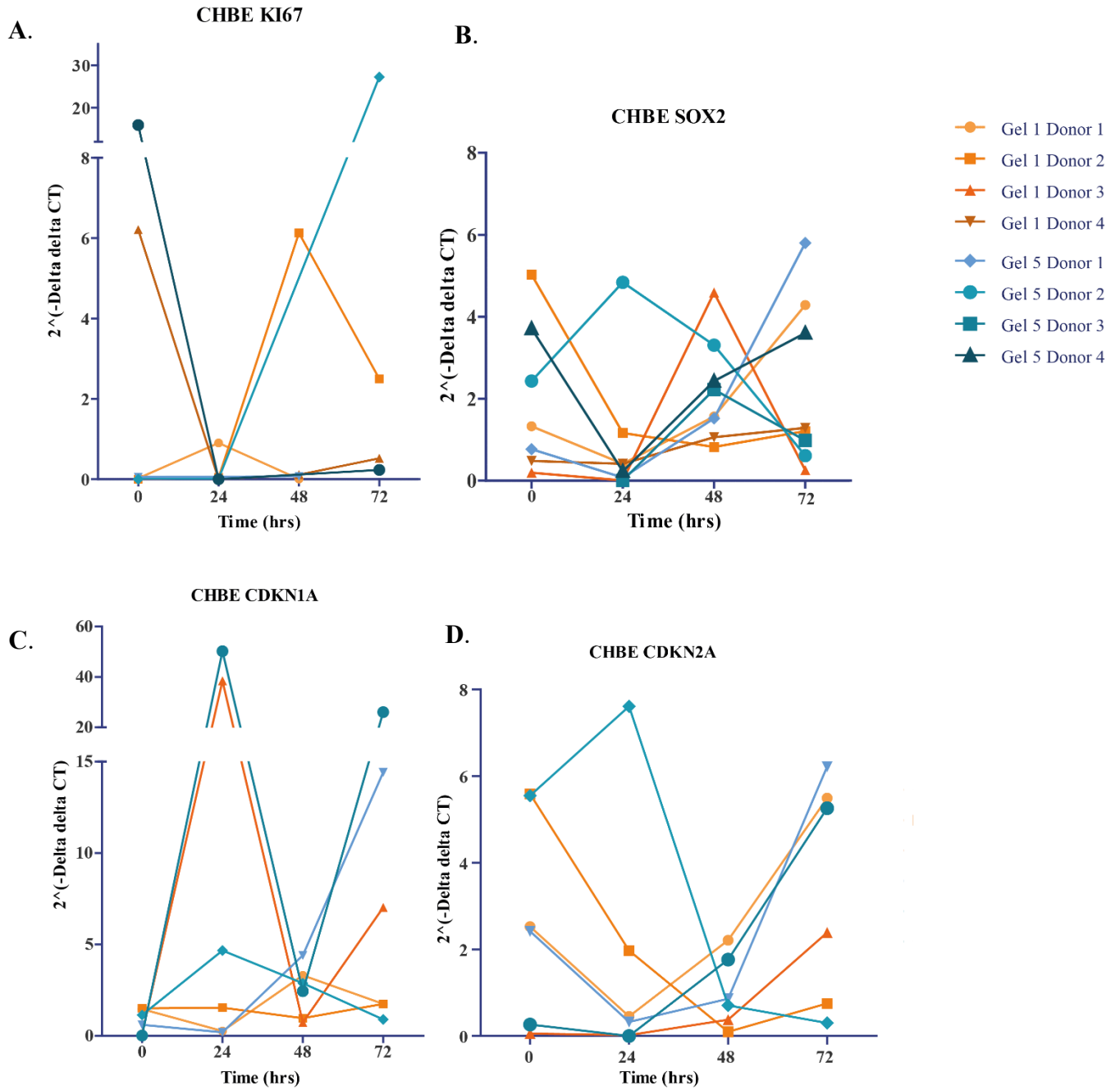


Figure 4.8: Expression levels of the proliferation markers Ki-67- KI67(A), SRY-Box transcription factor 2- SOX2 (B), Cyclin-dependent kinase inhibitor 1A- CDKN1A (p21, C) and cyclin-dependent kinase inhibitor 2A- CDKN2A (p16, D). RT-qPCR gene expression normalised to PMET gel stimuli on bronchospheres without photocuring (light). 0-72 hour time series performed following from encapsulation of COPD human bronchial epithelial cell (CHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, $n=4$ (A-B) and $n=3$ (C-D) respectively.

4.3.5 NHBE TP63, FOXJ1 and TGFβ1

Healthy bronchospheres displayed general upregulation of TP63 mRNA within 48-hours (**Figure 4.9A**). This upregulation inversely correlated with the downregulation seen with FOXJ1 mRNA (**Figure 4.9B**) although there was an outlier seen with donor 2 data set. In contrast, variable TGFβ mRNA expression was seen at 24 and 48 hours with an overall lower expression seen with the 1 minute photocured data sets (**Figure 4.9C**).

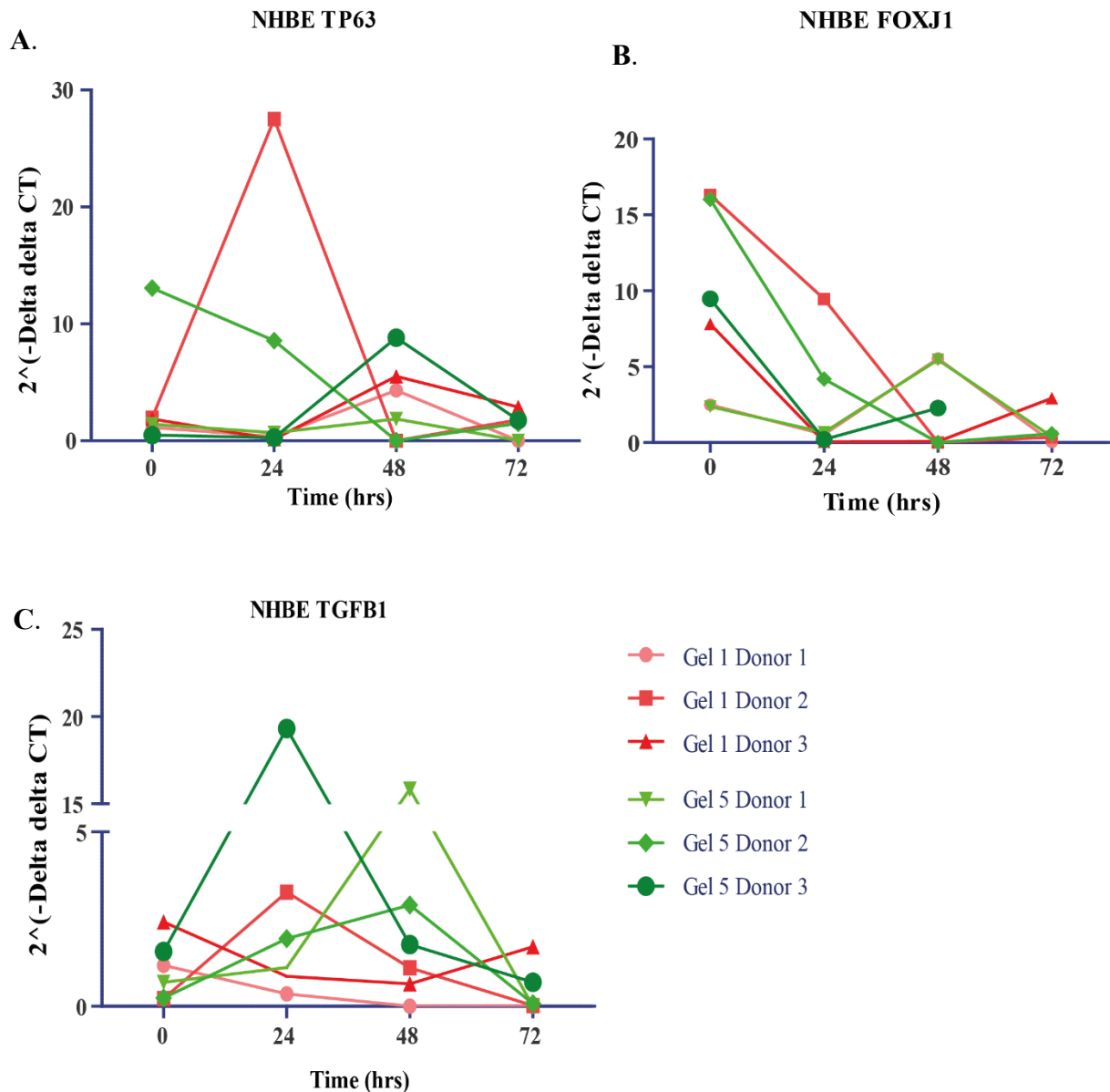


Figure 4.9: Expression levels of tumour protein P63- TP63(A), Forkhead Box J1- FOXJ1(B), transforming growth factor 1- TGFβ1(C). RT-qPCR gene expression normalised to PMET gel stimuli on bronchospheres without photocuring (light). 0-72 hour time series performed following from encapsulation of normal human bronchial epithelial cell (NHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, n=3.

4.3.6 CHBE TP63, FOXJ1 and TGFβ1

Similar to the NHBEs, CHBE bronchospheres displayed a 48 hour upregulation of TP63 mRNA with the bronchospheres exposed to 5 minutes photocuring showing a greater induction than seen with 1 minute photocured gels (**Figure 4.10A**). FOXJ1 mRNA expression followed this pattern (**Figure 4.10B**), whilst TGF-β1 mRNA showed steady mRNA expression by 72 hours (except donor 3) (**Figure 4.10C**).

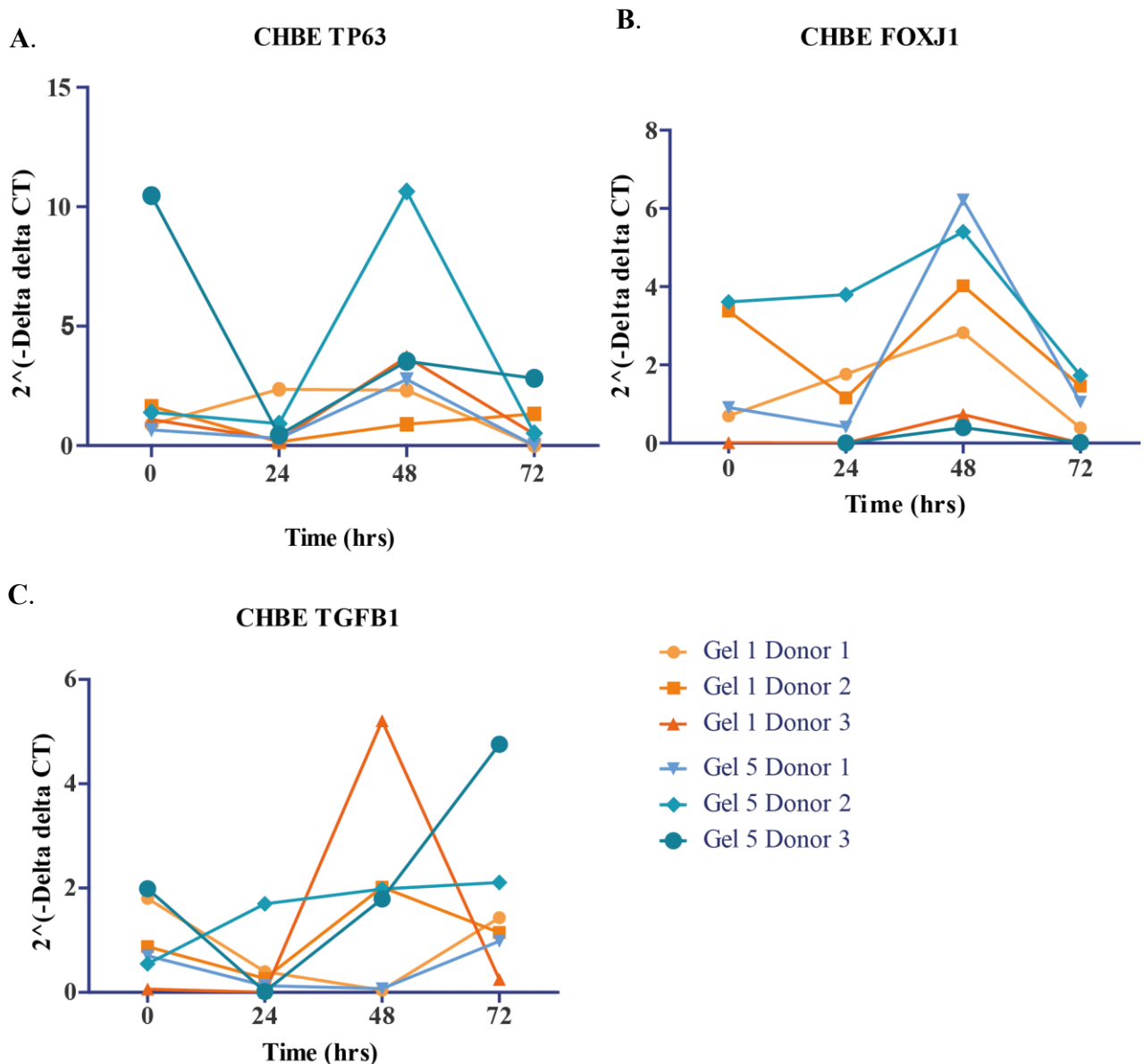


Figure 4.10: Expression levels of tumour protein P63- TP63(A), Forkhead Box J1- FOXJ1(B), transforming growth factor 1- TGFβ1(C). RT-qPCR gene expression **normalised to PMET gel stimuli on bronchospheres without photocuring (light)**. 0-72 hour time series performed following from encapsulation of COPD human bronchial epithelial cell (CHBE)-derived organoids in PMET gel. Photocuring was performed for either 1 minute (Gel 1) or 5 minutes (Gel 5), with bronchospheres from each donor subjected to both conditions, n =3.

4.4 Discussion

4.4.1 MUC5AC AND MUC5B

The gel-forming secretory mucin MUC5AC has been labelled as an inflammatory response mucin, produced in response to external stimuli as it is upregulated in mucobstructive diseases like COPD and cystic fibrosis (Whitsett, 2018, Okuda et al., 2019). Evidence for the induction of MUC5AC mRNA expression due to stiffening alone was seen at 48 hours particular after embedding in the 5 minute photocured gel. This induction of MUC5AC mRNA was greater and more prolonged in bronchospheres derived from CHBE cells than those derived from NHBES. Hypersecretion of MUC5AC can also be an indication of goblet cell hyperplasia (Hovenburg et al., 1996, Fahy and Dickey, 2010, Okuda et al., 2019). Single cell RNA-sequencing analysis of bronchospheres exposed to different degrees of stiffness may identify whether this occurs and is different between NHBES and CHBEs. This is also seen at protein level by ICC analysis. MUC5AC protein expression was distributed both within the bronchosphere and in particular MUC5AC was secreted into the lumen and often co-localised with MUC5B. Importantly, most donors produced more MUC5AC and MUC5B mRNA and protein, as detected by ICC, over time i.e., after embedding in photocured matrix.

Mucus plugs containing MUC5B are associated with impaired mucociliary clearance in fibrotic respiratory diseases such as IPF (Conti et al., 2016a, Nakano et al., 2016, Whitsett, 2018, Okuda et al., 2019). Since MUC5B is the more abundant mucin in the airways, particularly in the bronchi and smaller bronchioles and is localised to the cellular membrane, if there is a surplus production it would be more evident than MUC5AC. Photo-exposure of the PMET gel for 5 minutes induces a YM closer to that seen with severe forms of fibrotic lung disease, therefore being a stronger stimulus than the 1 minute photocured PMET gel. NHBES bronchospheres secreted more MUC5B protein compared to the control following embedding in 5-minute photocured gels, due to the generation of the pseudofibrotic microenvironment. Interestingly, the time-course of MUC5B expression at mRNA level was earlier in bronchospheres derived from CHBEs compared to NHBES before waning by 72hrs.

The overexpression of MUC5AC and MUC5B by the majority of donors supports the hypothesis proposed at the start of the chapter, in that change in matrix stiffness itself (increase in stiffness) could generate a disease phenotype in the absence of other external

stimuli. The mechanism(s) by which MUC5AC and MUC5B is induced by increase in stiffness may be explained by activation of extracellular signal-related kinase (ERK) dependent pathways through compressive stress induction, particularly MUC5AC as seen in past western blot data with NHBE ALI cultures (Li et al., 2012). However further investigation is required. This would include exploring the interactions seen between the cell and the surrounding matrix, whereby mechanical cues sensed is transduced to biochemical cues within. There are other pathways and mechanisms that have been of recent interest, concerning such mechanobiological signalling e.g. the hippo pathway involving the roles of transcriptional coactivators YAP/TAZ (Pocaterra et al., 2020) as well as Piezo ion channels which can influence cellular behaviour by mediating mechanical changes in the matrix back to the cell (Murthy et al., 2017). Additional proteins which are involved in perpetuating chronic inflammation, such as chitinase-like YKL-40 may also be investigated, as previous data show upregulation of MUC5AC mRNA as YKL-40's presence also increases in 16HBE (human bronchial epithelial cell line) culture (Liu et al., 2013). The colocalization of stimulated mucins from the increased stiffness may also highlight the need for further optimisation of this project's disease model; introducing a range of stiffnesses to reflect the small and large airways as well as the initial source of cells used, to fine-tune and mimic specific locations of the lung associated with each disease (as explained further in the summary).

Previous research studies correlate with the results seen in the qPCR data whereby samples derived from donors who have dysregulated ECM and a degree of fibrosis (due to COPD and IPF) result in an increase in mucus production. Increased expression (IHC) of MUC5AC have been reported in the bronchial submucosal glands of COPD patients compared to healthy donors and smokers with 'healthy' lung function (Caramori et al., 2009) with elevation in conjunction with increasing severity or the degree of smoking history (Caramori et al., 2004). This highlights the relationship between MUC5AC and the presence of inflammatory mediators like cigarette smoke. In the study from *Caramori* and colleagues, MUC5B was also found to increase but this may be attributed to higher baseline concentrations (Caramori et al., 2009). Data from the SPIROMICS cohort also show greater expression of MUC5AC in the bronchial epithelium of current and former smokers with COPD and that this correlated inversely with FEV1 versus healthy controls (Radicioni et al., 2021). Additionally, 3D in-vitro modelling of the airway epithelium via air-liquid interface (ALI) cell culture also agreed with the qPCR assay's results; MUC5AC was found to increase

18-fold in bronchial epithelial cells from smokers (cigarette smoke as harmful inhaled particles causing prolonged inflammation and a potent driver towards pulmonary fibrosis) compared to non-smokers (Groiss et al., 2022).

4.4.2 KI67, SOX2, CDKN1A AND CDKN2A

KI67, a known proliferative marker, is expressed in the nuclei and present amongst dividing cells. SOX2, a transcription factor is also associated with proliferation, binds with co-factors e.g., OCT4 to promote cell differentiation and pluripotency (Gontan et al., 2008, Que et al., 2009, Tompkins et al., 2011). Upregulation of KI67 and SOX2 mRNA was seen in both NHBs and CHBs, however, the induction of KI67 mRNA appeared to be more variable than that of SOX2 mRNA. Biological variation between donor data sets does also play a part in results and it is most likely that a bigger sampling number is required to form a true conclusion since no true pattern was observed. This may reflect differential proliferation occurring in different cell types within the bronchospheres and could be addressed using scRNA-sequencing. In parallel to NHBs, SOX2 upregulation in CHBs is noted but a shift is seen towards 48 hours which may be due to delayed mechanotransduction signalling after initial photocure in **Figure 4.8B**. The 5 minute photocured matrix amplifies the fibrotic modelling further with majority of donors showing continued expression at 72 hours (donor 2 potentially as an outlier). The reasoning of delayed response to the more rigid matrix may be partially due to impaired signalling occurring within the organoid. Additionally, the YM of the 5 minute photocured matrix is in the range of ~138kPa (138 +/- 23 kPa); an albeit more severe fibrotic microenvironment that is more of a stimulus to the COPD bronchospheres in contrast to the 1 minute photocure counterpart. These organoids could theoretically have memory of the local microenvironment they were exposed to *in vivo*. KI67 presence was also variable amongst donor samples; even though there was a consistent downregulation at 24 hours, no trend was seen at 72 hours.

This data suggests that increasing the matrix stiffness relays a signal from the surrounding stiffened matrix, stimulating the proliferation of the various cell types present in the bronchosphere (Whitsett, 2018, Eenjes et al., 2021a). Increased expression of Ki67 positive cells have been reported in the airway epithelium of patients diagnosed with COPD GOLD stage 2 but interestingly, not GOLD stage 3 or stage 4 or in current smokers. This has been suggested to result from the presence of cellular exhaustion as severity progresses (Tam et al., 2019). This increase was also reported in a separate study, in conjunction with receptor patched homolog 1 (PTCH1) of the hedgehog signalling pathway, linked with pulmonary fibrosis (Kugler et al., 2015). Although no recent studies could be located concerning SOX2 and lung fibrosis specifically, past reports also include the co-expression of SOX2 and

MUC5B in the dysregulated mucosal epithelium airway lining of patients with IPF, implying its role in the drive towards unregulated epithelial cell differentiation (Plantier et al., 2011). The displayed upregulated trend of SOX2 seen with the NHBEs and the CHBEs (with a 24 hour shift in upregulation) correlate with this induced proliferative behaviour from, in this project, using only matrix stiffness as a stimulus.

Additionally investigated in parallel are the two cell cycle inhibitors, CDKN1A (p21) and CDKN2A (p16), prominent markers in cellular senescence and quiescence (Barnes et al., 2019). CDKN1A mRNA expression over time increased, following exposure to photocuring and following the expression seen with SOX2. CDKN1A (p21), inhibits cell cycle progression by regulating cyclin dependent kinase complexes at G1. This can be due to several issues, including cellular and ECM stress (Barnes et al., 2019). Stiffening exerted by the microenvironment may lead to cycle checkpoints being activated to arrest cell division, not just at G1 but also at the S stage where DNA is synthesised (Barnes et al., 2019). CDKN2A's inhibitory effect (caused by oxidative stress within the cell (Barnes et al., 2019)) appeared to be less influential, as downregulation is seen from 24 hours onwards (potential outlier with donor data set 2) in **Figure 4.7D**, whilst SOX2 was still expressed by the 48-hour mark. As CDKN2A's pathway converges with CDKN1A activation to initiate cell arrest, it may be that a cumulative effect is occurring with both inhibitory markers to counteract proliferation, although this is donor specific as some SOX2 expression was seen by 72 hours. Even though past data in literature alludes to a stiffer matrix driving proliferation, most results are based on 2D or monolayer-based cell culture. The 3D structural shape of the bronchosphere accompanied by the non-degrading PMET gel may also be a limiting factor to how much 'growth' there can be, in terms of size- therefore influencing the results concerning the proliferation markers.

The results match qPCR data of peripheral lung tissue of COPD patients (particularly those with severe COPD) whereby elevated CDKN1A and CDKN2A levels have been reported (Acquaah-Mensah et al., 2012) as well as being amongst the top 10 differentially expressed genes using microarray sequencing data from COPD biopsy samples versus those from healthy control subjects (Yang et al., 2020). With the qPCR peripheral tissue data, the stiffness generated from photocuring the PMET gels (e.g., 1 minute) could correlate with the small airway and peribronchial fibrosis seen COPD (Barnes, 2019). This link between fibrotic respiratory diseases and cellular senescence is well known. Repetitive insults to the lung epithelium due to cigarette smoke can lead to continuous oxidative stress- a known

inducer of senescent markers (Birch et al., 2015). Telomere shortening with upregulated proliferation can also induce these markers, relating to IPF (Alder et al., 2008); IPF - modelled bleomycin treated murine epithelial cells and fibroblasts and human IPF tissue sections both exhibiting higher expression of CDKN1A and 2A mRNA and protein (RT-qPCR and IHC respectively) (Schafer et al., 2017).

The inhibitors of cellular proliferation CDKN1A and CDKN2A (**Figure 4.8C** and **4.8D** respectively) displayed opposite patterns of expression seen to the proliferative markers investigated in CHBEs, similar to NHBEs. Direct association can be hypothesised between SOX2 and CDKN1A. High level of upregulation at 24 hours for CDKN1A was present whilst SOX2 became inhibited at this timepoint. This switched by 48 hours, allowing for SOX2 to be expressed. Downregulation of CDKN2A was seen at this point too before all correlation dissipated at 72 hours.

There are suggestive impaired signalling pathways in COPD, due to the epigenetic changes from continuous noxious particle exposure (e.g. cigarette smoke (Liu et al., 2010a, Ryan et al., 2014)) and these may affect bronchosphere structure and cellular composition e.g. more mucus and truncated or fewer cilia (Yaghi and Dolovich, 2016, Gohy et al., 2019, Thomas et al., 2021). The expression of proliferation markers in distinct cell types may have functional consequences for those cell types, even if mRNA for inhibitory checkpoint proteins is upregulated in the bronchospheres overall and vice versa. The use of scRNA-sequencing may help resolve this issue.

4.4.3 TP63, FOXJ1 AND TGF β 1

Basal cell hyperplasia is one of the hallmarks of emphysema and bronchitis, linked to the airway modelling of COPD (Hadzic et al., 2020). TP63 expression of the NHBEs (**Figure 4.9A**), highlighted at 48 hour reflects this (Gontan et al., 2008, Ochieng et al., 2014), with change occurring from 0 hours and the initiation of a stiffened matrix. There was considerable variation in the response amongst the NHBE donors, with no distinction between 1 minute and 5 minutes photocuring. Such thickening of the basal progenitors then translates to growth being somewhat cell specific, as **Figure 4.9B** displays ciliated cells with marker FOXJ1 becoming progressively downregulated from 0 hours in the NHBE bronchospheres; in stark contrast to the goblet cell marker MUC5B in **Figure 4.2B**. The stiffened matrix from the photocure may, in turn, have an overall impact on the cilia even though they are internal structures in this bronchosphere model. Reduced number of cilia is a common trait of COPD, alongside reduced beating frequency and truncated length (Yaghi and Dolovich, 2016, Thomas et al., 2021). The same upregulation at 48 hours for TP63 also relates back to the SOX2 (**Figure 4.8B**) expression pattern, inferring increased proliferation of mainly goblet and basal cells. Uncontrolled proliferation of basal cells is linked to the tissue remodelling seen in fibrosis with p63 positive basal cells being reported to undergo epithelial to mesenchymal (cell) transition (EMT) due to being in close proximity of fibroblastic foci. These cells act as progenitors or driver cells in IPF with the epithelium being characterised by the loss of epithelial marker e-cadherin and the gain of the fibroblast/mesenchymal marker n-cadherin (Jonsdottir et al., 2015).

The expression of the proliferative marker KI67 is also described, together with trophoblast cell surface antigen 2 (TROP2), which is highly expressed in hyperplastic basal cell regions, by immunostaining of lung tissue samples from COPD donors but only moderately expressed in smokers, when compared to non-smoker controls (Liu et al., 2016). Once more, EMT markers were also upregulated in basal cell lysates, with protein levels of e-cadherin significantly decreasing as TROP2 increases whilst the expression of the mesenchymal marker vimentin is also increased (Liu et al., 2016).

TGF- β 1, part of the TGF- β family and a known profibrotic signal in airway remodelling (Takizawa et al., 2001, Saito et al., 2018, Zhou et al., 2018), showed mixed gene expression profiles at 24 and 48 hours, with generally the 5-minute stiffer stimuli producing a

higher response in the NHBEs (**Figure 4.9C**). This coupled with upregulated TP63, and a comparative downregulation of ciliated cells suggests a level of de-differentiation taking place as well as a shift towards EMT (Turner et al., 2011, Pain et al., 2014, Osei and Hackett, 2020). The expression of TGF- β was elevated in COPD airways and is associated with reprogramming of the multilayer epithelium and myofibroblast differentiation that together ultimately result in limiting airflow (Saito et al., 2018).

The expression of the basal cell marker P63 was also upregulated at 48 hours in CHBEs as seen in NHBEs (**Figure 4.10A**). The expression profiles seen in bronchospheres derived from CHBEs displayed upregulated FOXJ1, with 5 minutes stimuli exhibiting higher fold change from the 1 minute photocure and the control (**Figure 4.10B**). The sustained up-regulation of TGF- β 1 expression at 72hrs could amplify the remodelling induced by the change in microenvironment/stiffness, especially in the severe fibrosis-like YM of the 5 minute photocured matrix (**Figure 4.10C**). All 3 peaks in **Figure 4.10** at 48 hours correspond with the peak SOX2 expression at 48-hour (**Figure 4.7B**) suggesting that basal cell proliferation occurs before differentiation. The rise in the FOXJ1 population in CHBEs may also mark the start of cell trans-differentiation to increase the number of goblet cells (Turner et al., 2011). As the NHBEs mark the rise of MUC5B, **Figure 4.4A** alludes to the cellular increase in goblet cells with inflammatory responsive MUC5AC in CHBE bronchospheres, indicating goblet cell metaplasia (Hovenburg et al., 1996, Boucherat et al., 2013). Ciliated cells have been reported to act as goblet cell progenitors in the airways when induced with IL-13 (Turner et al., 2011), a cytokine implicated in pulmonary fibrosis (Zhu et al., 1999, Wijssenbeek et al., 2018). The data suggests that altering the matrix may also act as an inducer of fibrosis, leading to the increase of MUC5AC mRNA.

4.4 Summary

Grouped together, the results show indications of induced phenotypic behaviour of pulmonary fibrotic disease in the bronchospheres; increased mucus expression and cell proliferation from solely varying the stiffness of the surrounding matrix. Elevated levels of paralogs of mucin mRNA have been found in both healthy and COPD-derived human bronchial epithelial cells, in comparison to bronchospheres encapsulated in uncured PMET gel counterparts. MUC5AC, identified as a response mucin in respiratory disease like COPD, was expressed in CHBE bronchospheres and exaggerated with the 5 minutes stimuli. The longer photocure created a stiffer matrix, acting as a stronger pro-fibrotic marker than the 1 minute photocure. The opposite trend was seen with MUC5B instead, associated with mucosal plugs in IPF pathogenesis from excessive secretion, further upregulated with 5 minutes photocure in NHBE bronchospheres. Evidence suggests that transdifferentiation may be occurring regarding the CHBEs, in parallel as the ciliated cell marker FOXJ1 expression increased along with that of TGF- β 1 (a profibrotic/inflammatory marker). This data also suggests that epithelial cell de-differentiation may be occurring with FOXJ1 downregulation and upregulation of TP63 in NHBEs (signs of thickening of basal cell layer) are displayed in comparison.

SOX2 and cell cycle inhibitors CDKN1A and 2A were also both upregulated, with CHBEs having a delayed response to the stiffened matrix induced at day 0, in comparison to NHBEs. Increased expression of proliferative markers- notably SOX2 may be the cause of increased mucins (as shown in confocal imagery), inferring goblet cell hyperplasia. Impaired inhibitory pathways in CHBEs are displayed as both 1A and 2A expression increased in tandem with SOX2. However basal cell hyperplasia is also reflected via TP63 upregulation in both NHBEs and CHBEs.

Upon review, there are many publications dedicated to research using lung organoids - specifically in culturing techniques (Barkauskas et al., 2017), whereby similar bronchospheres or least spheroids are manufactured. Choice of this method for in-vitro modelling, extends to other organ biomimics and can even incorporate more than one cell type (e.g., dual cultures and tricultures) (Osei and Hackett, 2020, Güney et al., 2021). Research has also studied the regional stiffness changes found within the lung and its influence on neighbouring cells, mainly as reference points (Booth et al., 2012) or in

comparison to other organs (Busch et al., 2021). However, there is limited research investigating the effects of matrix stiffness on epithelial function and differentiation fate. The use of commercial basement membrane extracts and decellularised scaffolds are widely used to imitate the ECM (Booth et al., 2012, Hedström et al., 2018, De Hilster et al., 2020), but this is normally paired with single cell culture instead of using native 3D cellular structures. The advantage of my project allows for downstream analysis to occur (e.g., genomic and proteomic), looking at the direct effects of the ‘new’ scaffold, PMET gel, on self-assembled differentiated organoids like bronchospheres from healthy and non-healthy donors.

This research is best combined with existing methods as more optimisation is required. Increasing the number of biological samples was not as straightforward as planned, due to funding restrictions and access to patient samples. Relying on samples acquired from previous projects, stored in liquid nitrogen was employed instead but viability at organoid growth stage was not guaranteed- seemingly due to the variation seen amongst patients. Conclusions are dictated by the results (that the matrix stiffness is an influence, particularly demonstrating elevated expression of mucins and proliferative markers); however, I am also aware of my assay’s limitations. For the data produced to be more robust and meaningful, it would be advantageous to increase the biological replicates for both sample groups, healthy and disease (COPD). This would help to determine true outliers in data, e.g., Donor 3 Muc5AC mRNA high baseline expression in **Figure 4.1A** in contrast to the rest of the donor groups.

Many problems were encountered, especially at the RNA extraction stage and the subsequent PCR analysis. Because of the non-degradable nature of the gel (which, in turn helps to stabilise the initial stiffness measured throughout the assay time course), a laborious and harsh method was used during RNA extraction- combining manual tissue homogenisation with liquid nitrogen and following with Phenol/Chloroform (Gasparian et al., 2015). As a result, the amount of RNA captured was relatively low compared to standard monolayer cell lines. This, in turn, impacted on the amount of cDNA synthesised and limited the number of genes that could be investigated (replicates were used per gene, for each qPCR run). However, an average of 3 housekeeping genes (HK18S, GAPDH and EEF1A1) for normalisation was applied to each sample and each sample normalised to the sample encapsulated in uncured PMET gel- so if there were differences in initial RNA (and cDNA) seen between the two groups, they are first normalised by internal checkpoints to validate any changes in each sample.

The bulk of the cell samples were also obtained from the large airways/ bronchi. This may have influenced which type of secreted mucin was detected due to the stem-cell like nature of the initial basal cells, particularly with the increase in mRNA expression of MUC5B with the bronchospheres from NHBES indicating hallmarks of IPF. To fine tune the data produced, sample would ideally be sourced from the small airways instead, where COPD is normally dominant. The addition of other cell types (e.g., airway smooth muscle, fibroblasts or alveolar epithelial cells) would also be beneficial, exploring cell-cell interaction as the matrix is altered.

Supporting data figures like the immunocytochemistry images were also limited in the number of mRNA markers labelled, primarily due to the long optimisation period, finally developed at the latter stage of the PhD project, required for good functionality. Standard ICC protocols could not be used, since the architecture of the spheroid created meant that the depth of fixation, permeabilization and antibody staining had to be extended. Even the washings per stage needed to be increased and the additional step of tissue clearing, highlighted in recent protocols (Nikolić et al., 2017, Dekkers et al., 2019), were mandatory in order to obtain high quality data. The process of reversing each organoid encapsulation had to be developed, with manual mechanical extraction via needles occurring per gel, per condition, per timepoint. Whole mount staining was the preferred method of ICC, forgoing the use of cover slips and using image chamber gaskets instead, to preserve the 3D structure created when in culture. Paraffin and cryo -based sectioning were not compatible with the gel's chemistry.

Adaptations to the assay itself could also be explored. Some trends described within the qPCR results seemed to lack good correlation as expected e.g., the upregulation in cell differentiation in some cell types and corresponding proliferative markers Ki67 and SOX2 – this could be explained by the time course not having enough increments so small nuances in expression could have been missed, or that the time course was not long enough and following the trendline after 72 hours would be beneficial to understand what is occurring. There was also the potential issue of different cell types having different time-courses which needs to be studied further.

With the reduced amount of expected cDNA created and the limited number of markers explored (both bench RT qPCR and ICC), important events may have been missed. Investigating EMT markers like collagen I expression as additional markers of fibrosis would

have also been beneficial. Cells exhibited upregulated expression at various stages and uncoordinated expression of some proliferation markers is also displayed, with unstable housekeeping gene expression, stressing the need for further analysis. As the organoid is a multicellular structure, demonstrated in data analysed here as well as emerging research on newly discovered cell types (Travaglini et al., 2020), the approach of single cell analysis should be considered and is introduced at the end stage of my PhD.

Chapter 5:
Mechanisms and
impact of
Mechanotransduction

5.1 Single cell RT-qPCR

5.1.1 *Introduction*

Complex, pseudostratified structures such as bronchosphere organoids are composed of multicellular types and subtypes. Each having different functions and responding independently to external stimuli amidst cell-to-cell and cell-to-ECM crosstalk. In earlier chapters, I used a hydrogel to induce specific microenvironment conditions that mimicked human disease states which accounted for the dimensionality and viscoelasticity of the ECM (decreasing as the hydrogel stiffens). However, standard RT-qPCR may miss nuances occurring from exerting mechanical stress on individual cell types.

Analysing the data produced from RT-qPCR data in the previous chapter, the correlation between pathways were not as strong as I expected. Limiting amounts of cDNA in turn restricted the number of genes that could be investigated. However, with the use of scRT-qPCR, an individual cell's mRNA can be amplified during cDNA synthesis and then exposed to a larger number of gene probes.

Due to the recent pandemic causing the restriction and closure of most facilities, in and out of the UK, the original plan to conduct scRT-qPCR at ICL and continue with scRNA-seq for in-depth genomic analysis at the University of Groningen with the additional opportunity of learning new skills and methodologies was unable to go ahead. Instead, scRT-qPCR was conducted as the main focus to try and explore individual cell dynamics under set mechanical properties.

5.1.2 *scRT-qPCR methods*

The following scRT-qPCR analysis was performed on one biological sample from a healthy donor subject and from an aged matched single patient with COPD (donor 2: NHBE, donor 1: CHBE, see page 61). Using trend cues from bench RT-qPCR expression, the duration of the assay was shortened to 48 hours as most genes displayed the greatest upregulation at this time point. The PCR array used the Dynamic Array™ IFC chip in conjunction with the Biomark™ HD system from Fluidigm® (Fluidigm®, California, USA). The 48.48 IFC array allowed for 2304 reactions to be performed in total across 48 single cells (cDNA) with automated qPCR processing and outperforms a typical lab bench PCR assay, undertaken by one lab user.

The updated gene list (**Table 5.1**) included TaqMan probes which targeted mechanosensitive signalling genes such as **YAP1** (Yes associated transcriptional regulator, an important member of the Hippo pathway) and **CTNNB1** (Beta Catenin 1). Mechanotransductive cytoskeletal and cell adhesion-associated proteins are also assessed via the TaqMan probes **VCL** (vinculin), **DOCK5** (Dedicator Of Cytokinesis 5), **KRT4** (Keratin 4) and **CEACAM5** (CEA Cell Adhesion Molecule 5). Differentiation marker proteins have been further utilised, alongside **MUC5AC**, **MUC5B**, **FOXJ1** and **TP63**. I also examined the expression of **SNTN** (Sentan) for ciliated cells, **CSF3** (colony stimulating factor 3) and **CXCL10** (C-X-C Motif Chemokine Ligand 10) for basal cells and **MUC2** for goblet cells. Full details are described in Chapter 2 (pages 70-74).

No.	Gene	Name	Function
1	KI67	Marker of Proliferation Ki-67	Nuclear protein linked to cellular proliferation
2	SOX2	SRY-Box Transcription Factor 2	Member of the SRY-related HMG-box (SOX) family of transcription factors. Involved in embryonic development regulation as well as cell fate
3	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	Cyclin-dependent kinase inhibitor- regulator of cell cycle progression at G1
4	CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	Cyclin-dependent kinase inhibitor- regulator of cell cycle progression at G1 and G2 phases
5	TGFβ1	Transforming Growth Factor Beta 1	Secreted ligand. Member of the TGF-beta (transforming growth factor-beta) superfamily of proteins
6	MUC5AC	Mucin 5AC	Gel-forming glycoprotein (mucin) of respiratory tract epithelia. Marker of goblet cell
7	MUC5B	Mucin 5B	Another gel-forming glycoprotein (mucin) of respiratory tract epithelia. Marker of goblet cell
8	TP63	Tumor Protein P63	Member of the p53 family of transcription factors. Basal cell marker
9	FOXJ1	Forkhead Box J1	Ciliated cell marker
10	EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1	Isoform of the alpha subunit of the elongation factor-1 complex. Responsible for ribosomal enzymatic delivery of aminoacyl tRNAs. <i>Housekeeping gene</i>
11	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Part of the glyceraldehyde-3-phosphate dehydrogenase protein family. <i>Housekeeping gene</i>
12	HK18S	RNA, 18S Ribosomal	Ribosomal RNA. <i>Housekeeping gene</i>
13	ACTB	Beta Actin	One of six different actin proteins. <i>Housekeeping gene</i>
14	RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Ribosomal protein (component of the 60S subunit). <i>Housekeeping gene</i> .
15	TBP	TATA-Box Binding Protein	Part of the group of proteins for Transcription factor IID (TFIID). <i>Housekeeping gene</i>
16	HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Transferase (a catalyst, converting hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate via transfer of the 5-phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate). <i>Housekeeping gene</i>
17	RPL13A	Ribosomal Protein L13a	Member of the L13P family of ribosomal proteins (component of the 60S subunit). <i>Housekeeping gene</i>
18	YAP1	Yes1 Associated Transcriptional Regulator	Downstream nuclear effector of the Hippo signalling pathway. Role in development, growth, repair, and homeostasis
19	ACE2	Angiotensin Converting Enzyme 2	Angiotensin-converting enzyme (2) family of dipeptidyl carboxydipeptidases

20	MUC2	Mucin 2	Glycoprotein member of the mucin protein family
21	CEACAM5	CEA Cell Adhesion Molecule 5	Cell surface glycoprotein. Part of the carcinoembryonic antigen (CEA) family of proteins. Associated in cell adhesion, intracellular signalling and tumour progression
22	CHGA	Chromogranin A	Part of the chromogranin/secretogranin family of neuroendocrine secretory proteins
23	KRT4	Keratin 4	Part of the keratin gene family. Expressed during differentiation of epithelium
24	SNTN	Sentatin	Located within cilia- cilia apical structure protein
25	CSF3	Colony Stimulating Factor 3	Part of the IL-6 superfamily (cytokines)
26	CXCL10	C-X-C Motif Chemokine Ligand 10	Chemokine of the CXC subfamily and a ligand for the receptor CXCR3
27	CTNNB1	Catenin Beta 1	Part of a complex of proteins that form adherens junctions (AJs)
28	CRIM1	Cysteine Rich Transmembrane BMP Regulator 1	Transmembrane protein (six cysteine-rich repeat domains and an insulin-like growth factor-binding domain). Associated with tissue development
29	DOCK5	Dedicator Of Cytokinesis 5	Part of the dedicator of cytokinesis protein family
30	VCL	Vinculin	Cytoskeletal protein (cell-cell and cell-matrix junctions)

Table 5.1: List of genes to be investigated using scRT-qPCR in bronchospheres derived from a single healthy donor and from a patient with COPD. Definitions sourced from the Genecard database (Stelzer et al., 2016, Safran M, 2022).

5.1.3 Results

5.1.3.1 scRT-qPCR of NHBE bronchospheres

0hrs

In **Figure 5.1A**, it was not expected to observe large differences in gene expression following 1 and 5 minutes photocuring, with the NHBEs. However, in the same figure, some differences in gene expression at baseline (t=0) were seen, potentially reflecting rapid changes associated with matrix stiffness. For example, the violin plots for some of the housekeeping associated genes such as **GAPDH**, **RPLPO**, **ACTB** displayed a thicker density, a wider distribution along the log₂EX axis. This variation was reflected in standard bench RT-qPCR as the Ct value is not seen to be stable when the stimuli condition was altered (PMET gel matrix). Further variation in width, although less significant when compared to the housekeeping genes, was seen with **Ki67**, **YAP1**, **TGFβ1**, **SOX2** and **CTNNB1**. The housekeeping genes **EEF1A1** and **RPLPO** displayed 2 subpopulations as indicated by the violin plot with a bimodal expression. Other housekeeping genes also varied rapidly in expression, for example **HK18S** was very abundant in expression, although less so in the 5 minutes PMET gel at 0hrs. As a comparison, the same genes investigated in the bronchospheres from the 25% Matrigel® culture (**Figure 5.1B**) were relatively stable across the 48 hour time period.

24hrs

At 24 hours, the width distribution of mRNA expression varied between 1 minute and 5 minutes of photocured gel, with genes **CTNNB1**, **CXCL10**, **FOXJ1** and **Ki67** (to some extent) (**Figure 5.2A**). After 5 minutes of photocuring, the expression of **CTNNB1**, **CXCL10** and **FOXJ1** mRNAs were upregulated, compared to the 1 minute photocured gel counterparts. The bimodal pattern of the 2 subpopulations of the housekeeping genes **EEF1A1** and **RPLP0** observed at 0hrs, were still seen at 24hrs. In contrast, most genes were stable in the pseudocontrol culture (at 25% Matrigel®) except for **Ki67** and **TP63** mRNAs which showed some upregulation (**Figure 5.2B**).

48hrs

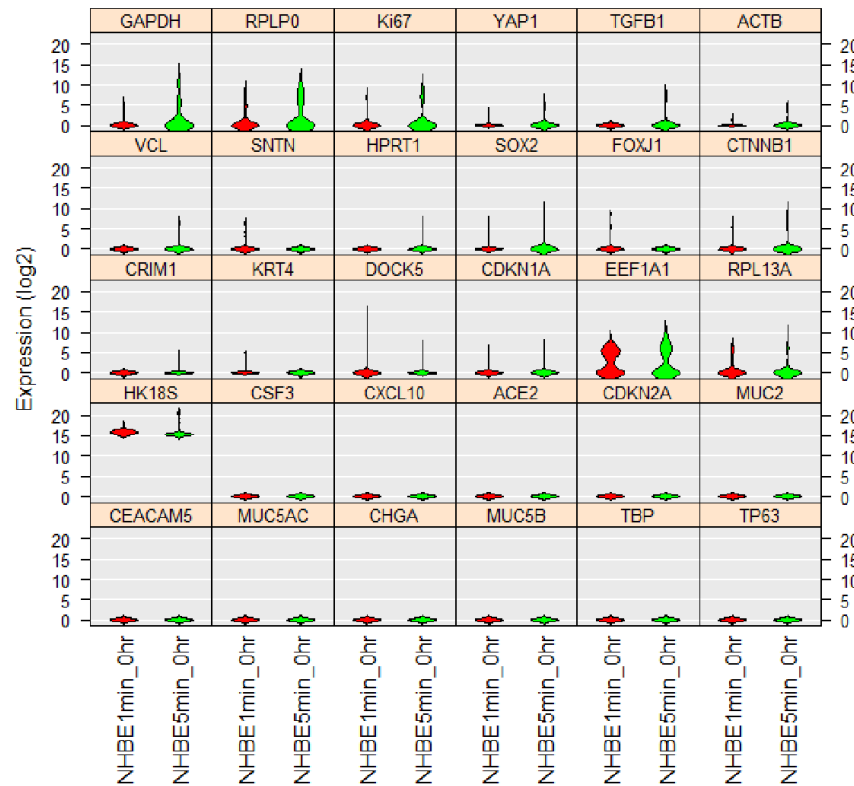
Reduction in the expression of most genes was seen after 48hrs (**Figure 5.3A**) except for the housekeeping genes **HK18S**, **RPL13A**, **EEF1A1** and **RPLP0**. Constant low-level expression was seen with **Ki67** (from the previous 24hrs) and **SOX2** (upregulated in 1 minute

PMET gel from 24hrs). This profile differed to the 25% Matrigel profile (***Figure 5.3B***) whereby gene expression was relatively stable again.

Figure 5.1: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from NHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of NHBE after 1 min vs 5 min of photocuring and measured at baseline (0 hours). (B) Gene expression of NHBEs at 25% Matrigel at 0, 24 and 48 hours (control).

A.

Violin Plot of Gene Expression



B.

Violin Plot of Gene Expression

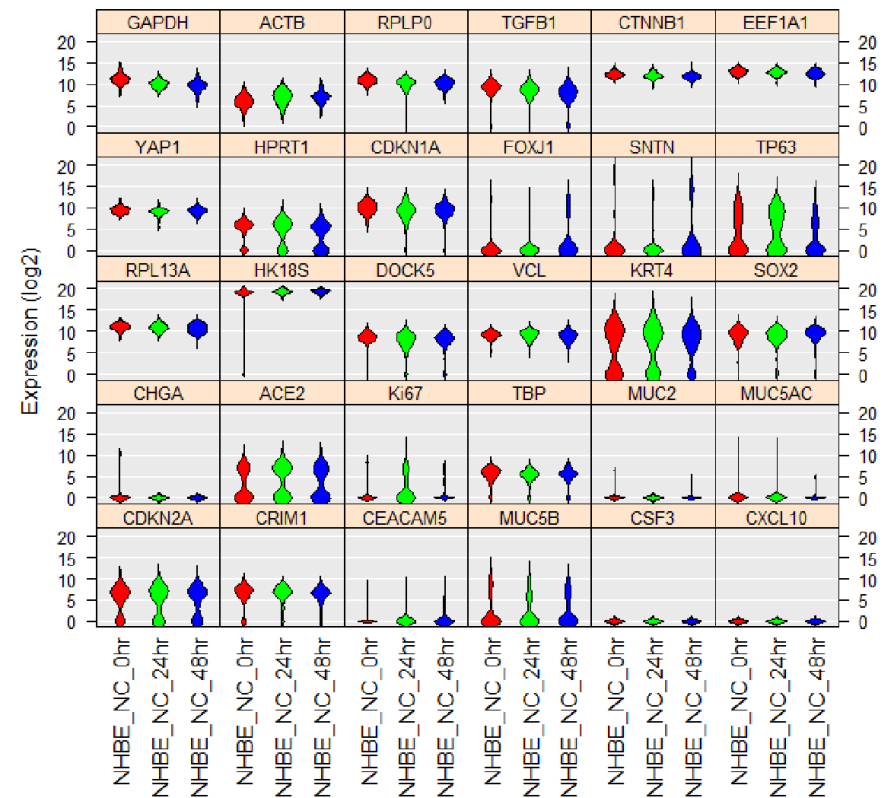
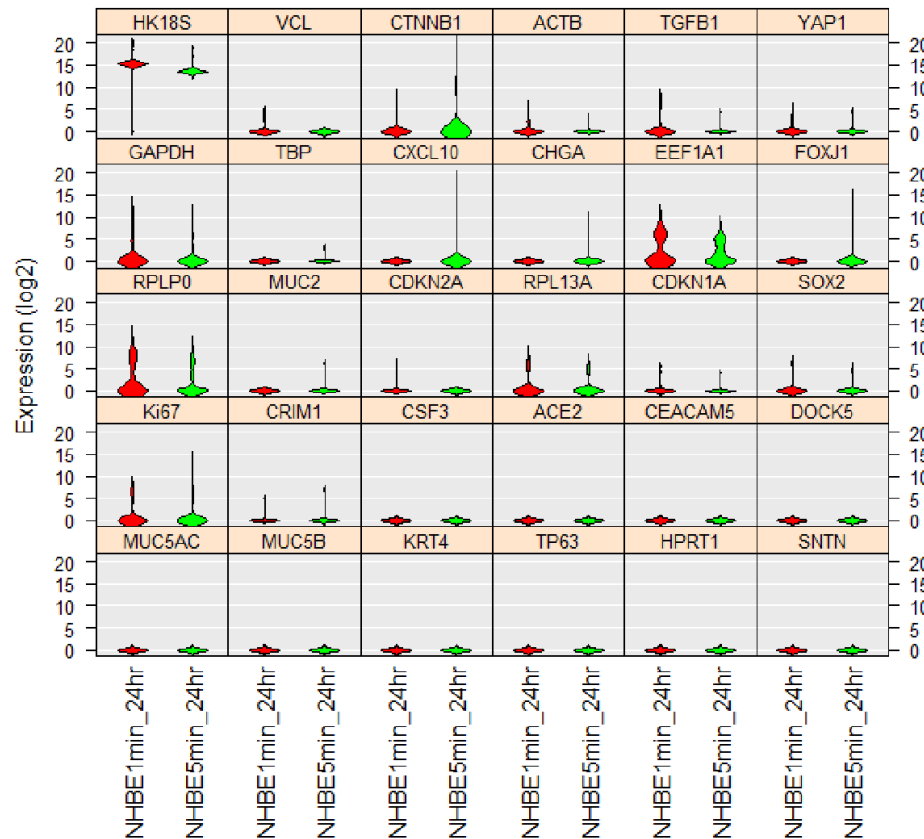


Figure 5.2: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from NHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of NHBE after 1 min vs 5 min of photocuring and measured after 24 hours. (B) Gene expression of NHBEs at 25% Matrigel at 0, 24 and 48 hours (control).

A. Violin Plot of Gene Expression



B. Violin Plot of Gene Expression

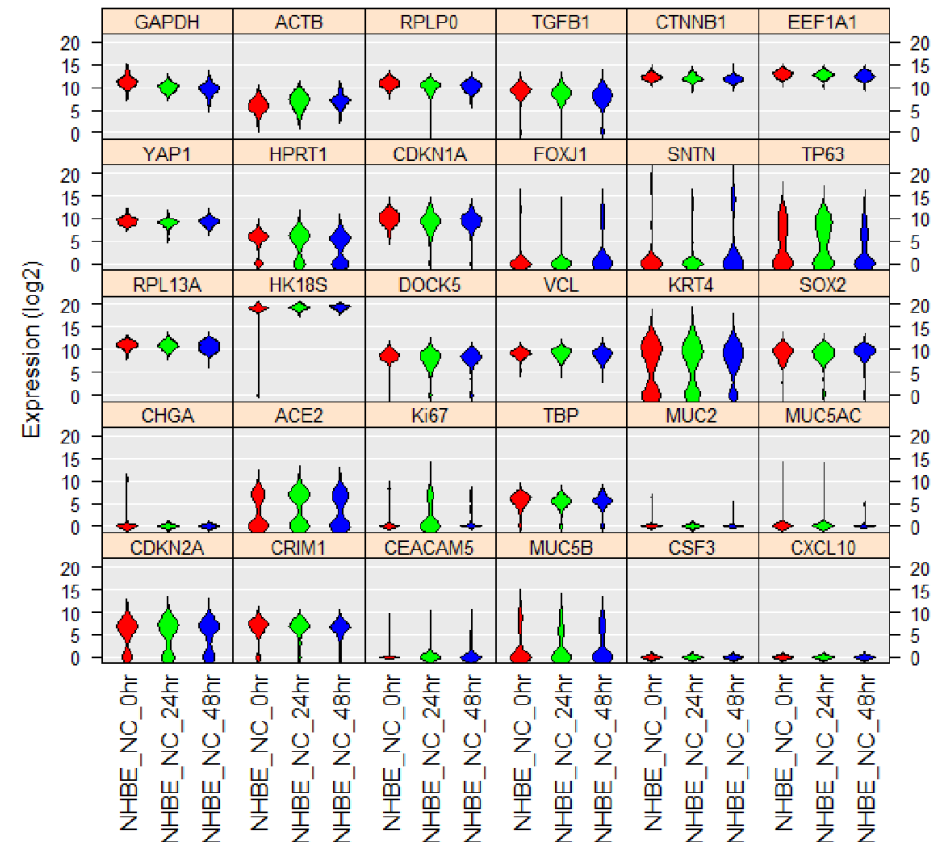
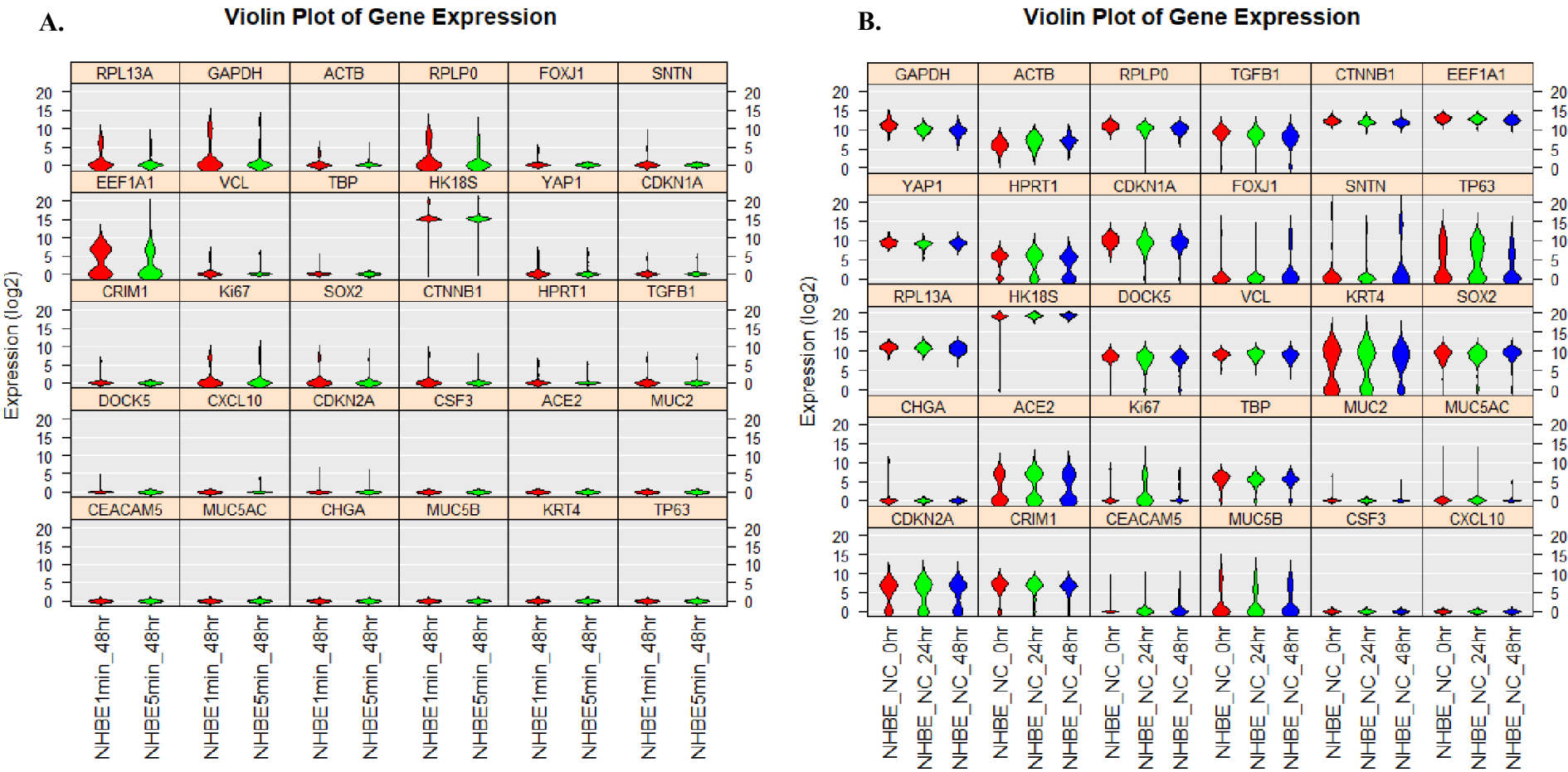


Figure 5.3: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres derived from NHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of NHBE after 1 min vs 5 min of photocuring and measured at 48 hours. (B) Gene expression of NHBEs at 25% Matrigel at 0, 24 and 48 hours (control).



5.1.3.2 Single cell RT-qPCR of CHBE Bronchospheres

0hrs

Housekeeping genes **RPL13A**, **EEF1A1**, **RPLP0**, **GAPDH** and **HK18S** in *Figure 5.4A* all displayed sub populations from 0hrs after photocuring, in contrast to the cells devolved from 25% Matrigel conditions (where just one population was seen). Housekeeping **TBP** went against this trend, with mRNA expression of just one population (singular width) being higher in 5 minute photocured PMET instead of 1 minute, alongside **MUC5B**. The mechanosensitive genes (**YAP1**, **VCL**, **CTNNB1** and **DOCK5**) were reduced in expression, in one of the subpopulations displayed within the 5 minute plots. The same trend was seen with plots of **SOX2**, **Ki67**, **ACTB**, **CDKN1A** and **KRT4**. The same genes investigated at 0 hrs, from the bronchospheres cultured at 25% Matrigel® (*Figure 5.4B*) remained stable in comparison.

24hrs

Genes associated with mechanotransductive pathways (**CTNNB1**, **YAP1** and **VCL**), proliferation (**Ki67** and **SOX2**) and cell cycle inhibition (**CDKN1A**) showed increased expression (*Figure 5.5A*) when organoids were encapsulated within the 5 minute photocured PMET gel matrix. The increase in **CDKN1A** (p21) coincided with the upregulation of **SOX2** and **Ki67** with a more pronounced expression at 5 minutes compared to 1 minute. Bimodal subpopulations were still seen in **RPLP0** and **EEF1A1**. Downregulation of **CEACAM5** of the control culture (*Figure 5.5B*) and upregulation of **Ki67** were noted at the same 24 hours.

48hrs

Expression was mostly limited to housekeeping genes (**HK18S**, **EEF1A1**, **RPLP0**, **RPL13A**, **GAPDH**) in *Figure 5.6A*, with **YAP1**, **Ki67** and **SOX2**. Stable gene expression was reported at 48 hours (*Figure 5.6B*), alongside some upregulation of **ACE2**.

Figure 5.4: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres. Organoids were derived from CHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of CHBE after 1 min vs 5 min of photocuring and measured at baseline (0 hours). (B) Gene expression of CHBEs at 25% Matrigel at 0, 24 and 48 hours (control).

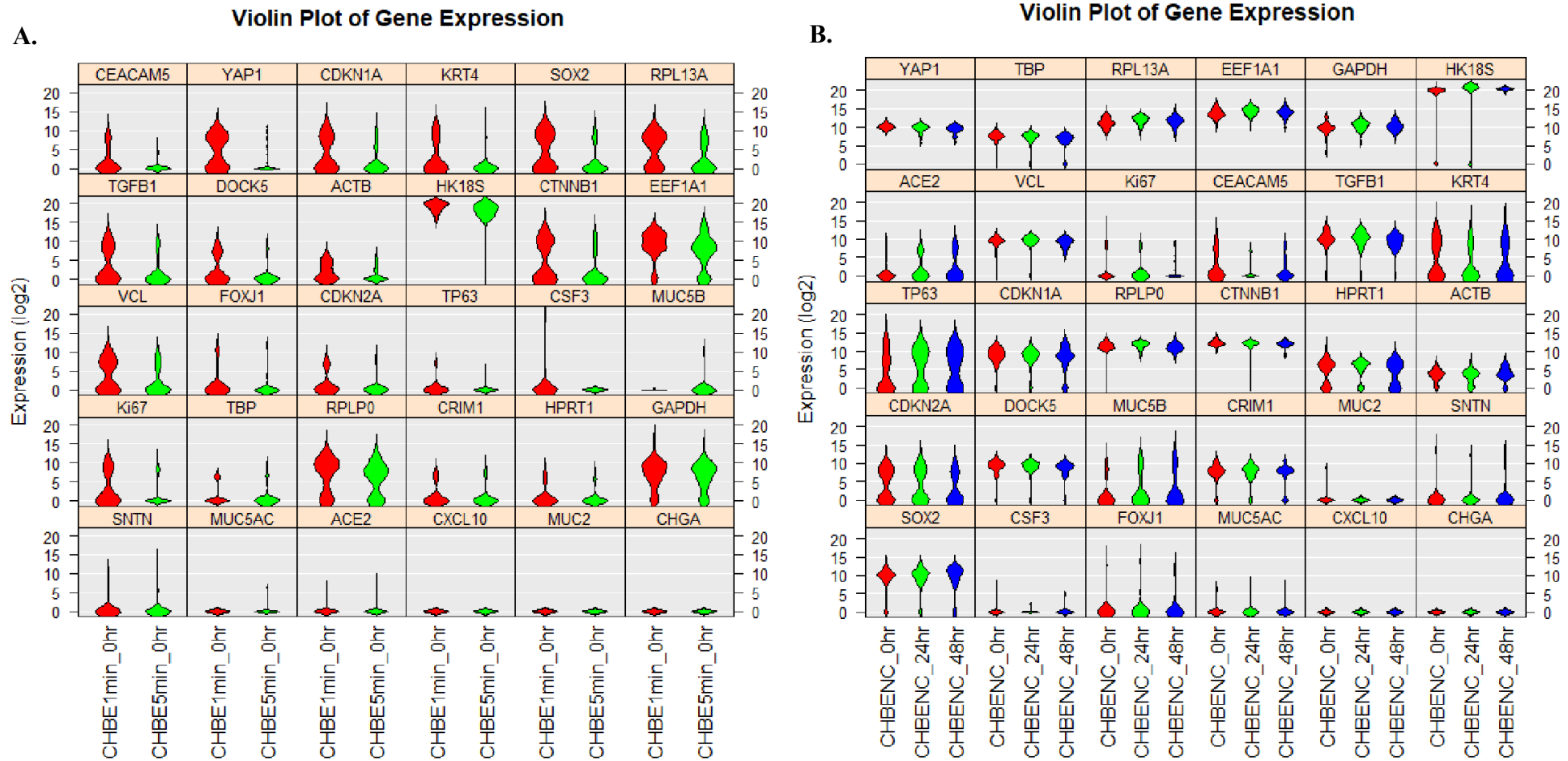


Figure 5.5: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres. Organoids were derived from CHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of CHBE after 1 min vs 5 min of photocuring and measured at baseline (0 hours). (B) Gene expression of CHBEs at 25% Matrigel at 0, 24 and 48 hours (control).

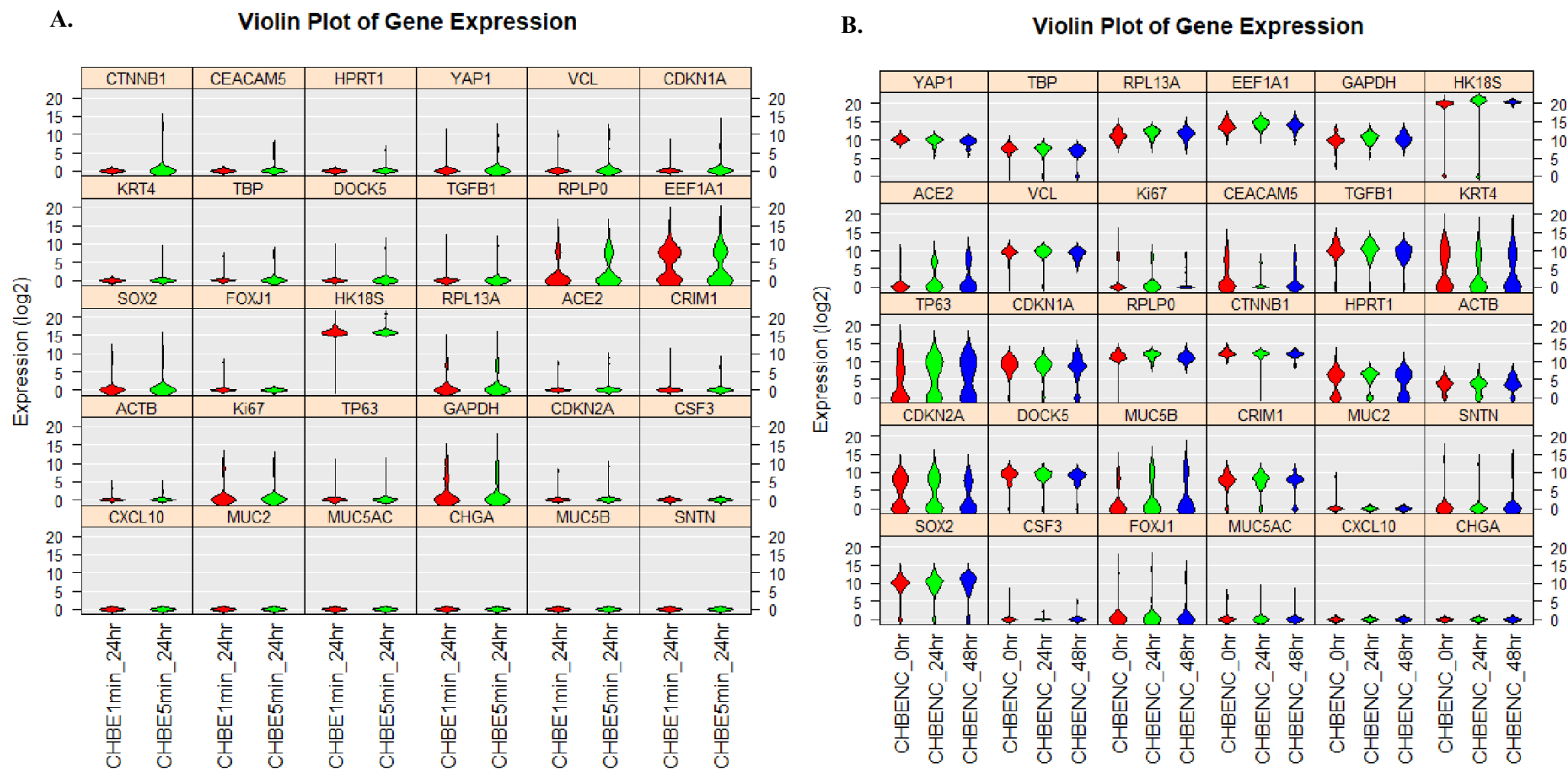
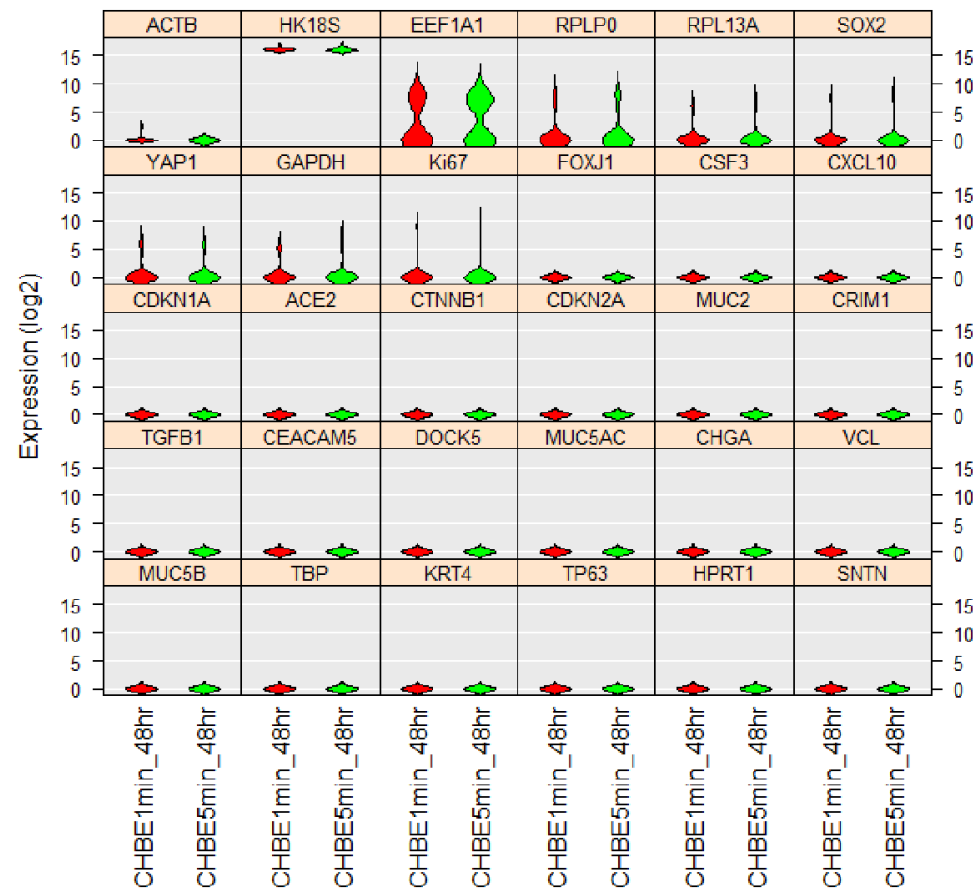


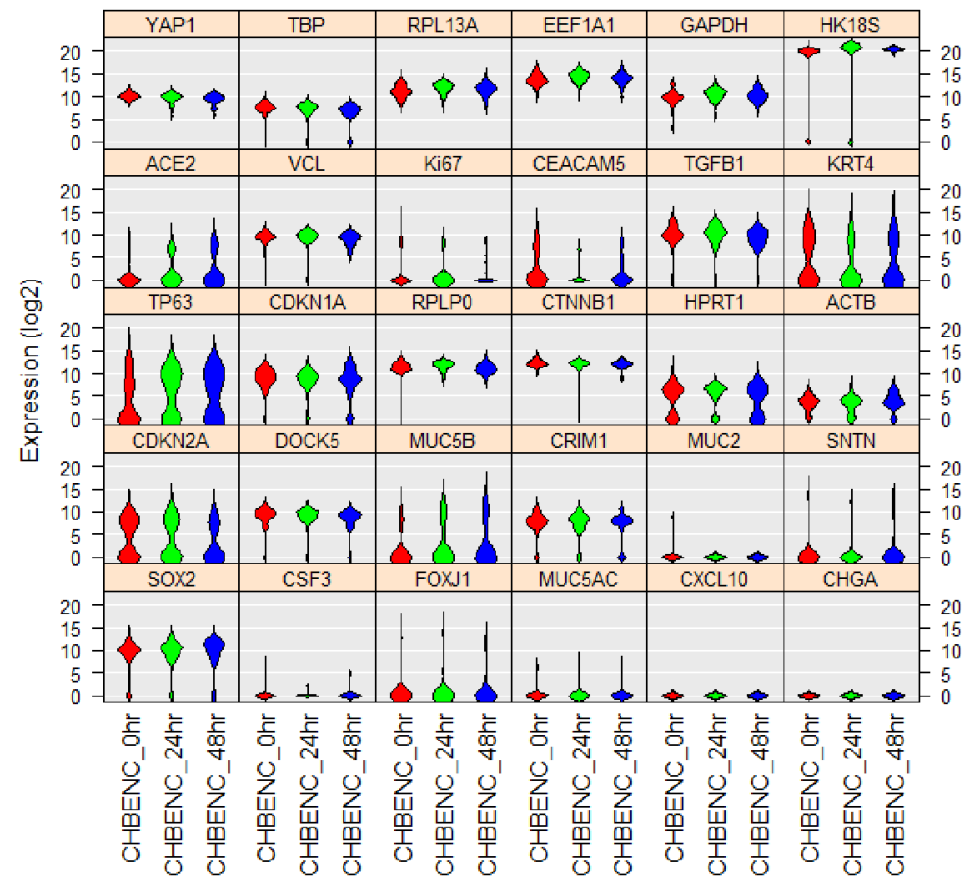
Figure 5.6: Comparison of violin plots from single cell (sc)-qPCR analysis of bronchospheres. Organoids were derived from CHBEs, photocured for 1 minute and 5 minutes. Bronchosphere RNA was extracted, amplified and cDNA was made per cell. Fluidigm® 48.48 Dynamic Array™ IFCs were used to perform RT-qPCR. (A) Gene expression of CHBE after 1 min vs 5 min of photocuring and measured at 48 hours. (B) Gene expression of CHBEs at 25% Matrigel at 0, 24 and 48 hours (control).

A.

Violin Plot of Gene Expression



Violin Plot of Gene Expression



5.1.4 Discussion

Single cell analysis allows researchers to explore in depth, developing a fuller picture of an individual cell's profile within the mix of a complex heterogeneous multicellular system – accounting for function, lineage, any fluctuations, or disturbances that is unique to one cell type vs another. This technique is ever evolving, leading to whole organ single cell atlases utilising transcriptomic, proteomic, metabolomic, genetic and epigenetic data sets – undertaken by various labs and research groups worldwide (Luecken et al., 2022). These can be accessed via databases e.g., the human cell atlas data portal (www.humancellatlas.org), copdcellatlas.com and cellxgene.cziscience.com. These tools help to decipher cell-to-cell variation and interplay within a seemingly 'homogenous' system as well as helping to identify new cell types or states (Sauler et al., 2020, Travaglini et al., 2020), expanding the spatial reconstruction of the organ in focus.

There are many techniques used following isolation of single intact cell, such as scRT-qPCR and scRNA-seq (e.g., 10x Genomics). The integrity of the data produced also depends on the method used to capture the single cell suspension from their native 3D network; ranging from micromanipulation, flow-activated cell sorting to optical tweezers (Saliba et al., 2014).

The data trends acquired from scRT-qPCR of NHBE single cells displayed upregulation of genes associated with the cell cycle (proliferation, senescence) alongside mechanosensitive markers, but expression appeared capped after the first 24 hours. Single cells from CHBE donors already had greater activity at 'baseline' (0 hours) than their healthy counterparts, eliciting greater expression within the 0 hour timepoint in comparison than the whole 48 hour assay. As the stiffness of the PMET gel was altered via increasing the length of time of photocuring, changes in mRNA expression were noted amongst some of the genes studied. This suggests the existence of subpopulations according to the housekeeping genes once the matrix was stiffened in both NHBE and CHBE cells.

At 0 hours (**Figure 5.1A**), **Ki67** and **SOX2** violin plots after 5 minutes photocure were denser in comparison to their 1 minute counterparts. Their role in cellular proliferation may indicate initial growth immediately after the green light exposure or the exhibition of a higher baseline status and functionality in the 5 minute PMET gel to begin with.

YAP1 and **CTNNB1** are key players in mechanotransduction, with denser widths of their violin plots seen in **Figure 5.1A** (**YAP1** and **CTNNB1** slightly denser at 0 hrs) and **Figure 5.2A** (**CTNNB1** at 24 hrs). **YAP1** is a key transcriptional regulator mechanosensitive protein of the hippo signalling pathway and when complexed with TAZ and sequential co-activators can initiate dedicated pathways when stimulated by ECM mechanics. The violin plot data surrounding **YAP1** briefly infers with past studies in literature regarding fibrosis, further driving the increased expression of **YAP1** and therefore adding to the positive feedback of fibrosis- although more pertaining to IPF, active fibroblasts (Liu et al., 2015) and epithelial cells which may undergo EMT because of the stiffened matrix (Xu et al., 2016). There are also other reports (albeit conflicting) whereby the deficiency of YAP/TAZ may lead to a COPD (emphysema)- like profile (Mitani et al., 2009, Chakraborty et al., 2017, Xiao et al., 2018). **YAP1** expression after the 5 minute photocure confers with the former report, as the stiffness of the gel is initially increased from this point, but perhaps due to the low number of single cells used, this conclusion will require further investigating.

CTNNB1, a regulator of the canonical Wnt1/**CTNNB1** signalling pathway, encodes for the structural docking protein β -catenin, found at focal adhesions and cell-cell adherens junctions with E-cadherin (Tian et al., 2011). Cytosolic activation of β -catenin results in translocation into the nuclei, targeting cellular processes such as tissue morphogenesis (Tian et al., 2011). Induced expression from both mechanosensitive genes analysed here was expected perhaps to a larger degree, particularly by the 5 minutes stiffer PMET gel since the immediate surroundings experienced by the organoid had been altered to be considerably stiffer. However, this assumption is mainly based on data from 2D systems where mechanosignalling has been extensively investigated. It may be that the native 3D conformation in this system altered the typical mechanism response expected. These two proteins are not mutually exclusive in their signalling cascades (as described within Chapter 1) and interact in tandem to allow for downstream transcription in the cell if changes are ‘sensed’ in the ECM (Piersma et al., 2015). Such continuous signalling would lead to the progression of fibrosis via increased ECM deposition and a subsequent positive feedback loop of matrix stiffening (Herrera et al., 2018).

TGF β 1’s violin plot was also denser after 5 minutes of photocure (**Figure 5.1A**) and is a potential marker of future downstream effects on cell proliferation and/or senescence which may result in fibrosis in the human airway and contribute to tissue remodelling factors e.g. protease inhibitors (Celedón et al., 2004, Herrera et al., 2018, Saito et al., 2018, Barnes et

al., 2019). **TGFβ1** has also been implicated in facilitating the nuclearization of both **YAP1** (complexed with SMAD-2) and β-catenin during mechanotransduction (Cha et al., 2016, Hussain et al., 2017). This is plausible to suggest that the upregulation of TGFβ1 at 5 minutes compared with 1 minute is due to the photoexposed stiffened matrix having caused the YM to increase.

The bimodal expression of the known housekeeping genes **EEF1A1** and **RPLPO** suggests different cell types growing at different rates, in comparison to only one population seen in the ‘control’ samples (25% Matrigel®). This trend was also seen throughout the subsequent plots over the 48 hours measured (**Figures 5.1A, 5.2A and 5.3A**) which supports the phenomenon seen with bench RT-qPCR as the Ct values analysed were not stable once the gel’s stiffness has been altered. On the contrary, **HK18S**’s mRNA expression remained stable and abundant but in hindsight, this particular gene should not have been amplified during cDNA synthesis as the results may be biased. Its abundance is already seen in bench RT-qPCR data, with relatively high Ct values (Ct<13). Adding an amplifying step, scRT-qPCR Ct values read incredibly high (Ct<4) that the software used to analyse scRT-qPCR data consistently reported these readings as false positives. As PMET gel synthesis can be easily replicated (using a transwell mould), it is assumed that the crosslinking is homozygous throughout the gel. With bimodal expressions showing different populations, it may be that there are heterozygous ‘hotspots,’ allowing for varying stiffness and therefore influencing set cell types too.

At 24 hours (**Figure 5.2A**), the small variation in the mRNA expression of **CXCL10** was noted between the two gel states. **CXCL10**, a chemokine ligand which binds to CXCR3, mediates the recruitment of inflammatory cells such as CD8⁺ and CD4⁺ T cells (Roman and Mutsaers, 2018, Wang et al., 2018b, Yang et al., 2019). Elevated expression of **CXCL10** has been reported in bronchiolar epithelial cells of COPD patients and acts in regulating tissue remodelling of the lung (Sauty et al., 1999, Hardaker et al., 2004). Increased expression of the ciliated cell marker **FOXJ1** may indicate stimulated growth of ciliated cell subtypes particularly in light of the upregulation seen in the proliferative marker **Ki67**. These ciliated cell subtypes include intermediary goblet cells termed Deuterosomal cells (Ruiz García et al., 2019, Davis and Wypych, 2022) which possess cilia, possibly signifying goblet cell hyperplasia. However, no data on mucin markers are present to corroborate this theory. The matrix state after 5 minutes of photocuring also stimulated the expression of **CTNNB1** after 24 hours due to stiffening of the immediate microenvironment which may result in a

‘fibrotic’ milieu -having the potential to induce fibrosis around the bronchosphere (Piersma et al., 2015).

At 48 hours (**Figure 5.3A**), the combination of a relatively constant mRNA expression of some housekeeping genes **HK18S**, **RPL13A**, **EEF1A1** and **RPLP0** and of **Ki67** and **SOX2** suggests enhanced proliferation of cells within the bronchosphere following both photocuring times. In contrast to the photocured gels, the ‘control’/25% Matrigel® (**Figures 5.1B/5.2B** and **5.3B**) maintained a constant organoid growth profile. The upregulation of **Ki67** and **SOX2** throughout the assay may indicate some growth of cell subtypes that we are unable to map in the current study, which may be identified using scRNA-seq analysis. The increase in proliferation marker **Ki67** corroborates with reported presence within the COPD epithelium (Liu et al., 2016, Tam et al., 2019) in past literature, which was used in supporting the bench RT-qPCR data in chapter 4. Increased levels of Ki67-positive cells were found in airway epithelium from current smokers exclusively diagnosed with COPD GOLD stage 2 in comparison to the non-COPD control, alongside positive correlation of the pulmonary fibrosis marker **PTCH1** of the hedgehog signalling pathway (Tam et al., 2019). In relation to the basal cell marker TP63, trophoblast cell surface antigen 2 (**TROP2**) was also found in accordance with elevated levels with Ki67, in COPD lung tissue. It was expected to see a degree of increased expression with coordinated basal cell markers, reflecting data captured by bench qPCR but this did not occur. Lack of correlation may be due to reduced sample number or choice of method preparation, as further explained.

CHBEs

Single cells from CHBE bronchospheres displayed a more distinct profile than that of the NHBEs at 0 hours (**Figure 5.4A**), with higher genomic expression seen with 1 minute PMET matrix in most genes. The same bimodal population trend from the NHBE data set was seen here with the CHBEs; with the housekeeping genes **RPL13A**, **EEF1A1**, **RPLP0**, **GAPDH** and **HK18S**, but not with **TBP**, suggesting different cell types or subtypes are populating the organoid at various rates. **MUC5B**, upregulated after 5 minutes photocuring, is associated with fibrotic diseases e.g. IPF, forming plugs due to hypersecretion (Conti et al., 2016a, Nakano et al., 2016, Okuda et al., 2019) as it is the more dominant mucin in apical secretions, demonstrated typically in air-liquid interface of NHBE cell culture (Kesimer et al., 2009) and also in the RT qPCR results reported in Chapter 4. The change from 25% Matrigel® to 5 minutes photocure can act as a pro-fibrotic stimulus, with the embedded

bronchospheres experiencing a rapid and dramatic increase in stiffness reflected by the change in YM. However, this may be due to an upregulated baseline reading as opposed to being a direct impact from the stiffening of the gel. **MUC5B** is also implicated in COPD due to enhanced presence in induced sputum (Kirkham et al., 2008) and upregulated levels in bronchial lumen (Caramori et al., 2009). Despite this, MUC5AC is usually considered as a pathological marker of COPD due to its secretion from goblet cells following the induction of inflammation by inhaled smoke and other environmental pollutants. Increased expression of MUC5AC is seen in patients with COPD within bronchial submucosal glands and the surface epithelium (Caramori et al., 2009). Hyperplasia of goblet cells will also increase the production of the mucin paralog (MUC5AC). Therefore, it was expected to see a width increase in the MUC5AC violin plot as well (at 0 hours and increasing as the assay progressed) in the CHBE sc-RT qPCR data, reminiscent of the bench qPCR data but this was not the case.

Reviewing the remaining plots (**Figures 5.5A** and **5.6A**), a general consensus of downregulation was seen with 5 minutes photocure when compared to 1 minute. As the width decrease in the violin plots was noticeable on the higher end of the Log₂_{ex} scale for the mechanosensitive genes **YAP1**, **VCL**, **CTNNB1** and **DOCK5**, the proliferation of this cell subtype had also been affected. Similar changes were seen in **SOX2**, **Ki67**, housekeeping **ACTB**, cell cycle regulator **CDKN1A** and differentiation and potential basal cell marker **KRT4** plots. The reduced expression of these genes after the 5 minute photocure compared to the 25% Matrigel® may be due to the cells ‘shocked’ by being exposed to a matrix stiffness similar to that seen in severe IPF rather than COPD, acting as a sufficient inhibitor. CHBE bronchospheres would be optimal in the 1 minute gel as this correlates with ‘milder’ fibrotic lung and its assumed native microenvironment. The 5 minute model favours the extreme end of the fibrosis, experienced by IPF patients as opposed to COPD. This data highlights why it is important to use biologically relevant culture systems for disease modelling since 25% Matrigel® initially allows the organoids to proliferate exceedingly well but at the cost of losing native growth profiles akin to that seen *in situ* in the airways.

After 24 hours, there was a reduction in gene expression (**Figure 5.5A**), especially for **CTNNB1**, **YAP** and **VCL** at 1 minute photocure, whilst the impact of the 5 minute photocure was reduced. Ideally, these data should be repeated using scRNA-seq and with a greater number of organoids to check the validity of these results. **VCL/Vinculin** is a cytoplasmic 116kDa actin binding protein found abundantly at cell-cell and cell-matrix adhesion sites,

such as focal adhesions (Brown and Izard, 2015, Bays and Demali, 2017). When bound to focal adhesion kinase and other associated focal adhesion proteins such as Talin, vinculin is able to relay any extracellular force to the cell's actin cytoskeleton experienced via bound membrane integrins (Martino et al., 2018). The increase in ECM stiffness from prolonged photocuring would induce binding to the cellular cytoskeleton resulting from force transmission, priming the cell for downstream functionalities such as directional migration (Martino et al., 2018). As the PEGDA in the PMET gel model is bioinert, the 'priming' is assumedly short-lived since there is only Matrigel® present that can interact with the organoid within the synthesised PEGDA polymers. Also, the encapsulation of the bronchosphere itself combined with the non-degradable nature of the gel makes cell migration improbable. The role of **VCL** in other epithelial cell functions is worth further study.

CDKN1A (p21), **SOX2** and **Ki67** showed pronounced expression at 5 minutes compared to 1 minute photocuring which indicates cell cycle arrest and cell senescence (stronger stress stimulus due to increased stiffness) that may counteract proliferation. Bimodal subpopulations were still seen with **RPLP0** and **EEF1A1**. After 48 hours (**Figure 5.6A**), few of the 30 genes investigated were expressed with the majority being housekeeping genes (**HK18S**, **EEF1A1**, **RPLP0**, **RPL13A**, **GAPDH**), with the exceptions of mechanosensing (**YAP1**) and proliferation (**Ki67** and **SOX2**) genes. The differences between the two gel conditions appeared to be negligible after day 2, as the change in expression was replicated by both stimuli. Compared to the NHBE data, where particularly proliferation and mechanosensing genes are downregulated at this stage, the CHBEs appeared to display a higher proficiency in successfully adapting to change in its microenvironment in both stiffness conditions; or at least exhibiting some biomemory to its native ECM to regulate cellular behaviour accordingly. Although PEGDA is bioinert, the COPD bronchospheres may be producing their own ECM and therefore mitigating any inhibitory influence from the gels.

Gene expression for the majority of genes in the CHBE organoids was constant across the 48 hours in the control 25% Matrigel® (**Figures 5.4B/5.5B and 5.6B**). However, there was a noticeable drop in **CEACAM5** expression, a cell surface glycoprotein and adhesion marker, from 0 to 24 hours with a gradual recovery by 48 hours. Furthermore, there was a marked increase in **Ki67** at 24 hours-both indicating some proliferation amongst the differentiated cell types found within the bronchosphere.

A gradual increase over 48 hours was seen with **ACE2**. This was expected as **ACE2** is mainly expressed in the respiratory tract, myocardium and gastrointestinal mucosa (Leung et al., 2020) and recent findings from the COVID-19 pandemic shows that **ACE2** is upregulated in current smokers and in patients with COPD in comparison to healthy patients (Leung et al., 2020). Whilst the results from the 25% Matrigel® culture condition was anticipated, the same expression was also expected in all of the other culture conditions, especially with the stiffer 5 minute photocured gel, as this would mimic the airway matrix setting, stiffened over time due to prolonged remodelling from debilitating diseases like IPF.

Regarding limitations to this assay, unlike the bench RT-qPCR data analysis, single cells from the uncured PMET gel could not be used as the control. The cells, once dissociated, were unable to be retrieved or FACS sorted due to their unforeseen sticking to FACS tubes and tissue culture (TC) plastic. As a result, the overall expression of the genes investigated was compared to the 25% Matrigel® control culture and found to be quite downregulated. This may be due to the cell dissociation process- a combination of enzymatic and mechanical dissociation to try and remove as many viable single cells from the PMET gel matrix as possible. The cells were then subjected to single cell sorting into individual wells via FACS, which could have impacted further on the cell's viability and affected the detection of sparse cell subpopulations. Additionally, as previously mentioned briefly, **MUC5AC** and **MUC5B** mRNA expression were projected to be upregulated as key markers of mucus hypersecretion and goblet cell hyperplasia, however their absence in all NHBE violin plots and most of CHBE single cell data when derived from PMET gel embedded bronchospheres may again be due to the 'harsh' single cell dissociation process. The initial part of the dissociation process was replicated with the bronchospheres extracted from 25% Matrigel. However, retrospectively it would have been beneficial to expose these bronchospheres to the same full dissociation process as the other conditions when isolating, to try to reduce any variance seen between the conditions.

Another major caveat to the dataset above is the singular number of biological donors. Referring to bench RT-qPCR, the variation from donor to donor can be vast even without considering healthy vs 'disease'. This therefore makes the singular cell results more of a snapshot of what scRT-qPCR can produce, in preparation for scRNA-seq. Even though each individual cell is to some extent, a replicate within the whole organoid investigated, exploring just 48 cells per condition is a low sample selection. Without the imposed restrictions from last year concerning lab access and international travel and the limited time to conduct these

assays, the biological donor replicate number would have been addressed. The same also goes for the process used to dissociate the bronchospheres before FACS sorting. Research in this field whereby the starting cell culture are organoids is limited, therefore optimisation would have been quite beneficial for the process required, to try and increase the percentage of viable cells before cDNA synthesis. As stated above, this could have had a knock-on effect on the results captured, especially concerning particular genes like **MUC5AC**, **MUC5B** and **MUC2**. **MUC2**'s presence has been implicated in COPD where its expression is upregulated and bound to cilia membranes where detected (Caramori et al., 2004). Expected results included a significant increase in mucins in comparison to the control but this was not apparent.

5.2 Proteomic analysis

5.2.1 Introduction

The proteins YAP and TAZ have gained popularity in the last few years, due to its presence in the nuclei, triggering multiple mechanisms particularly organ hypergrowth. Originally discovered in the *Drosophila melanogaster* homolog Yorkie (YKi), the same downstream mediator effects are seen in humans (Huang et al., 2005, Pocaterra et al., 2020).

YAP and TAZ are transcriptional coactivators, sharing 46% of their paralogous genes (Pocaterra et al., 2020). They have major roles in the Hippo pathway, which in turn, regulates a series of complex signalling networks. YAP1 and TAZ need to bind to TEAD (transcriptional enhancer factor domain) family members to switch on gene expression. When these DNA -binding factors are activated, transcription is regulated, mainly affecting cell fate mechanisms such as proliferation since they can override senescence and in some cases, reprogramming and differentiation (Pocaterra et al., 2020).

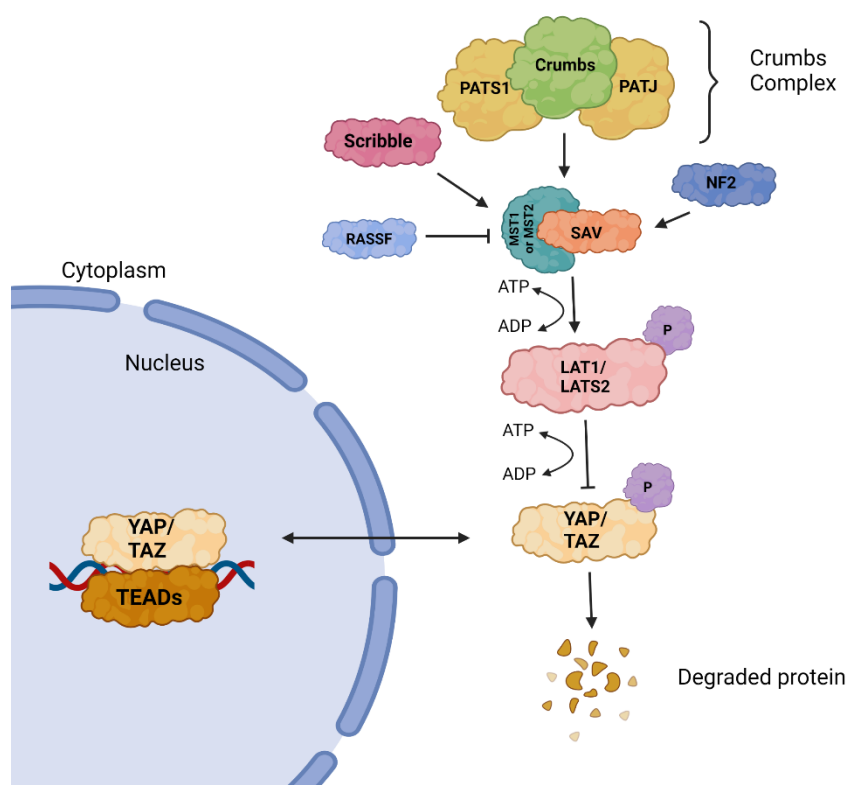


Figure 5.7: Hippo pathway schematic, displaying relevant protein components which lead to either the formation of the YAP/TAZ-TEAD complex in the nuclei, or the destruction of phosphorylated YAP/TAZ in the cytoplasm. Image reproduced from (Halder et al., 2012) and created in Biorender.com.

Within the Hippo pathway, YAP and TAZ are regulated via a cascading sequence; firstly, dedicated serine/threonine kinases (known as MST1 and MST2) and the scaffolding protein salvador family WW-domain- containing protein 1 (known as SAV1) activate the corresponding large tumor suppressor kinases 1 and 2 (known as LATS1/2) by phosphorylation (Ma et

al., 2019, Pocaterra et al., 2020). Interaction of the scaffolding proteins MOB kinase 1A and 1B (known as MOB1A and MOB1B) with YAP/TAZ enables their phosphorylation and inhibition. In the absence of phosphorylation, the YAP/TAZ complex can shuttle through nuclear pores to bind to TEAD factors and act as transcriptional transducers (Ma et al., 2019, Pocaterra et al., 2020). However, the regulation of YAP and TAZ can be activated by alternative means, including physical cues from the surrounding microenvironment and any deviation from normal healthy epithelial architecture can promote YAP/TAZ signalling (Cordenonsi et al., 2011, Dupont, 2016, Pocaterra et al., 2020).

Changes to the extracellular matrix stiffness fall into this category, exerting pressure not just on cell-cell adhesions but on tight junctions on the cell's membrane and on cell-ECM integrins and adherens. The cell's cytoskeleton is bound to cell membrane integrins, which are part of multiprotein microstructures called focal adhesions (Martino et al., 2018). These, in turn, directly link to the ECM and act as a relay of any mechanical cues sensed (Pocaterra et al., 2020). This also translates to increasing the contractibility of the cytoskeleton, promoting YAP/TAZ nuclear localisation. Actin filaments such as F-actin create tension in the cell when propagating physical force, complexed into stress fibres by dedicated proteins and follow some degree of actin re-organisation (including regulatory molecules and microfilaments) by sliding motor protein myosin II to facilitate numerous processes including remodelling and proliferation (Martino et al., 2018). The shape of the cell can also be a stimulus, as when cells are well spread across an ECM, the ability to proliferate is increased compared to cells in confined small spaces where apoptosis is more favoured (Halder et al., 2012). In terms of organoids, a high cell density is reached in the centre of the mass, particularly when a spherical, 3D shape has been formed in comparison to a monolayer on TC plastic. Cells at the edges of the organoid would allow for more proliferation, communicating through cell-cell junctions (in contact with the ECM, 'stretched' and therefore experience any tension gradients from mechanical forces and stress) versus cells in the middle or in the inner edge of the lumen (Halder et al., 2012). When YAP/TAZ is activated, it can drive ECM remodelling and hallmark features like epithelial to mesenchymal transitioning (EMT) in COPD (Lei et al., 2008, Halder et al., 2012, Pocaterra et al., 2020).

In this project, Western blots were used to probe for certain proteins which may have been upregulated from the switch of 'soft' gels to 'hard' gels (e.g., PMET gels). Western blots allow for proteomic semi-quantification of expression, across cell lysates. It is

hypothesised that the steep gradient across stiffness would create a mechanical cue, increasing the proteomic expression of mechanosensitive genes such as YAP and TAZ. NF- κ B (Nuclear Factor κ B) was also investigated as a protein complex, associated with being mechanoresponsive once dissociated from its inhibitor I κ B kinase and relocating to the nucleus to act as a transcription factor (Mendez and Janmey, 2012, Wang et al., 2018a).

With the recent pandemic, several genes and proteins have come into the spotlight, in order to try and elucidate the mechanism used when the viral infection of COVID-19 occurs. ACE2 is abundant on several tissues, like in the respiratory tract and the GI mucosa. Its presence has also been associated with the cardiovascular system. In respect to COVID-19, SARS-COV2 has affinity to ACE2 as a receptor and gateway into the mucosa for the virus to excel and proliferate (Leung et al., 2020, Baker et al., 2021). From the international push to manage and understand the disease, ACE2 has been found to be upregulated in patients with comorbidities such as COPD (Leung et al., 2020). Ex-smokers in comparison to non/healthy patients have demonstrated higher expression of ACE2, leading to notion of higher susceptibility to COVID-19 in COPD patients (Leung et al., 2020). Investigating ACE2 expression in these Western blot assays would determine the sub-hypothesis that stiffening the microenvironment around the bronchospheres will illicit such change similar to fibrosis in respiratory diseases like COPD and IPF, that ACE2 proteomic expression would be upregulated.

5.2.2 *Methods*

Bronchospheres were cultured for 24-27 days and then harvested from protein, via mechanical disruption once pelleted and RIPA lysis buffer. Once centrifuged and isolated from bronchospheres embedded in PMET gels photocured for 1 minute, 5 minutes, no light exposed and when cultured in 25% Matrigel® (donor sets n = 3, for healthy and COPD cells), protein samples were quantified, reduced, and ran in parallel for analysis. Using uncured PMET gel as the normalised control was performed to compare conditions whereby ‘no’ stiffness was applied to bronchospheres via the surrounding matrix (baseline). Total protein normalisation kits were used to label all protein instead of the traditional loading control, mainly due to the complications found during bench RT-qPCR. Full details are described in the Methods chapter (page 84). The following figures show data obtained from western blots to investigate the proteomic presence of YAP1, TAZ, NF- κ B and ACE2.

5.2.3 Results

5.2.3.1 Total Protein Normalisation- NHBEs

The images of chemiblots after staining for total protein – NHBE donors (**Figure 5.8**) and CHBE donors (**Figure 5.9**) – from using the VersaBlot™ Total Protein Normalization Kits (Biotium, USA), allow for normalisation of these lanes (per donor, per condition). These values (when configured) will then replace traditional measures, which normally use common housekeeping genes such as GAPDH and β -actin for probed protein signal intensity normalisation.

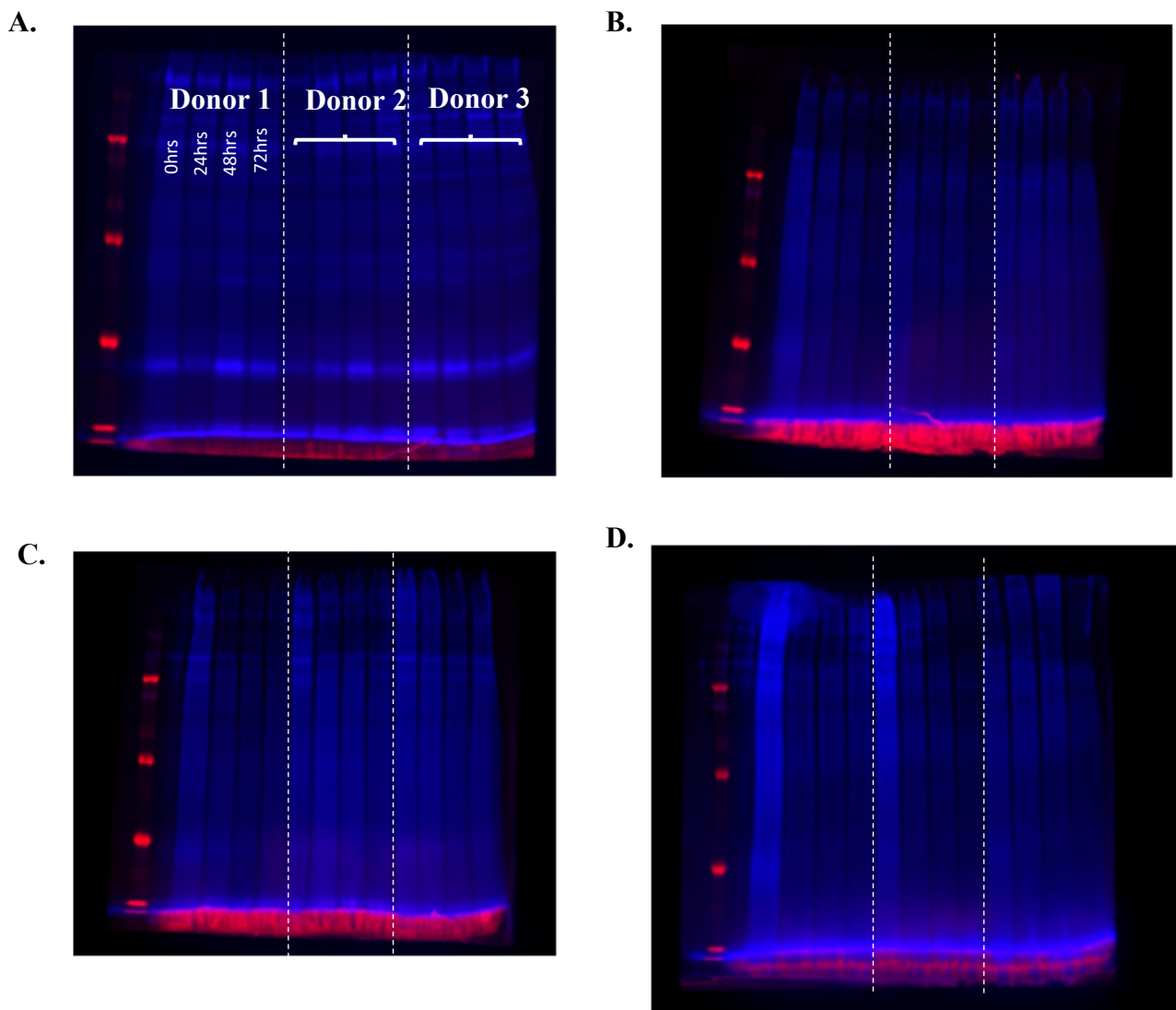


Figure 5.8: Total protein normalisation staining of NHBE Bronchospheres cultured in (A) 25% Matrigel, (B) PMET gel photocured for 1 minute, (C) PMET gel photocured for 5 minutes and (D) PMET gel uncured. N=3. Lanes per 24 hour cohort, per donor were averaged and used to normalise subsequent protein bands when probed on each chemiblot.

5.2.3.2 Total Protein Normalisation- CHBEs

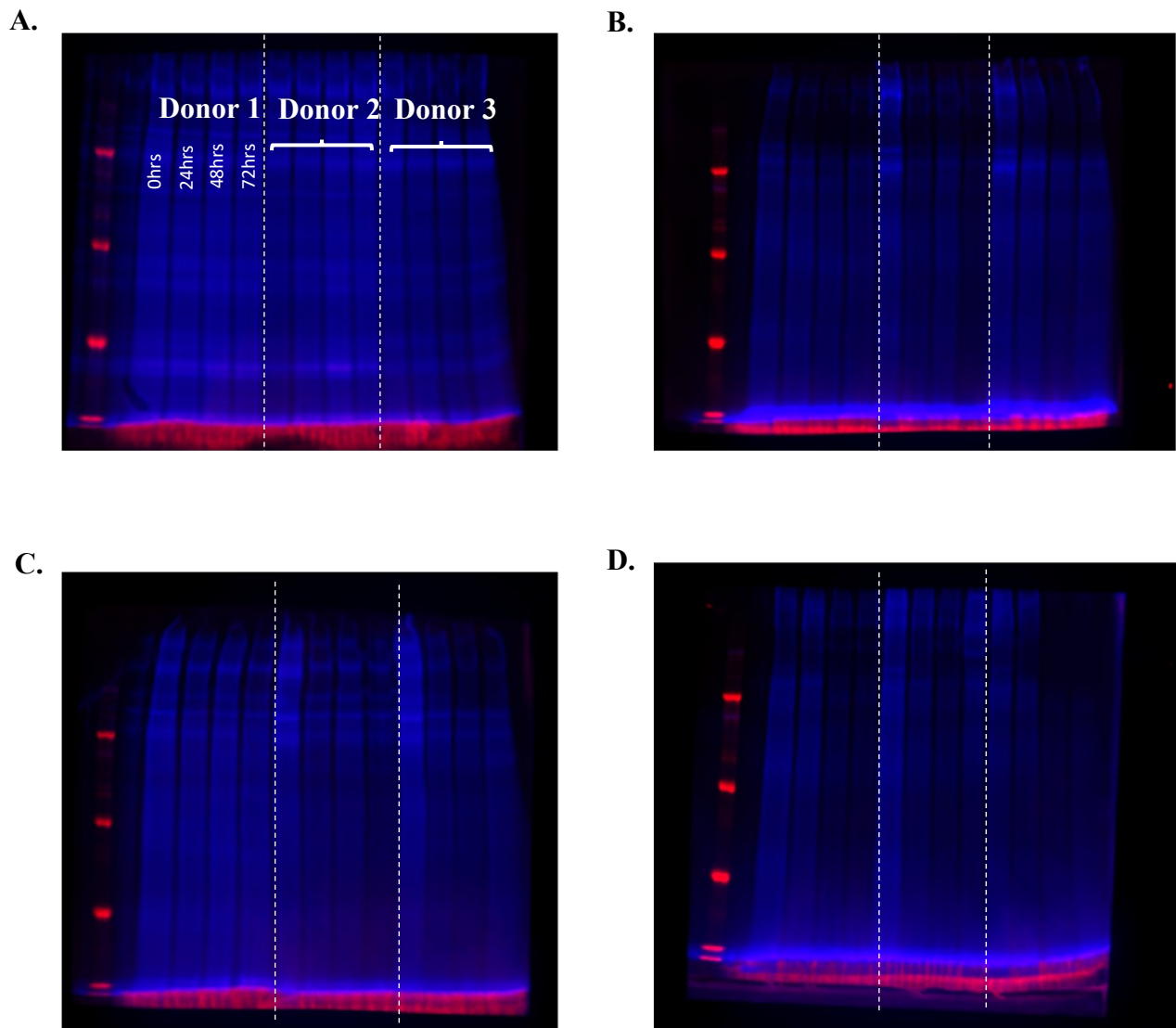


Figure 5.9: Total protein normalisation staining of CHBE Bronchospheres cultured in (A) 25% Matrigel, (B) PMET gel photocured for 1 minute, (C) PMET gel photocured for 5 minutes and (D) PMET gel uncured. N=3. Lanes per 24 hour cohort, per donor were averaged and used to normalise subsequent protein bands when probed on each chemiblot.

5.2.3.3 Chemiblot results- NHBE YAP1

In **Figure 5.10 (A-E)**, YAP1 was probed using NHBE protein lysates from the four conditions used to culture the bronchospheres (PMET gel photocured for 1 minute, photocured for 5 minutes, no photocure (control) and 25% Matrigel®). Signal intensity results were normalised to the conditions where no green light has been used for photocure (**Figure 5.10C**). Lysates from culturing in PMET gel for 1 minute showed an intense signal at 0 hours for all donors, followed by a graduated decrease across the next 72 hours. Lysates from culturing in PMET gel (photocured for 5 minutes) showed a near-equal intensity, which was lost within the following 24 -48 hours. The results are shown graphically in **Figure 5.10E**. The conditions where no light has been exposed, the YAP bands were fairly constant (**Figure 5.10C**).

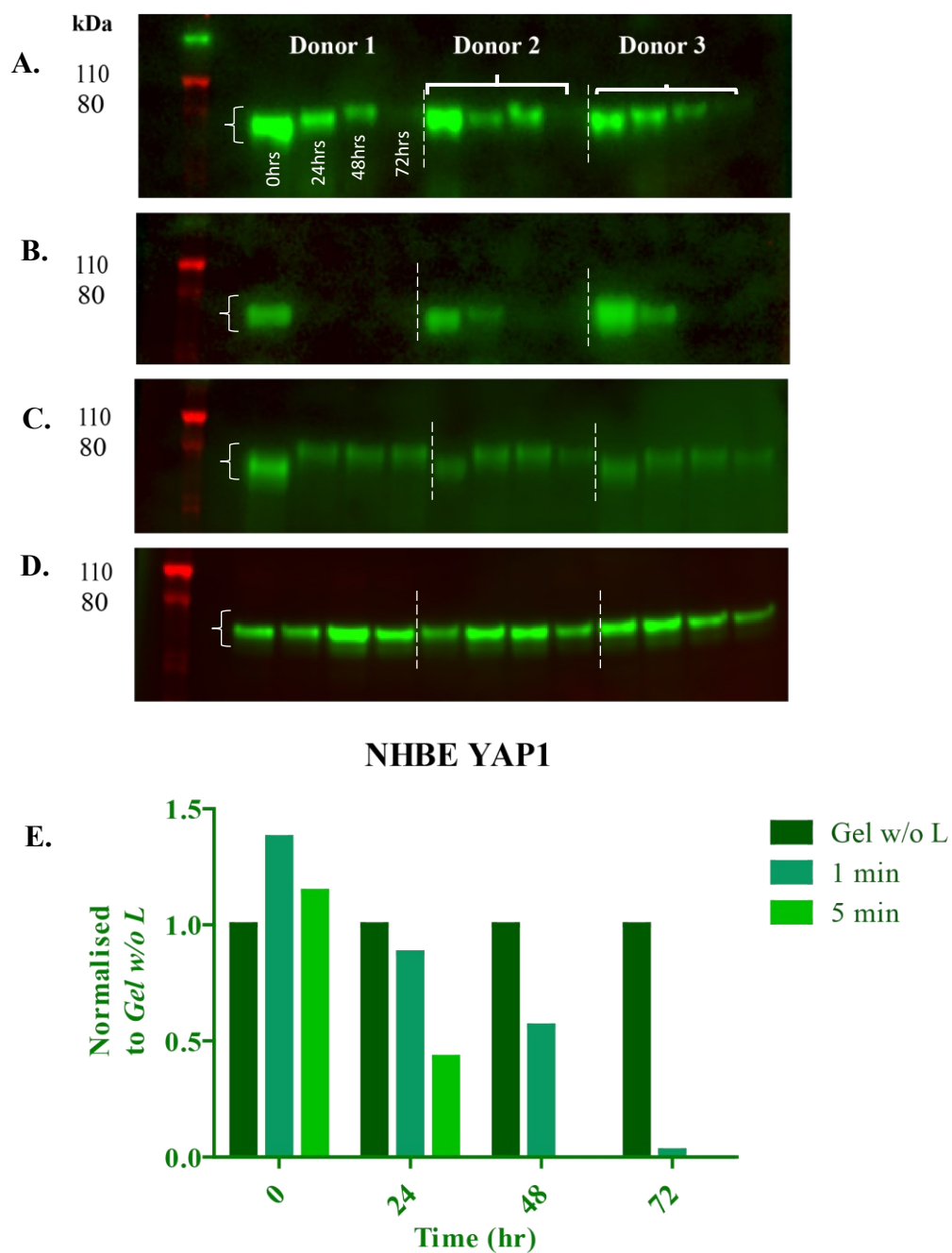


Figure 5.10: Western blots probed for YAP1 in NHBE bronchospheres cultured for 72 hours in (A) PMET gel photocured at 1 min, (B) PMET gel photocured at 5 min, (C) PMET gel with no light and (D) in 25% Matrigel. N=3. Bands appear between 65-75 kDa if present, when probed with HRP-secondary antibody. Results were normalised to PMET gel w/o light (E). YAP1 expression was seen in all conditions, although more regularly in PMET gel without light and at 25% Matrigel. 1 minute and 5 minute PMET gel display a gradual downregulation from 0 hours, with 5 minutes showing a faster decline.

5.2.3.4 Chemiblot results- CHBE YAP1

In **Figure 5.11 (A-E)**, YAP1 was probed using CHBE protein lysates from the four conditions used to culture the bronchospheres (PMET gel photocured for 1 minute, photocured for 5 minutes, no photocure (control) and 25% Matrigel®). Signal intensity results were normalised to the conditions where no green light has been used for photocure (**Figure 5.11C**). Lysates from CHBE bronchospheres cultured in PMET gel (photocured for 1 minute) and 25% Matrigel® had the most constant bands, across the 72 hour assay. However, the control and the bands from the 5 minute photocured gel were fairly inconsistent, across donors and the time course recorded. A graphical summary of the data is provided in **Figure 5.11E**.

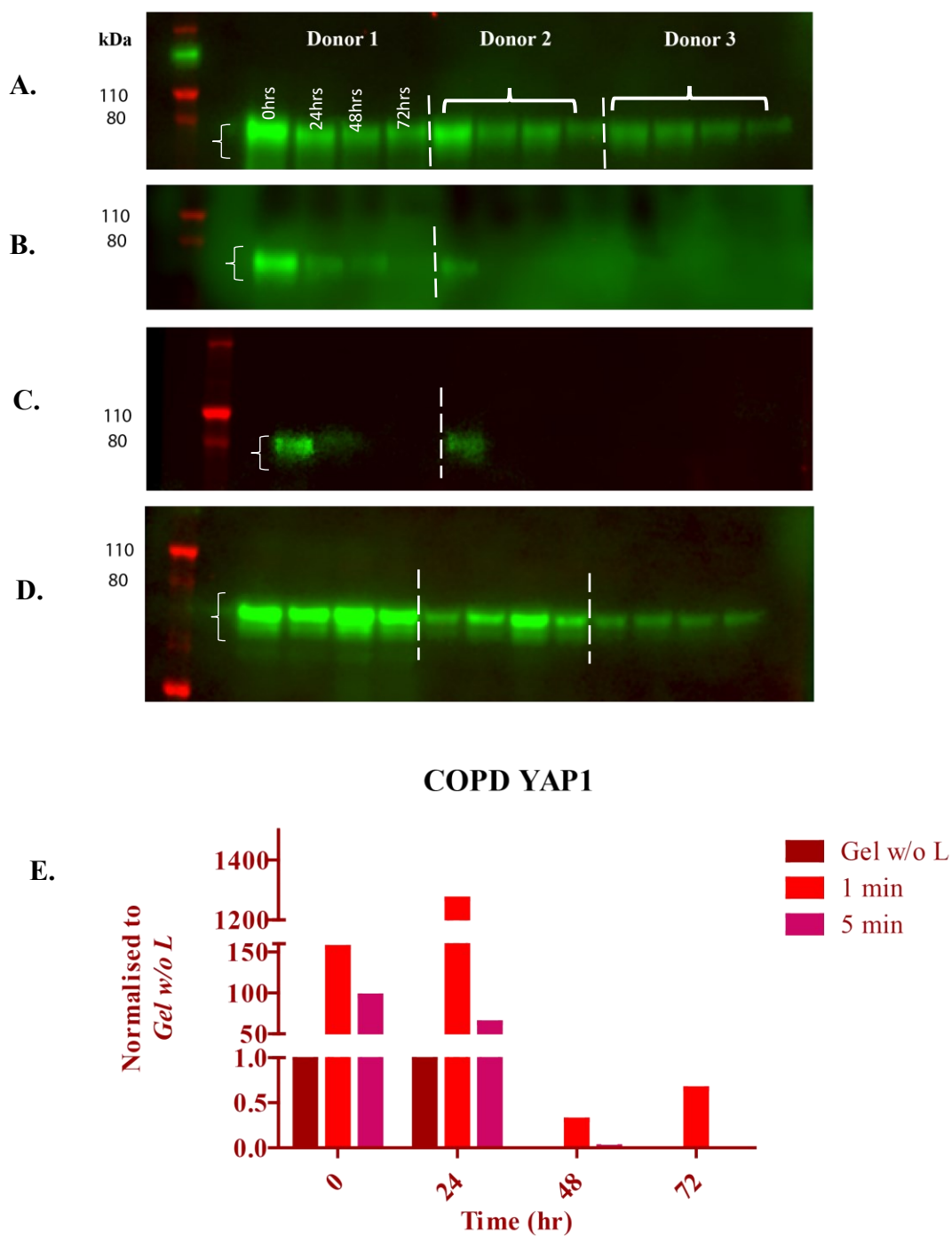


Figure 5.11: Western blots probed for YAP1 in CHBE bronchospheres cultured for 72 hours in (A) PMET gel photocured at 1 min, (B) PMET gel photocured at 5 min, (C) PMET gel with no light and (D) in 25% Matrigel. N=3. Each 24 hour cohort is screened for expression. Bands appear between 65-75 kDa if present, when probed with HRP-secondary antibody. Results were normalised to PMET gel w/o light (E). Expression is consistent at 1 min and at 25% Matrigel, in comparison to the varying expression at 5 minutes. Donor variability is particularly present at 5 minutes and at PMET without no light.

5.2.3.5 Chemiblot results- TAZ

Lysates from NHBE and CHBE bronchospheres were probed for TAZ, from all conditions used for culture (PMET gel photocured for 1 minute, photocured for 5 minutes, no photocure (control) and 25% Matrigel[®]). However only COPD bronchospheres cultured in PMET gel (1 min- **Figure 5.12A**) displayed some intensity across the time course recorded as well as lysates cultured from 25% Matrigel[®] (**Figures 5.12B and C**). The COPD lysate bands were inconsistent in comparison to the NHBEs, regardless of the condition used. A summary of the data presented for the Western blot results for TAZ expression in bronchospheres from healthy subjects and patients with COPD after photocuring is provided in **Table 5.2**.

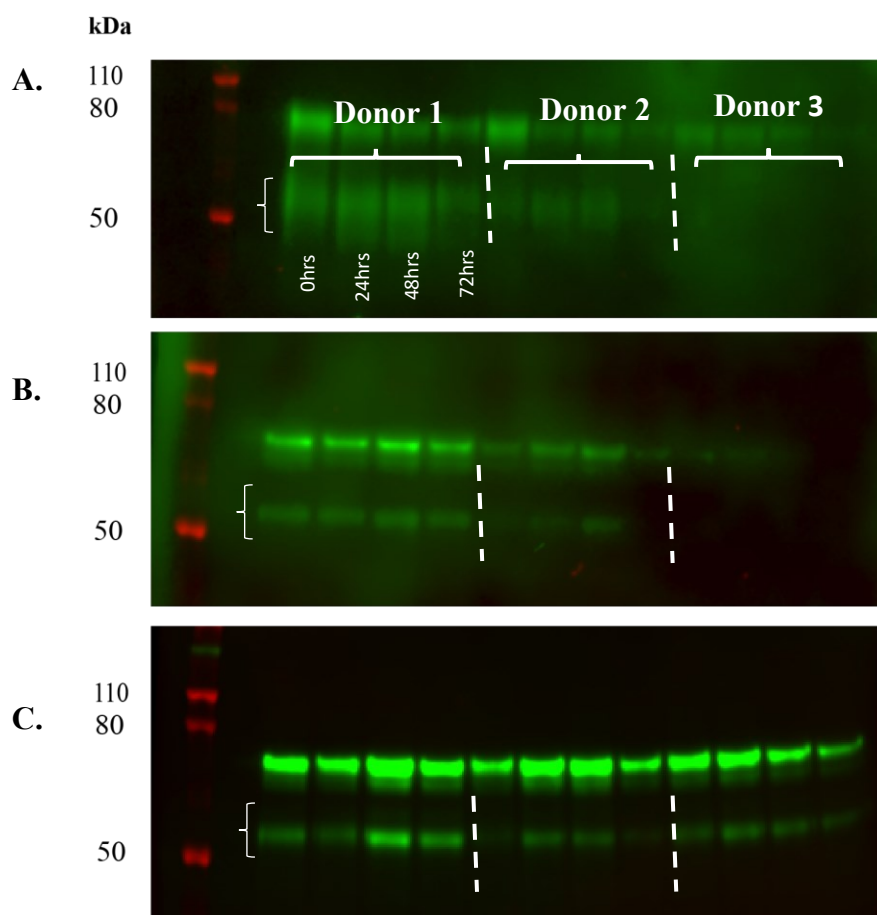


Figure 5.12: Western blots probed for TAZ in CHBE bronchospheres cultured for 72 hours in (A) PMET gel photocured at 1 min, (B) in 25% Matrigel and NHBE bronchospheres in (C) 25% Matrigel respectively, N=3. Bands appear just before 50kDa if present, when probed with HRP-secondary antibody. No normalisation is done via a control condition as no bands were present on PMET gel w/o light.

NHBE				
	0 hours	24 hours	48 hours	72 hours
1 minute	x	x	x	x
5 minutes	x	x	x	x
Gel w/o L	x	x	x	x
25% Matrigel	•	•	•	•
CHBE				
	0 hours	24 hours	48 hours	72 hours
1 minute	•	•	•	•
5 minutes	x	x	x	x
Gel w/o L	x	x	x	x
25% Matrigel	•	•	•	•

Table 5.2: Summary of WB results after TAZ antibody staining and HRP detection with all conditions involving healthy and COPD bronchospheres. TAZ protein is only detected in NHBE bronchospheres cultured in 25% Matrigel, CHBE bronchospheres encapsulated in 1 minute PMET gel and 25% Matrigel.

5.2.3.6 Chemiblot results- NF- κ B

NF- κ B bands were not visible for the majority of conditions investigated (PMET gel photocured for 1 minute, photocured for 5 minutes, no photocure (control) and 25% Matrigel®). They were only present with the NHBE bronchosphere lysates cultured without light and after 1 minute photocure in PMET gel (*Figures 5.13A* and *B* respectively). Results were normalised to the control (*Figure 5.13C*).

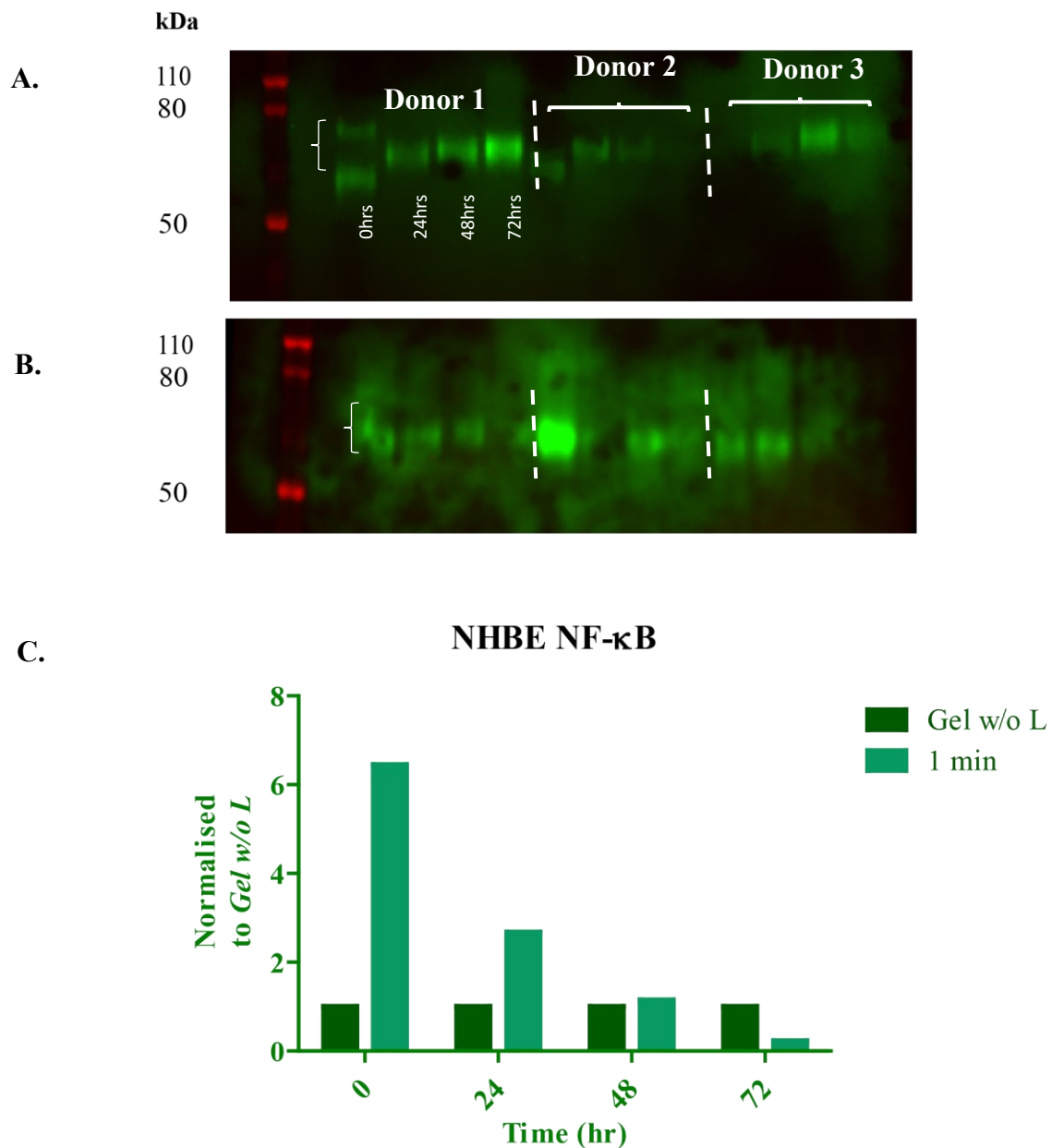


Figure 5.13: Western blots probed for NF- κ B in NHBE bronchospheres cultured for 72 hours in (A) PMET gel with no light and (B) PMET gel photocured at 1 minute. $N=3$. Bands appear at 65kDa if present, when probed with HRP-secondary antibody. Results were normalised to PMET gel w/o L (C).

5.2.3.7 Chemiblot results – ACE2

ACE2 was probed across all bronchosphere lysates, cultured from the four conditions: PMET gel photocured for 1 minute, photocured for 5 minutes, no photocure (control) and 25% Matrigel®. However, no bands were present (see **Table 5.3**) except for the NHBE bronchosphere lysates cultured in 5 minutes photocured PMET gel (**Figure 5.14A**) and CHBE bronchosphere lysates cultured in 1 minute photocured PMET gel (**Figure 5.14B**).

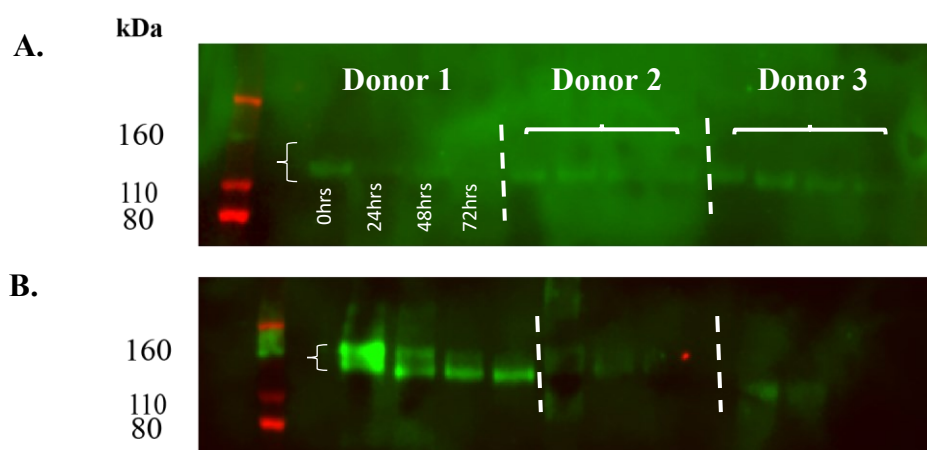


Figure 5.14: Western blots probed for ACE2 in NHBE bronchospheres cultured for 72 hours in (A) PMET gel photocured at 5 min and (B) and CHBE bronchospheres in PMET gel photocured at 1 min, respectively. N=3. Bands appear at 125-130kDa if present, when probed with HRP-secondary antibody. No normalisation is done via a control condition as no bands were present on PMET gel w/o Light membrane.

NHBE				
	0 hours	24 hours	48 hours	72 hours
1 minute	x	x	x	x
5 minutes	•	•	•	•
Gel w/o L	x	x	x	x
25% Matrigel	x	x	x	x
CHBE				
	0 hours	24 hours	48 hours	72 hours
1 minute	•	•	•	•
5 minutes	x	x	x	x
Gel w/o L	x	x	x	x
25% Matrigel	x	x	x	x

Table 5.3: Summary of WB results after ACE2 antibody staining and HRP detection with all conditions involving healthy and COPD bronchospheres. Only the ACE2 protein was detected in healthy bronchospheres encapsulated in 5 minute PMET gel and CHBE bronchospheres encapsulated in 1 minute PMET gel.

5.2.4 Discussion

YAP1 expression differed between the healthy and COPD donor sets. NHBE's YAP1 expression was more uniformed, a gradual downregulation across the 72 hour assay in all conditions (1 minute, 5 minutes of photocuring and no photocuring - see **Figures 5.10A-C**) except when the bronchospheres were cultured in 25% Matrigel® (**Figure 5.10D**). Intense bands were seen at 0 hours, like the CHBEs, however when normalised to bronchospheres in uncured PMET gel, the difference in expression was not as vast unlike the COPD counterparts. Again, 5 minutes of photocuring also showed increased expression when normalised but was short-lived, ending after 24 hours. This increase in YAP1 expression from the onset of induced stiffness is expected, inferring mechanotransduction signalling taking place as the ECM is altered.

In **Figure 5.11A**, YAP1 was roughly expressed by CHBE bronchospheres to the same degree when encapsulated in PMET gel and cured for 1 minute – although the bands were brighter at 0 hours, especially with donor 1 and 2, in comparison to the rest of 24 hour cohorts. Although inconsistent, more intense bands were still found at 0 hours, in comparison to 24, 48 and 72 hours, per donor, in PMET gel cured for 5 minutes and uncured. This may signify the relay of the onset stiffness from photocuring (especially from a stronger stimulus like the 5 minute photocure), resulting in an upregulated proteomic expression of YAP1. Mechanosensitivity appears to be greater at 1 minute, with the greatest upregulation seen at 24 hours on average, displayed by **Figure 5.11E**'s graph when normalised to no curing. PMET gel cured at 5 minutes also showed some upregulation, but to a lesser extent and across a shorter time span. The delay in upregulated YAP1 expression in CHBEs compared to the NHBEs also suggests some impaired signalling mechanisms due to the diseased state phenotype- in COPD, inducers include chronic inflammation and cigarette smoke-induced oxidative stress (Kirkham and Barnes, 2013, Chatterjee and Fisher, 2014, Barnes et al., 2015). Reactive oxidative stress (ROS) is already a factor in various signalling pathways. But when said pathways are overwhelmed by excess ROS and inflammation, disturbance occurs in cue transductions and cell functions are affected. Cells inherited from COPD donors may therefore be inherently impaired, originating from a dysregulated microenvironment with ROS and chronic inflammation affecting intracellular responses. An example are the studies showing 'delayed' regulatory reaction of glutamylcysteine ligase due to oxidate stress, in COPD-derived epithelium and macrophages compared to healthy donors (Harju et al., 2002).

Cell-integrin adhesions communicate directly with the surrounding ECM and thereby take physical cues accordingly. However, this integrin feedback is reduced when placing the organoid within a more synthetic hydrogel. Encapsulated in the PMET gel, the bronchospheres initially experience a big change in ECM stiffness- in the form of compression acting on the sphere, especially when coming from a softer 25% Matrigel layer. Integrins would normally relay the change in external force to the cell, for the cytoplasm to react accordingly and result in the influx of nuclear YAP. Although there is Matrigel present in the PMET precursor mix, which would interact with these integrins no matter how limited, presumably there is a lack of adhesive sites on the mainly inert ECM due to the presence of PEGDA. The upregulation of YAP and TAZ appeared to then not be sustained (to continually promote directed cellular behaviour) as seen with the NHBE bronchospheres in 1 minute photocured PMET gel and more rapidly with both CHBE and NHBE bronchospheres in the 5 minute photocured gel. The compressive nature of the gel and the ‘squeezing’ onto the spheroid may impact any ion channels and pores through mechanical stress. YAP can therefore relocate, allowing for nuclearisation and initially overriding the normal regulatory mechanism (phosphorylation of YAP) occurring. However, tempering of expression (expression decrease) is seen per 24 hours, with further investigation required to determine why. Additionally, because of the now limited space in the microenvironment as the PMET gel is also not degradable, the organoid may favour differentiation instead, assumingly leading to the increased expression of goblet cells (MUC5AC and MUC5B) (Gjorevski et al., 2016, Panciera et al., 2017).

Whilst YAP and TAZ are often mentioned as one complex in research literature, they play different roles within the lung, once bound to the TEAD family. YAP has been linked to SOX2 regulation, associated to the regulation of stem cells and progenitors in the lung. TAZ contributes to normal alveolarization by promoting surfactant protein C expression in epithelial cells when adhered to TTF-1 (Xie et al., 2018). Both are ubiquitously expressed in the body, including the lung, collaborating with the data seen in **Figure 5.9** and **5.10**, but not for **Figure 5.12** and **Table 5.2** when concerning TAZ. TAZ expression was only found in the CHBE bronchospheres embedded in PMET gel and cured for 1 minute as well as the 25% Matrigel conditions for CHBE and NHBE bronchospheres (**Figure 5.12A-C** respectively). The expression itself was not uniform, with most bands being faint in contrast to the probed YAP bands above on the chemiblot. No reliable conclusion can be made from insufficient

data (**Table 5.2**). Additionally, it may be possible that the YAP antibody previously used may cause false positive readings, due to the 45% homology between the PDZ domain on both coactivators (Pocaterra et al., 2020). Future studies should investigate the role of other mechanosensors in the bronchosphere response to changing stiffness.

The data obtained when investigating NF- κ B also remains unclear. Many of the bronchospheres examined did not appear to express the NF- κ B p65 protein, with positive results only seen with NHBE bronchospheres cultured for 1 minute in the cured PMET gel and in the gel without any curing performed (**Figures 5.13A** and **B** respectively). NF- κ B is implicated in COPD pathophysiology from past studies showing elevated NF- κ B expression and activation in patients with COPD patients vs non-smokers and smokers without lung obstruction ('healthy smokers') (Di Stefano et al., 2002, Isajevs et al., 2011). In animal studies, NF- κ B induces chronic airway inflammation with remodelling of the small airway walls. Activation of the NF- κ B pathway evokes the recruitment of inflammatory cells e.g., macrophages, neutrophils and further drives sustained inflammation with downstream links to tumorigenesis in lung cancer (Zaynagetdinov et al., 2016). NF- κ B can also be regulated via external force e.g., mechanical stress. Evidence for NF- κ B nuclear localisation (via additional mechanosensitive proteins e.g., Focal Adhesion Kinase) and for the release of its inhibitor I κ B α in chondrocytes and osteoblasts is seen in response to changes in high magnitude stresses experienced by the cell (Mendez and Janmey, 2012). Some comparison can be made between the results gained, as shown in **Figure 5.13C**. Bronchospheres embedded in PMET gel (1 minute photocure) displayed high expression at the start of the assay but steadily declined over the 72 hour period (when normalised to the uncured PMET gel). In this case, NF- κ B may have been activated by the embedding process (the change in gel stiffness when exposed to green light, initiating cellular mechanical stress) but this trend is not replicated in the stiffer gel at 5 mins, or with the COPD bronchospheres in both gel conditions. Overall, the results for NF- κ B are inconclusive and further work needs to be undertaken to determine whether NF- κ B is activated in this model.

The recently found link between ACE2 and COVID-19 has increased the interest in ACE2 over the past 2 years. An additional probe for ACE2 was undertaken to explore the tenet that COPD patients might have a higher expression of ACE2 (Leung et al., 2020) in comparison to that in healthy subjects and whether the stiffer gel can upregulate this

expression further. **Table 5.3** shows that ACE2 expression was not apparent in most of the conditions investigated, apart from healthy bronchospheres embedded in PMET after 5 minutes photocuring and COPD bronchospheres embedded in PMET gel after 1 minute photocuring which partially supports the hypothesis. 5 minute photocured PMET gel is stiffer and drives increased expression within the NHBE organoids (**Figure 5.14A**). The less stiff PMET gel at 1 min generated no expression of ACE2 and neither did the control condition of no curing taking place. This was seen with the CHBE bronchospheres too, except for CHBE organoids in 1 min PMET gel. It may be due to the adequate condition matching of the COPD bronchospheres and the 1 minute PMET gel, similar to the native stiffened ECM found with this respiratory disease. This results in the perhaps more mechanosensitive COPD organoids regulating their ACE2 expression accordingly at an earlier deviation point from healthy lung stiffness. The 5 minute photocured PMET gel stiffness is considered to be way past the range considered for the healthy lung. It would be expected that this ACE2 expression upregulation from the COPD organoids would have been seen with 5 minute PMET gel too, however this was not the case. Instead, this stimulus was enough to prompt ACE2 expression in the NHBE bronchospheres only- portraying a dramatic change in microenvironment was required to produce this phenotype.

In similar studies, western blot data show an increase in YAP expression, seen from IPF sourced fibroblasts, including knockout models of YAP/TAZ attenuating the influence of induced matrix stiffness (Liu et al., 2015). These results therefore show how YAP is directly linked to mechanotransduction signalling. Further analysis demonstrates this increase is not due to fibroblasts increasing their level of YAP expression, but alluded to proliferation occurring in these cells instead, leading to the upregulated expression of this protein (Liu et al., 2015). Although no proteomic measurement of proliferation markers Sox2 and Ki67 was included in this western blot assay, the results positively relate to the increase seen in the bench RT qPCR and some dimensional expression in the violin plots of the sc RT-qPCR data. YAP is seemingly maintained throughout the assay regarding CHBEs and may be related to airway smooth muscle thickening (so proliferation also inferred). However, in this assay, the source of cell lysate was from bronchial section of the airways and therefore may not be as relatable to the YAP expression seen in the alveolar space where stiffness is reduced due to emphysema and a reduction in expression is assumed (Bidan et al., 2015).

It is recognised that the overall conclusion is moderated by the limiting donor numbers used during the assay. However, western blots can be used as a gateway into proteomics. They were performed as a method to investigate whether the expression of some of the proteins associated with COPD and IPF could be altered by just manipulating the immediate cell's (organoid's) microenvironment towards that seen more fibrotic pathologies. There are few literature reports of the use of western blot analysis of organoids. This is most probably due to complex nature of an organoid's architecture, that more emphasis is placed on comprehensive analysis such as single cell proteomics, performed alongside scRNA-sequencing. The assay suggests that the increase in YAP1 is independent of its normal regulated signalling pathway through change in gel stiffness. However, unphosphorylated YAP protein was measured. If the extracted protein were not as limited, phosphorylated YAP would have also been probed to calculate a ratio between the two, to deduce true levels of YAP. As with the genomic analysis I performed, standard protein characterisation protocols had to be optimised to an organoid culture before proceeding further. An example of this was protein concentration assays e.g., Bradford assays. This may be due to the chemistry of photoinitiators within the PMET gel as well as the EDTA added to the cell lysis buffer for protection against matrix metalloproteinases (MMPs), interacting to affect the resulting colorimetric value. A more robust assay in terms of handling the presence of such reagents, such as the Pierce BCA protein assay was finally used.

Total protein normalisation (TPN) was used as the chosen normalisation method, as this is becoming more and more popular for semi-quantitative methods such as Western blots as housekeeping loading proteins have been increasingly found to not be as stable as previously thought (Wen et al., 2015). TPN was also used as a 'housekeeping' control in contrast to other common housekeeping genes such as GAPDH and β -actin which I had already observed to 'drift' during qPCR analysis (see *Chapter 4*). Some of the stained protein lanes differed in intensity and although this was more consistent per time point and per condition in some analyses, each time point per donor acts as a replicate for normalisation of each protein band generated after antibody probing. Having resolved these technical challenges, I was able to assess the effects of changing matrix stiffness on organoid protein expression in a much higher throughput manner.

5.3 YAP1 Immunocytochemistry

5.3.1 *Introduction*

YAP1 is a major protein in the Hippo signalling pathway and also has a significant role in cellular mechanotransduction being influenced by external cues from the associated microenvironment (Halder et al., 2012, Dupont, 2016, Panciera et al., 2017, Pocaterra et al., 2020). I hypothesise that YAP will sequester into the nuclei due to the stiffer matrix ‘sensed’ by the organoids when photocuring takes place and that this would initiate a set of transcriptional cascades involved in cell proliferation and cell differentiation. This was examined using YAP nuclear localisation detection by immunofluorescence staining. The previous western blot data suggests that YAP activation would be greater at 0 hours, which represents the onset of the mechanical stimuli, in comparison to the latter stages of the analysis at 48 and 72 hours.

5.3.2 Methods

Bronchospheres were fixed and permeabilised before being exposed to the primary anti-YAP antibody and detected using a fluorescent-tagged secondary antibody as described in full in Chapter 2 (pages 80-82). The samples were then tissue -cleared with 2,2'-Thiodiethanol (TDE) before the whole mount was visualised by confocal microscopy.

YAP intracellular and intranuclear staining was imaged across the whole 72 hour time course of the experiments. As the non-photocured PMET gel bronchosphere culture resulted in a sticky cell suspension which was difficult to harvest, a control bronchosphere culture in 25% Matrigel[®] was also imaged in parallel for descriptive analysis.

5.3.3 Results

5.3.3.1 Confocal Imaging – Effect of photocuring on Normal Human Bronchial Epithelial cells

ICC images of control NHBE bronchospheres grown in 25% Matrigel[®] showed faint YAP1 staining (red) at the periphery of the bronchospheres corresponding with E-cadherin (green) staining at 0 (**Figure 5.15A**) and 48 hours (**Figure 5.15B**). There was evidence of some purple (red YAP1 + blue DAPI) colour at baseline where bronchospheres overlapped. In contrast, bronchospheres embedded in PMET gel for 1 minute and studied at t=0 (**Figure 5.15C**) showed a greater intensity of YAP1 cell staining with evidence of nuclear staining (purple) from the merged images. After 72 hours (**Figure 5.15D**) the 1 minute PMET gel-exposed bronchospheres showed similar intensity of YAP1 staining as seen in control 25% Matrigel[®] (**Figures 5.15A and B**), although the definition at the periphery of the bronchospheres was less. Similar immunostaining results in the NHBE bronchospheres were also generated at 48 and 72 hours with the 1 minute photocure.

Comparable results were seen with bronchospheres derived from NHBEs that were exposed to 5 minutes photocuring (**Figure 5.16**). ICC images of control NHBE bronchospheres grown in 25% Matrigel[®] showed faint YAP1 staining (red) at the periphery of the bronchospheres corresponding with E-cadherin (green) staining at 0 (**Figure 5.16A**) and 48 hrs (**Figure 5.16B**). In comparison, bronchospheres embedded in PMET gel for 5 minutes and studied at t=0 (**Figure 5.16C**) showed a greater intensity of YAP1 cell staining with evidence of nuclear staining from the merged images. After 48 hrs (**Figure 5.15D**) the 5 minutes PMET gel-exposed bronchospheres showed similar intensity of YAP1 staining as seen in control 25% Matrigel[®] (**Figures 5.16A and B**) although the definition at the periphery of the bronchospheres was reduced.

Confocal Imaging – Normal Human Bronchial Epithelial cells (PMET gel 1 minute photocure)

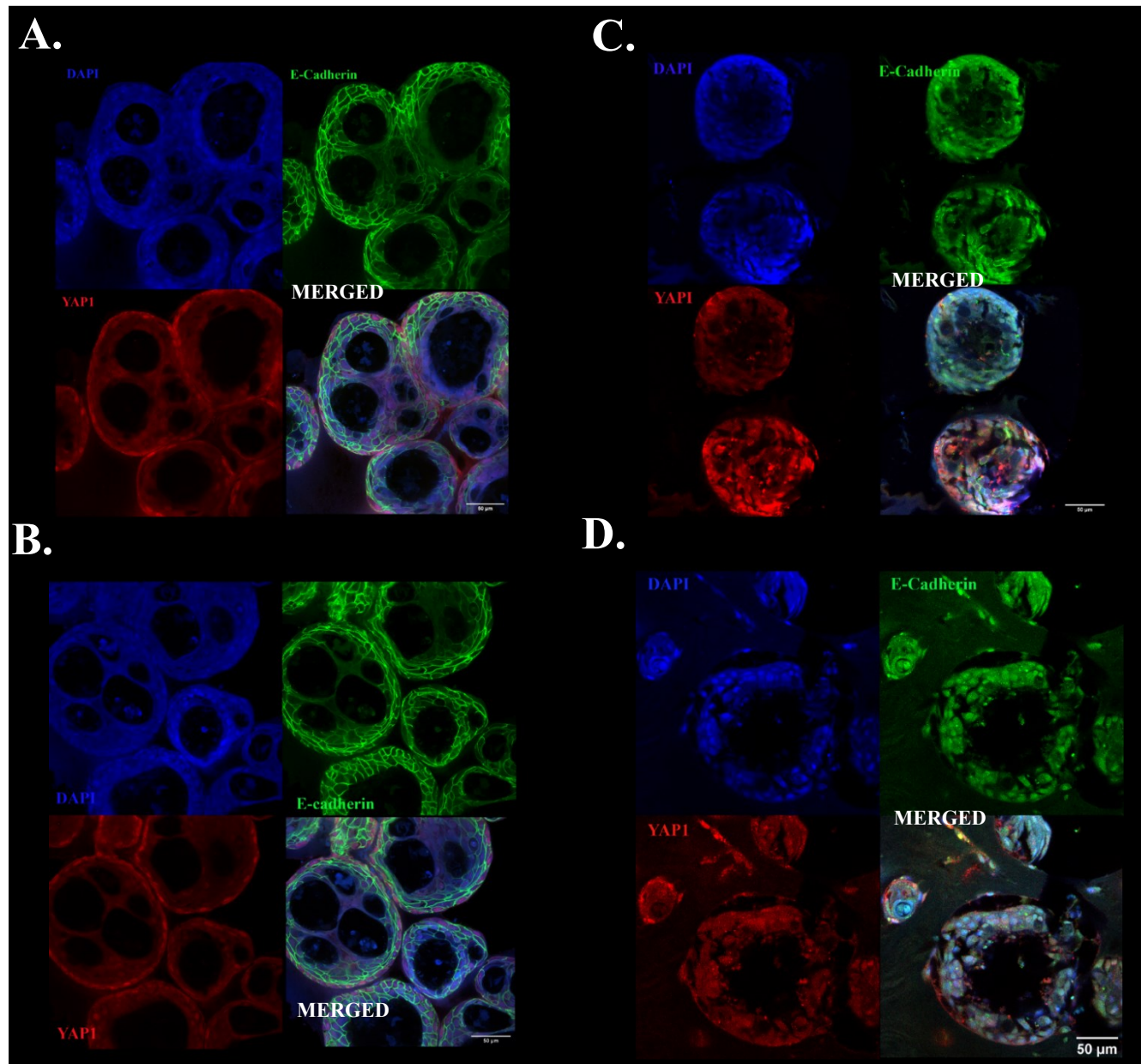


Figure 5.15: Representative ICC Confocal image of YAP1 staining in Bronchospheres, derived from NHBE donors. Control organoids (25% Matrigel) were fixed and whole mount stained with YAP1 antibody at 0 hours (A) and 48 hours (B). Bronchospheres embedded in PMET gel, photocured for 1 minute were also fixed and stained at 0 hours (C) and 72 hours (D) in parallel for comparison. Organoids were stained for nuclei (DAPI- blue), cell to cell junctions (e-cadherin- green) and YAP1(red).

Confocal Imaging – Normal Human Bronchial Epithelial cells (PMET gel 5 minute photocure)

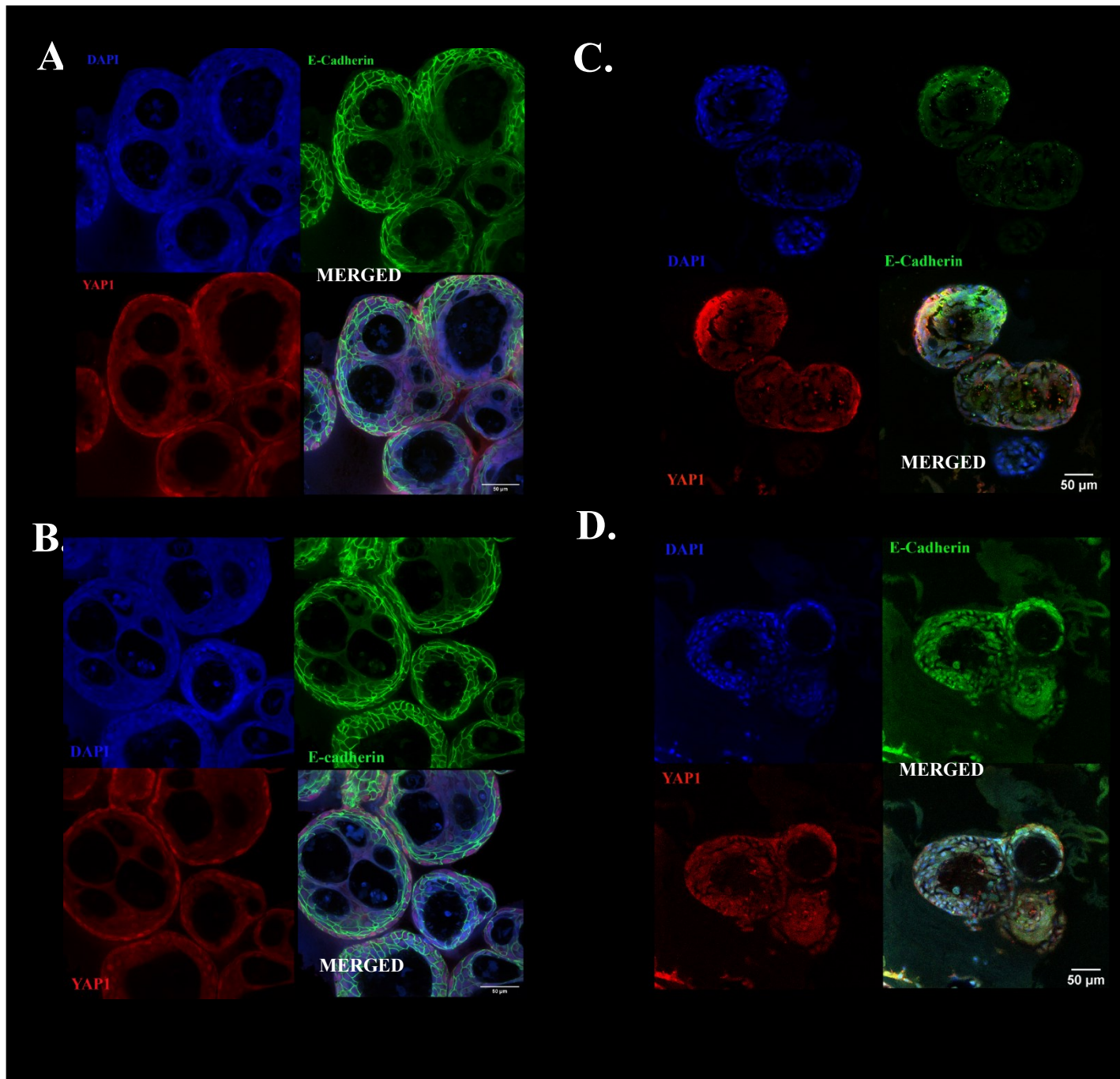


Figure 5.16: Representative ICC Confocal image of YAP1 staining in Bronchospheres, derived from NHBE donors. Control organoids (25% Matrigel) were fixed and whole mount stained with YAP1 antibody at 0 hours (A) and 48 hours (B). Bronchospheres embedded in PMET gel, photocured for 5 minutes were also fixed and stained at 0 hours (C) and 48 hours (D) in parallel for comparison. Organoids were stained for nuclei (DAPI- blue), cell to cell junctions (e-cadherin- green) and YAP1 (red).

5.3.3.2 Confocal Imaging – Effect of photocuring on COPD Human Bronchial Epithelial cells

The intensity of the peripheral YAP1 staining (red) was more prominent with the ICC control images of CHBE bronchospheres in 25% Matrigel after 48 hours (**Figure 5.17B** and **5.18B**), although less distinct than the 0 hour counterparts (**Figure 5.17A** and **5.18A**). Both were in presence of nuclei stained by DAPI (blue) and the accompanied E-cadherin staining (green).

In comparison, CHBE bronchospheres embedded in PMET gel with 1 minute photocure showed more distinction in the 48 hours images, with YAP1 directly overlaying the nuclei stained in DAPI (**Figure 5.17C**-0 hours, **Figure 5.17D**-48 hours). However, the opposite trend was seen with the corresponding CHBE bronchospheres embedded in PMET gel after 5 minutes of photocuring. Only at 0 hours was the YAP1 stain clearly visible, at the periphery (**Figure 5.18C**). But at 48 hours, the YAP1 stain became very faint and also more homogenous across the bronchosphere (**Figure 5.18D**). In both healthy and COPD donors, E-cadherin staining distinction was lost for the majority, when the matrix is altered.

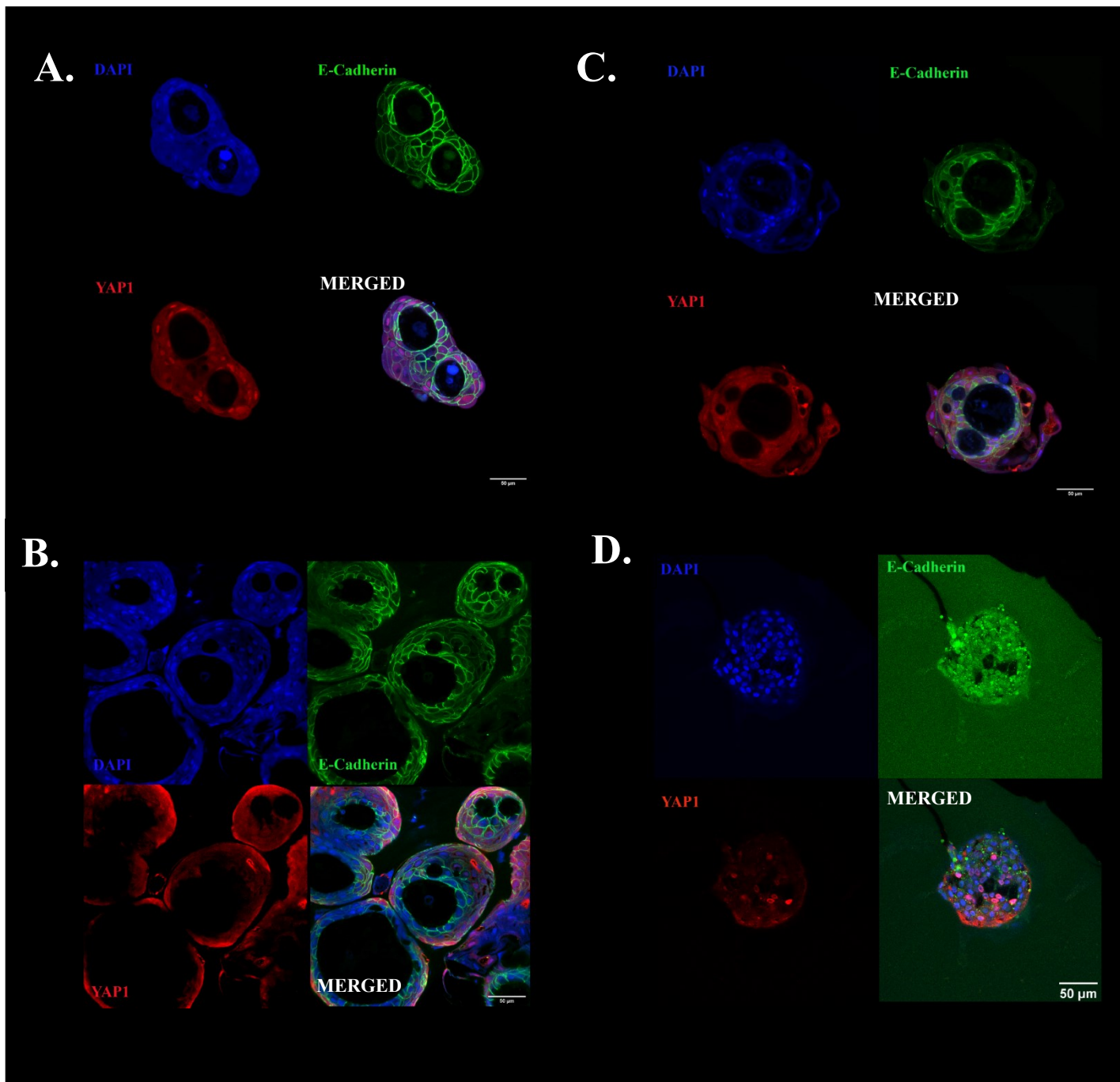


Figure 5.17: Representative ICC Confocal image of YAP1 staining in Bronchospheres, derived from CHBE donors. Control organoids (25% Matrigel) were fixed and whole mount stained with YAP1 antibody at 0 hours (A) and 48 hours (B). Bronchospheres embedded in PMET gel, photocured for 1 minute were also fixed and stained at 0 hours (C) and 48 hours (D) in parallel for comparison. Organoids were stained for nuclei (DAPI- blue), cell to cell junctions (e-cadherin- green) and YAP1 (red).

Confocal Imaging – COPD Human Bronchial Epithelial cells (PMET gel 5 minute photocure)

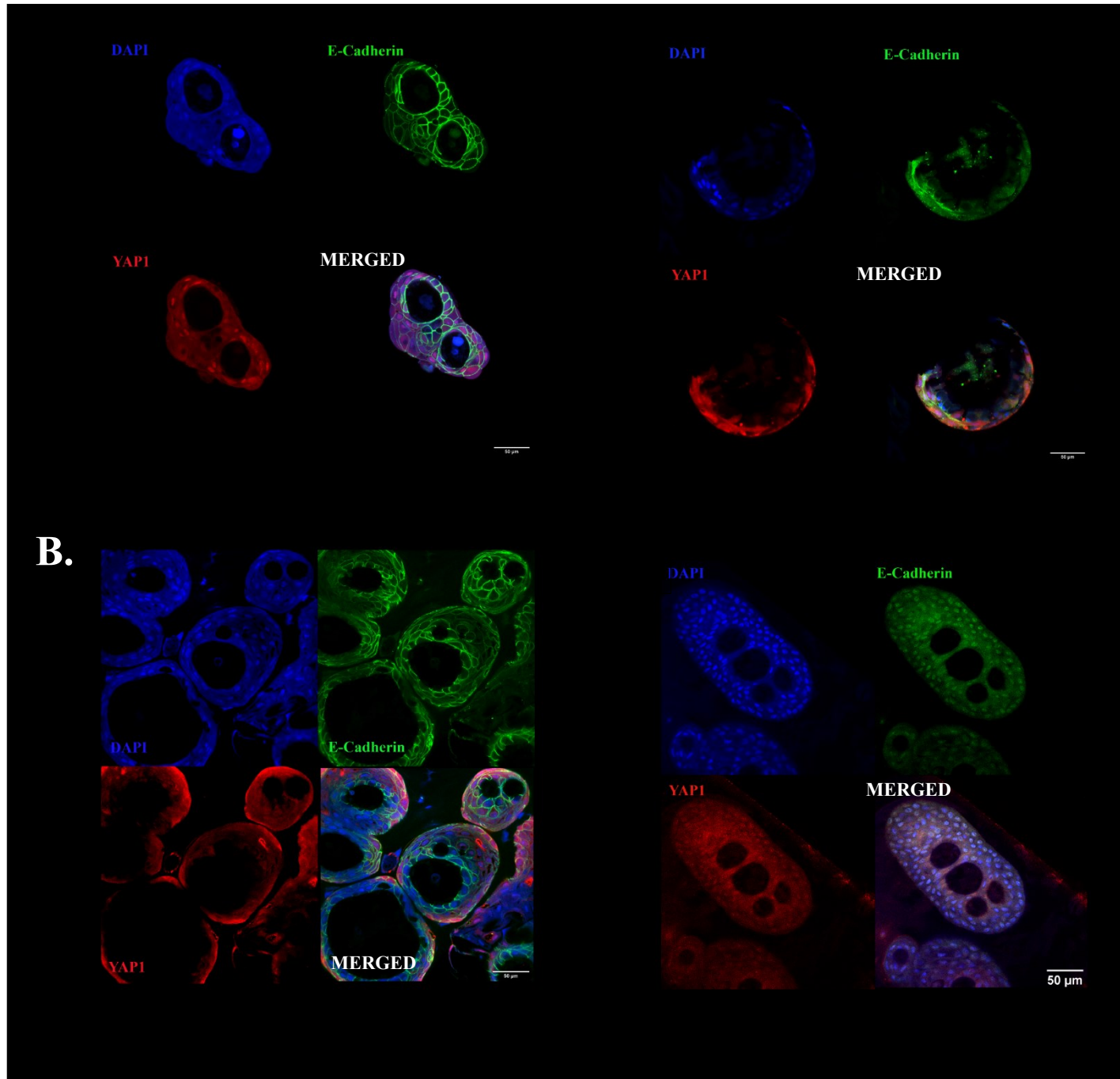


Figure 5.18: Representative ICC Confocal image of YAP1 staining in Bronchospheres, derived from CHBE donors. Control organoids (25% Matrigel) were fixed and whole mount stained with YAP1 antibody at 0 hours (A) and 48 hours (B). Bronchospheres embedded in PMET gel, photocured for 5 minutes were also fixed and stained at 0 hours (C) and 48 hours (D) in parallel for comparison. Organoids were stained for nuclei (DAPI- blue), cell to cell junctions (e-cadherin- green) and YAP1(red).

5.3.4 Discussion

Whole mount imaging was performed focusing on YAP1 and its localisation within the bronchospheres. Since the western blot data highlights the spike of this mechanosensitive transcriptional coactivator straight after the PMET gel has been optically manipulated, it is assumed that there would be a clear demarcation of YAP becoming nuclear instead of cytoplasmic, especially with healthy donors. With the ‘control’ NHBE bronchospheres cultured in 25% Matrigel (**Figures 5.15 and 5.16, A-B**), there was intense YAP staining around the border of the bronchospheres. The bronchospheres acting as one unit would initially experience physical insults at the perimeter of its structure before relaying the signal to its interior. E-cadherin staining is also more pronounced at the bronchosphere perimeter, which could be due to diffusion rate of the antibody into the organoid.

NHBE organoids in the PMET gel cured after 1 minute (**Figure 5.15 C-D**) lost their clear distinction between cytoplasmic and nuclear YAP, with more homogenous staining throughout the structure at 72 hours (although there seemed to be more of a YAP presence than in the control). NHBE bronchospheres encapsulated in PMET gel and photocured for 5 minutes (**Figure 5.16 C-D**) displayed a more distinct YAP staining at the bronchosphere border than seen in the 1 minute counterparts. The stiffer gel (5 minutes photocuring) was expected to generate a more physical cue, particularly on the immediate layer of cells in contact with the hybrid ECM. However, YAP1 staining remained largely homogenous throughout, with some increase of intensity at the organoid’s circumference but not as intense as expected. YAP1, regulated by the Hippo pathway, is involved in many interchangeable applications within the cell apart from the translation of mechanical cues e.g., reprogramming regarding regeneration, wound healing and anti-senescence (Ma et al., 2019, Moya and Halder, 2019, Pocaterra et al., 2020). The frequent interplay of the protein made it hard to pinpoint its location by general confocal imaging. Future studies should consider that any gel still bound to the organoid may also lead to autofluorescence in addition to acting as an additional barrier to antibody diffusion. This may bias the results due to unspecific and heterogenous labelling.

YAP staining in CHBE bronchospheres was more intense over time (48 hours) in the control 25% Matrigel, possibly reflecting some bronchosphere growth against the gel matrix

(*Figure 5.17 A-B, Figure 5.18 A-B*). A similar delayed increase of YAP staining was seen in the bronchospheres embedded in the gel photocured for 1 minute at 48 hours than at time 0 (*Figure 5.17 C-D*). In comparison to their healthy counterparts, CHBE bronchospheres would be naturally primed for a stiffer, less elastic ECM and the 1 minute photocured gel may imitate this fibrotic ECM setting. However, the 5 minutes photocure (*Figure 5.18C*) showed the enhanced YAP staining immediately after encapsulation into the stiffer gel, focused on the organoid's perimeter. This image confirms the results from the western blot data (*Figure 5.11B*). The stiffer 5 minute ECM may cause an initial surge of YAP into the nuclei, but this nuclear translocation was not maintained, again agreeing with the western blot results from *Figure 5.11*.

With respect to concomitant E-cadherin cell adhesion staining, differences were seen when photocure was introduced and this may potentially reflect the EMT (epithelial-mesenchymal transition) phenomenon occurring due to the change in matrix stiffness. The control images exhibited a clear honeycomb pattern of the cell-to-cell junctions where E-cadherin is located. When the matrix has been manipulated, no distinct adherens junctions that follow each cell's perimeter were seen. Instead, E-cadherin staining was nondescript and mimicked autofluorescence. Further staining with N-cadherin would have been beneficial to observe whether a shift of E-cadherin to N-cadherin is favoured during this EMT process, promoting a mesenchymal cell phenotype (Bartis et al., 2014).

As YAP is part of a mechanosensitive pathway, the acquired images matched the general expectation of YAP being present throughout most cells, with more stained YAP seen where the bronchosphere was in closest proximity with the PMET gel. It is then assumed that this increased presence would directly infer to an increase in nuclear YAP and a decrease in phospho-YAP, with mechanical cues 'travelling' across bronchosphere during transduction. Past proteomic data (western blots) show elevated YAP expression in whole lung lysates from IPF, and COPD donors compared to normal lung (higher ratio of yap versus phosphorylated yap) (Gokey et al., 2018). However, in relation to this generated data, clear demarcation was lost in many places, to specifically distinguish if associated YAP is nuclear or cytoplasmic as YAP appears to be quite homogenous throughout the bronchosphere once the gel has been manipulated. The loss in this demarcation also dampens the correlation between this data set and the previous proteomic analysis, whereby a clear increase is seen in YAP between the 1 minute and the 5 minute photocure conditions in the

western blot data set. This caveat of the ICC staining results could be counteracted by the use of a well characterised antibody that could target the phosphorylated YAP and visualise whether there was a difference between YAP being degraded by the original Hippo signalling pathway (by LATS1/2) or bypassing this regulation and be influenced by the mechanical stimulus from a derived ratio between the two protein states. Nuclear YAP in the latter case is not phosphorylated and binds to TAZ as well as its to TEAD. Multiple time points, especially between 0 and 24 hours may further help with ICC analysis, to ensure any changes in YAP concentration would not be missed. There were also some issues in increasing magnification on the current equipment used, with resolution being inversely affected- optimising the resolution and magnification in tandem with alternative equipment would have been beneficial to elucidate YAP's nuclearisation within the cell.

5.4 Zymography

5.4.1 *Introduction*

Airway and lung remodelling remains a key factor in COPD, with the imbalance of extracellular matrix (ECM) degrading enzymes being present in the lung parenchyma, aiding tissue reconstruction. Simultaneously, the additional deposition of new ECM glycoproteins and collagen add to the stiffening of the small airways contributes to the chronic inflammation and decreased elasticity seen in COPD pathogenesis (Chakrabarti and Patel, 2005, Suki et al., 2011, Bidan et al., 2015, Burgstaller et al., 2017).

Matrix metalloproteinase 2 and matrix metalloproteinase 9 (MMP2 and 9, also known as gelatinase A and B) are specifically implicated in COPD pathogenesis (Chakrabarti and Patel, 2005) due to the wide substrate availability in the ECM and their upregulation in COPD patients particularly those with comorbidities (Abd El-Fatah et al., 2015, Li et al., 2020, Mahor et al., 2020). MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs), inhibiting the zymogen conversion from the precursor form to the active form and thereby reducing MMP enzymatic activity, as well as binding to the MMP for subsequent regulation by macrophage engulfment (Lu et al., 2011). The ratio between the two proteins is important in terms of homeostasis. An increase of MMPs and a decrease of TIMPs leads to the degradation of the matrix membrane, whilst an increase of TIMPs and a decrease in MMPs allows for reconstruction to take place with the increased deposition of ECM. An imbalance of this ratio creates a continuous cycle of remodelling on the pulmonary architecture, leading to emphysema (Chakrabarti and Patel, 2005). A zymography assay was conducted to determine whether the stiffness of the surrounding matrix would influence the secretion of MMP 2 and 9 from the bronchospheres as a preliminary experiment to assess whether reporter peptides, if included in the PMET gel's network, would be cleaved during bronchosphere culture. These peptides were synthesised as MMP linkers attached to PEGDA monomers to study the effects of degradation but unfortunately, due to COVID-19 I was unable to perform the latter sets of experiments.

5.4.2 Methods

Bronchospheres were cultured and then embedded into the PMET gel and subsequently photocured for 1 minute and 5 minutes. During the following 72 hours, the supernatant from the apical and the basal side of the transwell was harvested every 24 hours and concentrated via ultra-centrifugation with a molecular weight cut-off of 30 kDa. Samples were then analysed using zymography gels. Full details of method are described in Chapter 2 (page 83).

5.4.3 Results

5.4.3.1 PMET gel (1 minute photocure) results

Representative zymograms are shown in **Figure 5.19**, using supernatant samples from cultured NHBE bronchospheres in the different conditions; PMET gel 1 minute photocure, 5 minutes photocure (apical and basal) and 25% Matrigel[®]. Separate MMP2 and 9 standards (71kDa and 92 kDa respectively) were also ran within each gel for reference. Although samples were collected from the 25% Matrigel[®] culture condition, the results were only observed and not analysed. Unfortunately, media from the uncured PMET gel culture condition were excluded from this assay as separation of reagents could not be adequately achieved.

Zymography analysis of the apical samples from the transwells used for culturing the NHBE bronchospheres after encapsulated in PMET gel (1 minute-**Figure 5.20A**) showed an increase in pro-MMP9 secretion over 72 hours. This was due to the very high levels seen in one patient. Basal secretion of pro-MMP9 activity was minimal and remained stable over the 72 hour duration of the experiment. Secretion of pro-MMP9 activity at the apical and basal side of the transwell containing CHBE bronchospheres donors were stable across the 72 hours (**Figure 5.20B**). Due to the spread of the individual data from patients, it was not possible to determine whether COPD bronchospheres secreted more pro-MMP9 activity than CHBE bronchospheres. No bands were produced from the encapsulated donor samples, against the MMP2 standard.

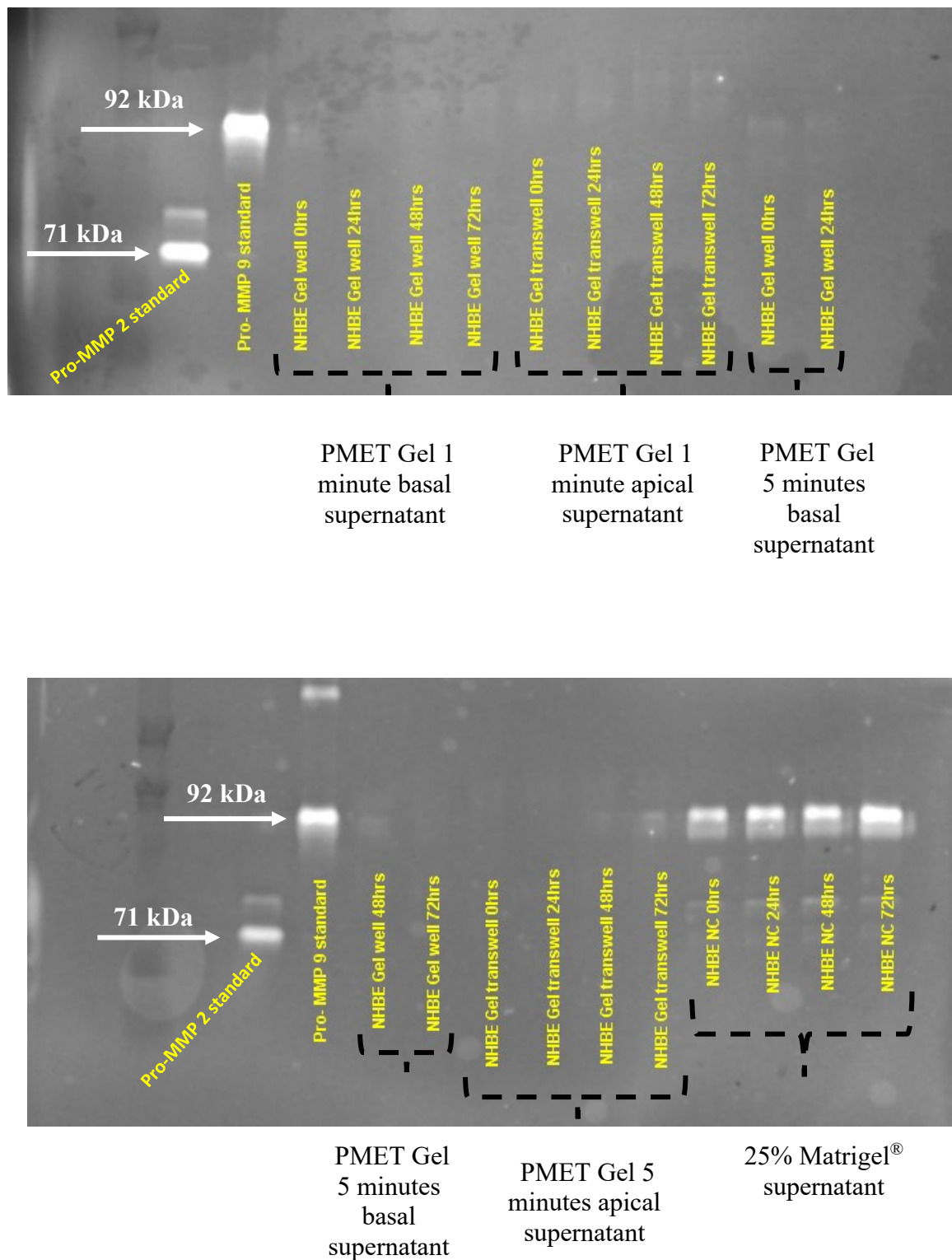


Figure 5.19: Representative zymograms of NHBE supernatant samples, harvested from bronchosphere culture conditions; PMET 1 minute photocure, PMET 5 minutes photocure and 25% Matrigel[®]. Apical and basal supernatants were also used from the transwell cultures- all referenced against the MMP9 standard which was ran in parallel (92 kDa). MMP2 standard was also ran (71 kDa).

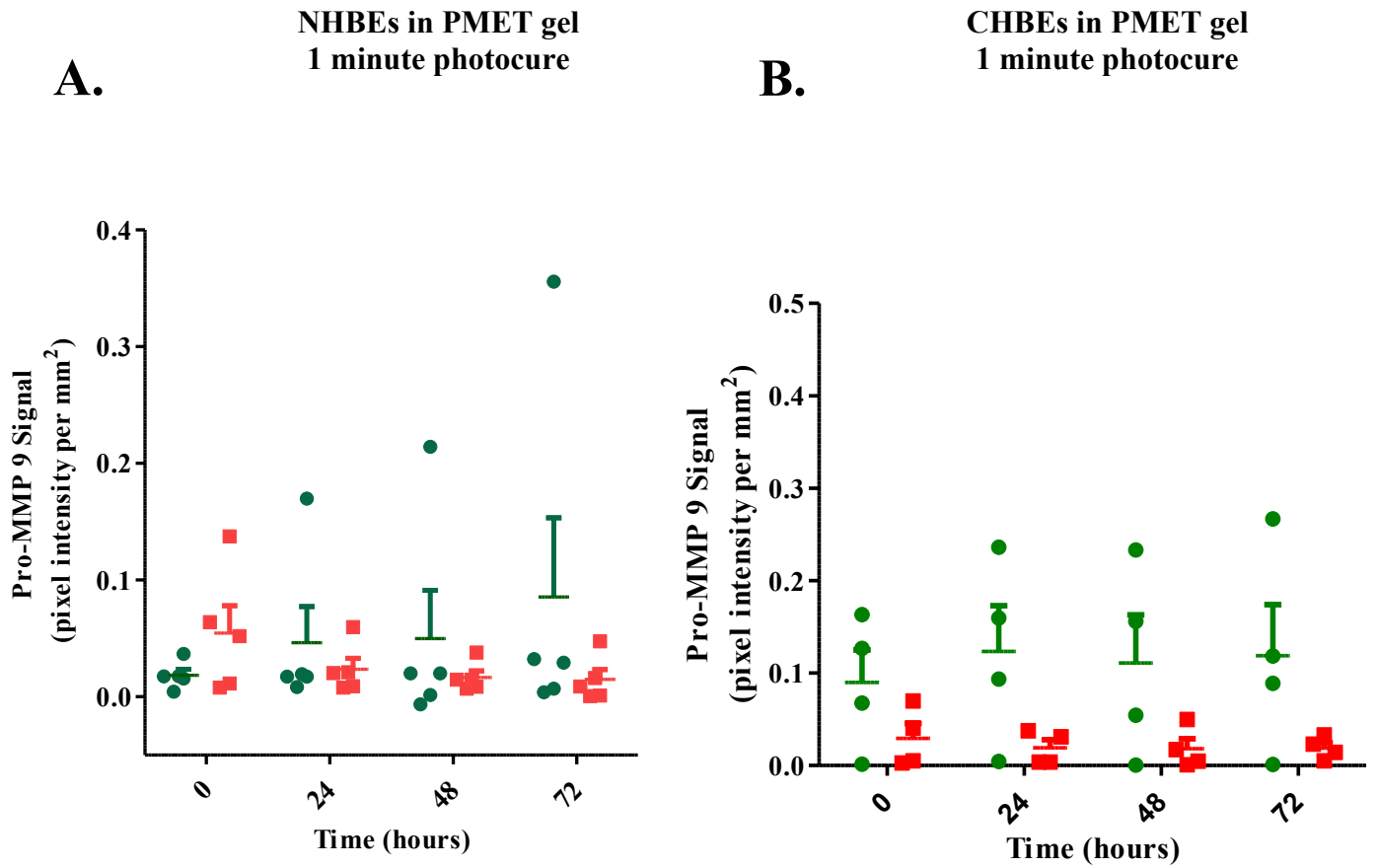


Figure 5.20: Densitometry data plotted from zymography gels showing degradation by pro-matrix metalloproteinase (MMP)9. Bands are from supernatant harvested from the apical and basal compartment of (A) normal human bronchial epithelial (NHBE) cell-derived bronchospheres (n=5) and (B) COPD bronchial epithelial (CHBE) cell-derived bronchospheres (n=4) embedded in PMET gel and photocured for 1 minute.

Key: ● apical supernatant, ■ basal supernatant.

Samples were collected every 24 hours for 72 hours from $t=0$. Mean of values and SEM plotted.

5.4.3.2 PMET gel (5 minute photocure) results

Representative zymograms are shown in **Figure 5.21**, using supernatant samples from cultured CHBE bronchospheres in the different conditions; PMET gel 1 minute photocure, 5 minutes photocure (apical and basal) and 25% Matrigel®. Separate MMP2 and 9 standards (71kDa and 92 kDa respectively) were also ran within each gel for reference. Although samples were collected from the 25% Matrigel® culture condition, the results were only observed and not analysed. Unfortunately, media from the uncured PMET gel culture condition were excluded from this assay as separation of reagents could not be adequately achieved.

Pro-MMP9 signal from the apical region of the NHBES showed a slight increase by 72 hours (**Figure 5.22A**), when encapsulated in PMET gel (5 minutes photocured). The profile is similar with the basal region, although there was a decrease at 48 hours. CHBE bronchospheres exhibited a steady low level at the basal side of the transwell used (**Figure 5.22B**) whilst the apical side had elevation of the same precursor MMP9. There was a similar level of pro-MMP9 activity in gels from bronchospheres embedded in 1 and 5 minute photocured gels. No bands were produced from the encapsulated donor samples, against the MMP2 standard.

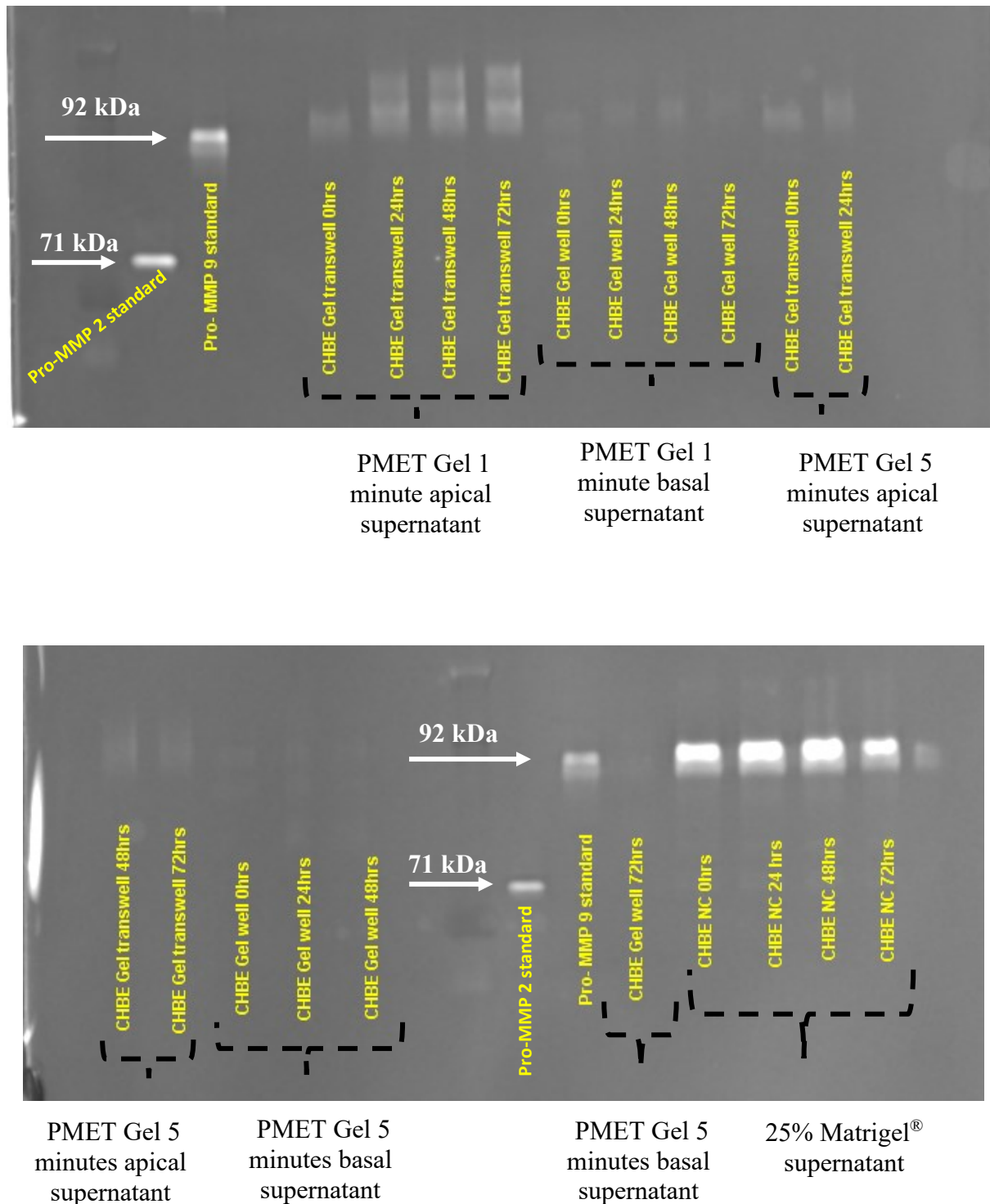


Figure 5.21: Representative zymograms of CHBE supernatant samples, harvested from bronchosphere culture conditions; PMET 1 minute photocure, PMET 5 minutes photocure and 25% Matrigel®. Apical and basal supernatants were also used from the transwell cultures- all referenced against the MMP9 standard which was ran in parallel (92 kDa). MMP2 standard was also ran (71 kDa).

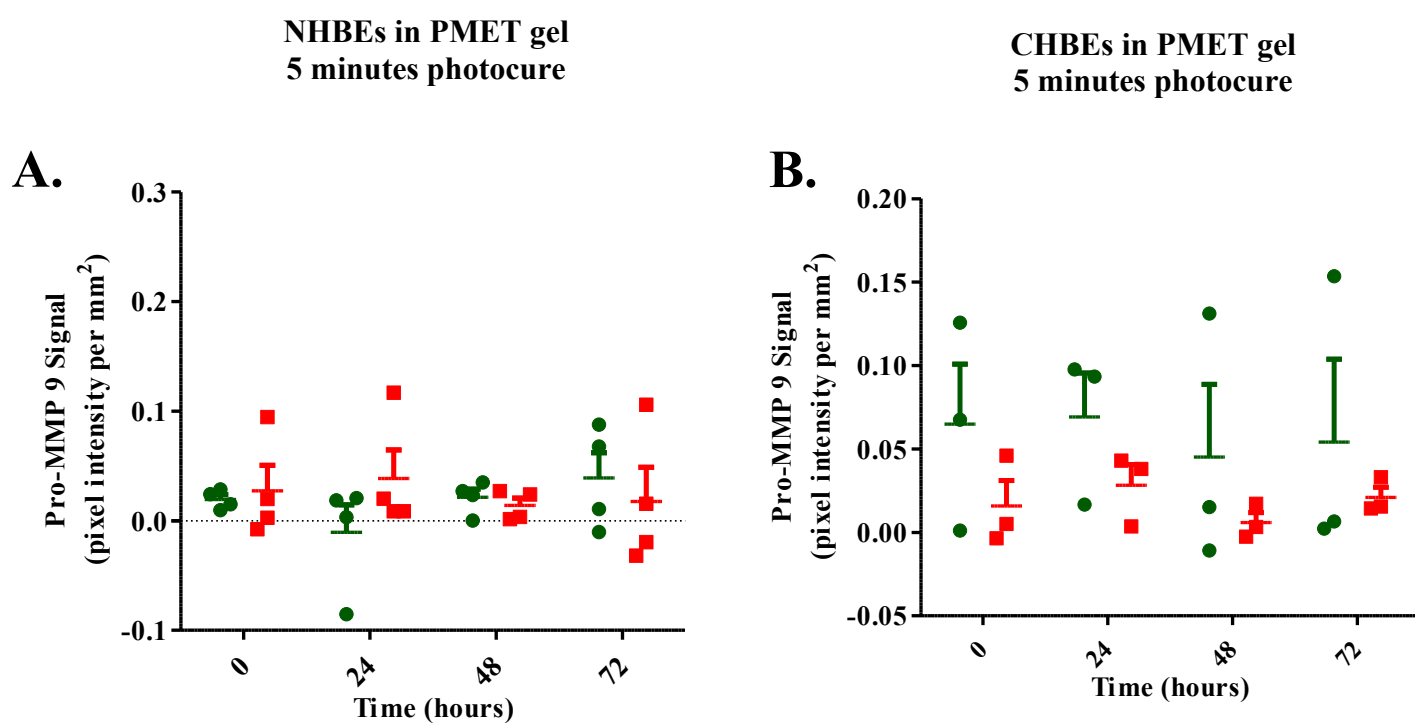


Figure 5.22: Densitometry data plotted from zymography gels, degraded by the presence of MMP 9 (Precursor form). Bands are from supernatant harvested from the apical and basal compartment of the (A) NHBE Bronchospheres ($n=4$) and (B) CHBE Bronchospheres ($n=3$), embedded in PMET gel and photocured from 5 minutes.

Key: ● apical supernatant, ■ basal supernatant.

Samples were collected every 24 hours for 72 hours from $t=0$. Mean of values and SEM plotted.

5.4.4 Discussion

MMPs are important for tissue remodelling within the ECM. Their presence is usually due to structural changes taking place e.g., for proliferation or even migration. There are 23 MMP subtypes defined by their substrate preference for ECM proteins and by their domain architecture (Lu et al., 2011). They are usually secreted as a precursor and an imbalance between TIMPS and MMPs can cause unchecked ECM modification influenced by external factors such as the mechanical force exerted onto the cell.

One NHBE bronchosphere sample set showed a time-dependent increase in apical secretion of pro-MMP9 from bronchospheres embedded in PMET gel cured for 1 minute but this was in contrast to the data from the other subjects. The increased pro-MMP9 from this one donor may be due to the cells initially reacting to transfer from an apparent (by eye and by Young's Moduli values) softer gel to the stiffer biosynthetic gel. Further studies are required to identify factors that distinguish this bronchosphere from others. There was no change in pro-MMP9 secretion to the basal side over time. A similar pattern of wide variability in pro-MMP9 release from different CHBE bronchospheres was observed together with a greater release to the apical side. There was a trend towards greater pro-MMP9 activity in the CHBE bronchospheres compared with that from the NHBE bronchospheres, but further experiments are required to determine whether this is indeed the case.

The pattern of results recorded were not as expected (such as no MMP2 seen secreted from the donors) and subsequent investigation would help to gain clarity. The increase in MMP9 has been particularly connected to chronic airway disorders like COPD, whereby constant exposure to e.g., cigarette smoke elevates the levels of this matrix metalloprotease (as well as MMP2). Previous published zymogram results show gelatinolytic bands of secreted MMP9 and MMP2 from submerged A549 and small airways epithelial cell (SAEC) submerged culture when exposed to cigarette smoke extracts over 24 hours and air-liquid interface SAEC cell culture when exposed to cigarette smoke extracts over 7 and 14 days (Agraval and Yadav, 2019). Such results were then linked to signalling cues for epithelial-mesenchymal transition to occur, as it is present in COPD, following from excess MMPs initiating ECM degradation (Agraval and Yadav, 2019). It was assumed that the change in gel stiffness, especially with the 5 minute photocured gel, would act as an inducer of the

MMPs and would be apparent when running the supernatant on zymograms. It was also expected that the supernatant of the CHBEs would have a higher level of MMP2 and MMP9 (pro or active form) than the NHBEs as they are already dysfunctional from normal and originate from a donor where unregulated remodelling is actively occurring. From the results, it may be the case that the gels (for both sets of donors) also require another co-stimulant to evoke the upregulation of MMPs such as pathogens, reactive oxygen species or harmful airborne particles, as per real-life scenarios and similar to the study conducted by *Agraval* and colleagues (Agraval and Yadav, 2019). The non-degradable nature of the gel could play a part in minimum zymogen activation occurrence and therefore would require some future optimisation to the assay used. The spheroid structure of the bronchosphere may also limit the secretion of the MMPs available in the supernatant as the lumen is not exposed, unlike more common air-liquid interface transwell models.

The difference seen between the two sections still remain unclear although measures were taken prior to running the zymography gels for example, a 30 kDa cut off ultra-centrifugal filters were used on the supernatants to achieve an equal starting point and volume. MMP2 and 9 molecular weight sizes (as well as their pro-MMP form molecular weights) are well above 30 kDa. It may well be that the media in the transwell is closer in proximity to the bronchospheres, instead of the well plate which surrounds the transwell. The volume in which the transwell holds is lower than that of a single well of a 24 well plate- invertedly increasing the overall concentration of the MMPs when ultra-centrifuged. Also, the cured PMET gel crosslinked architecture could have imposed a limiting factor on the MMP diffusion rate and amount of MMP secreted by cells, adversely affecting the enzyme secreted and measured. As zymography requires supernatant to be harvested, PMET gel without any curing (the preferred control) could not be added to the assay as there was no distinction between the gel solution and the medium. 25% Matrigel® culture done in parallel is an unsuitable control to use as Matrigel® can be heavily degraded and cleaved by organoids such as this whilst PMET cannot (only 25% Matrigel® is present and equally dispersed). Another limitation recognised in this assay is the measurement of TIMP levels as dysregulated remodelling can be due to an imbalance presence of inhibitors. With these values, a clearer view would be seen in comparison to the MMPs, creating a ratio per condition.

5.5 *Bronchospheres and COVID-19*

Due to the outbreak, multiple *in-vitro* models have come forth in research, in order to try and understand the mechanism of the virus and its infectiousness into the cell (Leung et al., 2020, Aloufi et al., 2021, Mulay et al., 2021). The organoids of the respective organs concerned with COVID-19 include the liver, brain, guts and more obviously, the lungs (Mallapaty, 2021). The model created with the bronchospheres in this project can be adapted to be used as valid models for COVID-19. Altering the composition of the media supplied to the bronchospheres and substrate by entirely removing the basement membrane Matrigel and incubating the fully formed organoid in EDTA, the polarity of the pseudostratified layer becomes inverted. The apical side of the bronchosphere, where the cilia reside and usually facing inwards into the lumen (**Figure 5.23**) becomes exposed in the surrounding medium, facing outwards instead, whilst the basal side faces inwards (**Figure 5.24**).

Since emerging data confirms that SARS-CoV-2 uses ACE2 receptors to gain entry into the cell (Mallapaty, 2021) and those cells would be present at the first line of defence e.g. ciliated cells, inverted bronchospheres now expose the cilia formed into the media. These bronchospheres can then be subjected to different strains of SARS-CoV2 and potential drug therapies to aid treatment. Contents of the lumen will also be excreted into the media, allowing for harvesting of any secretions for future assays. Such inversion can be done with bronchospheres as well as nasal epithelial organoids, using a relatively simple protocol (Co et al., 2019) referred to in Chapter 2 (see page 85) (**Figure 5.25** and **5.26**).

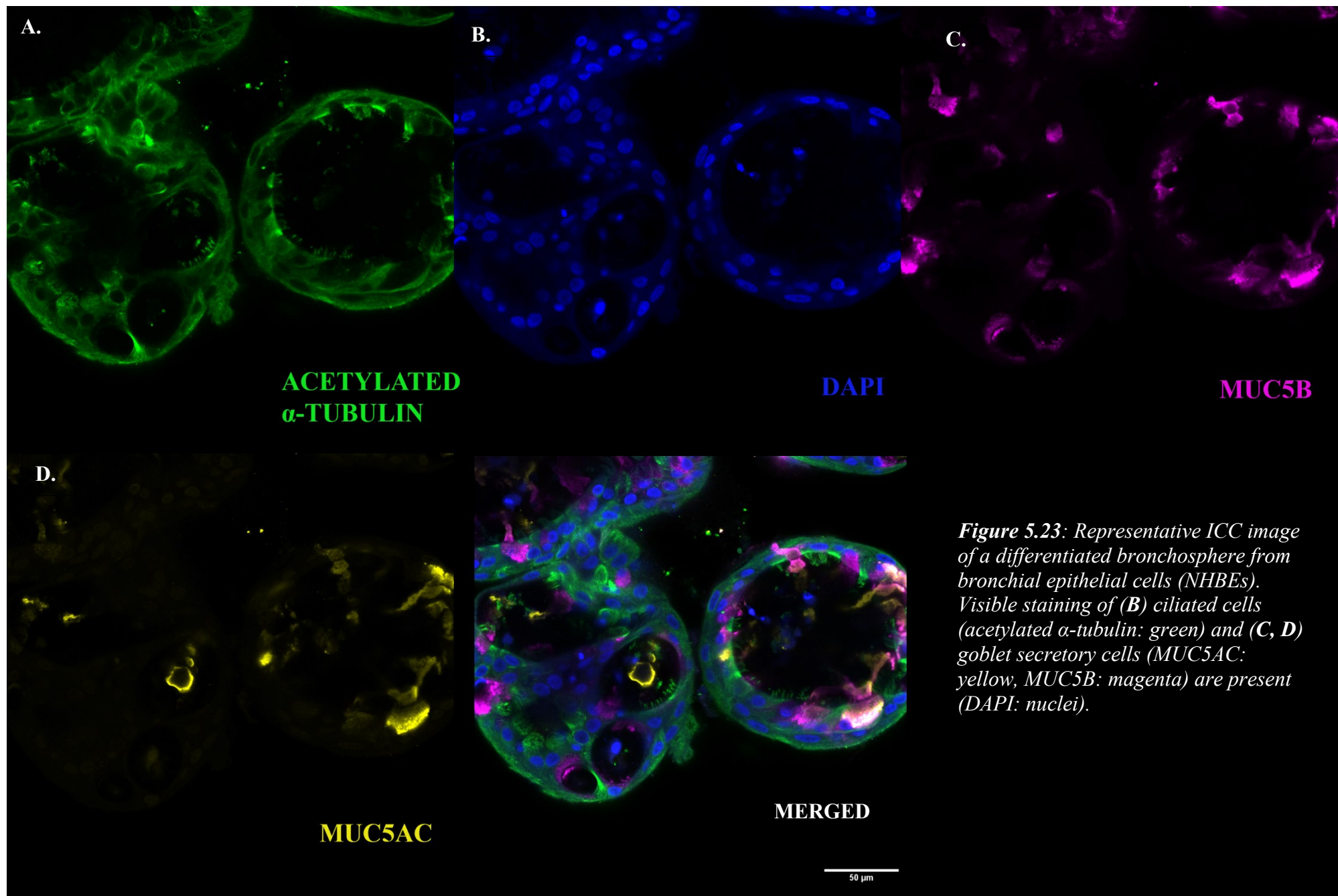


***Figure 5.23:** Representative ICC image of a differentiated bronchosphere from bronchial epithelial cells (NHBEs). Visible staining of (B) ciliated cells (acetylated α -tubulin: green) and (C, D) goblet secretory cells (MUC5AC: yellow, MUC5B: magenta) are present (DAPI: nuclei).*

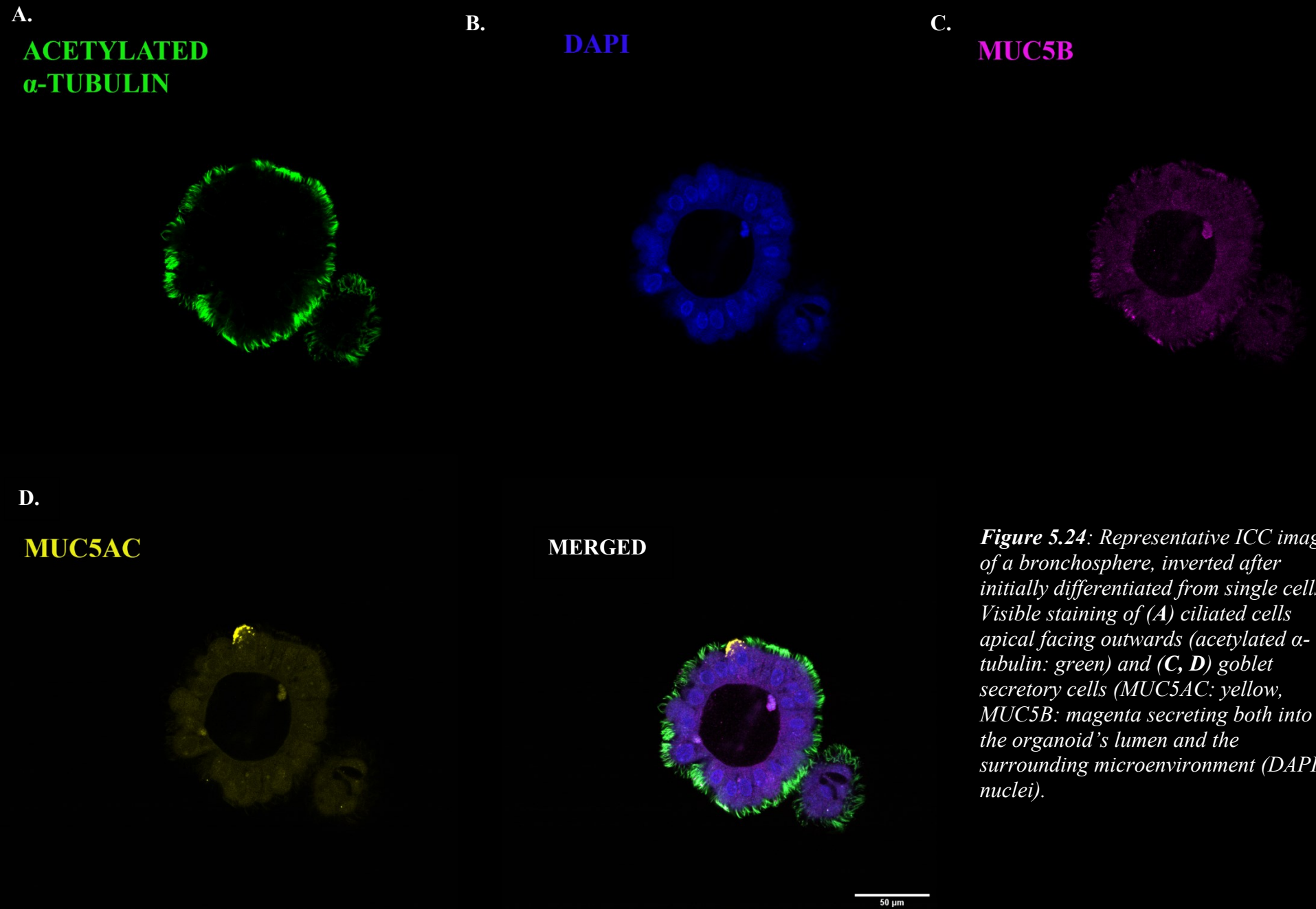


Figure 5.24: Representative ICC image of a bronchosphere, inverted after initially differentiated from single cells. Visible staining of (A) ciliated cells apical facing outwards (acetylated α -tubulin: green) and (C, D) goblet secretory cells (MUC5AC: yellow, MUC5B: magenta secreting both into the organoid's lumen and the surrounding microenvironment (DAPI: nuclei).

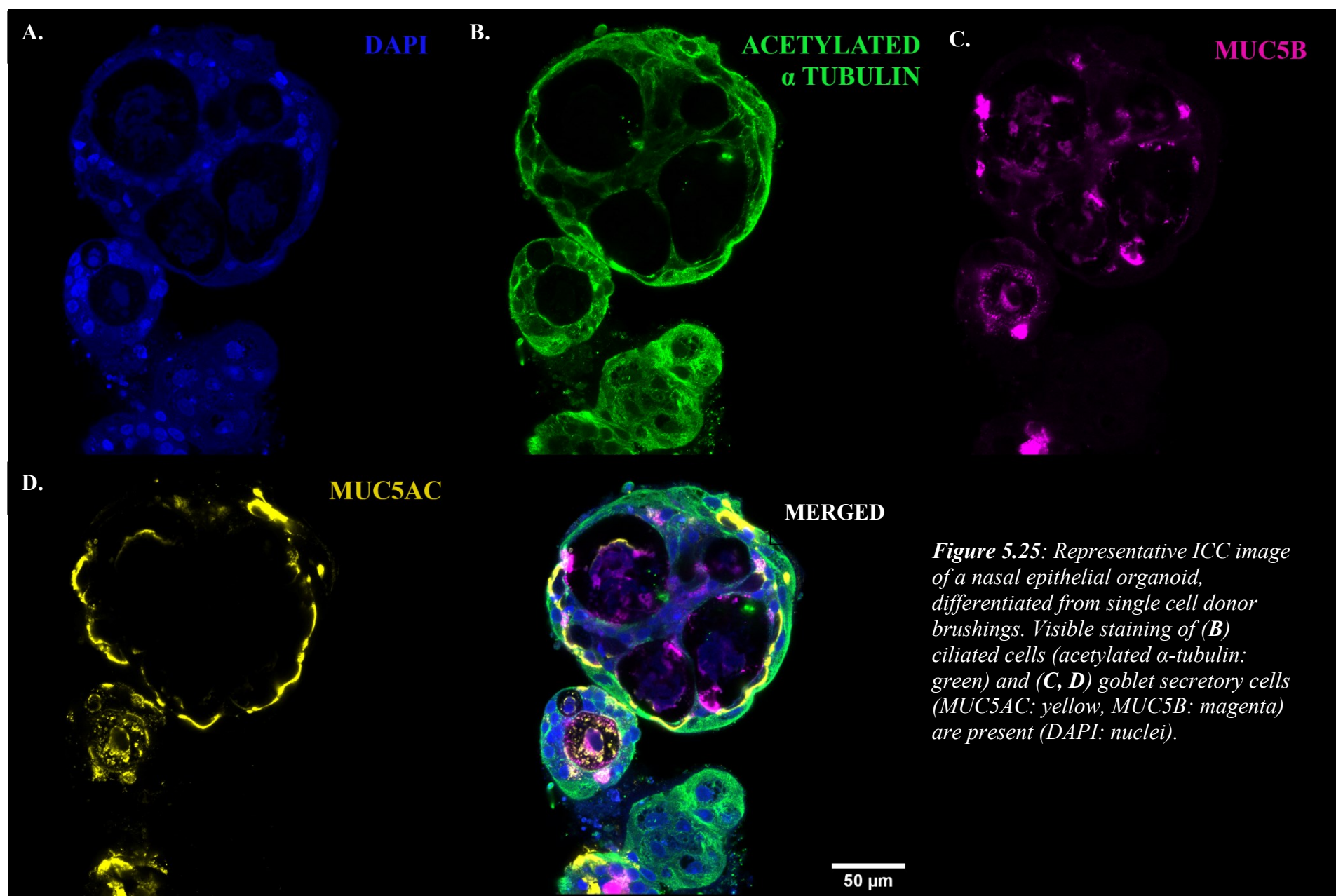


Figure 5.25: Representative ICC image of a nasal epithelial organoid, differentiated from single cell donor brushings. Visible staining of (B) ciliated cells (acetylated α -tubulin: green) and (C, D) goblet secretory cells (MUC5AC: yellow, MUC5B: magenta) are present (DAPI: nuclei).

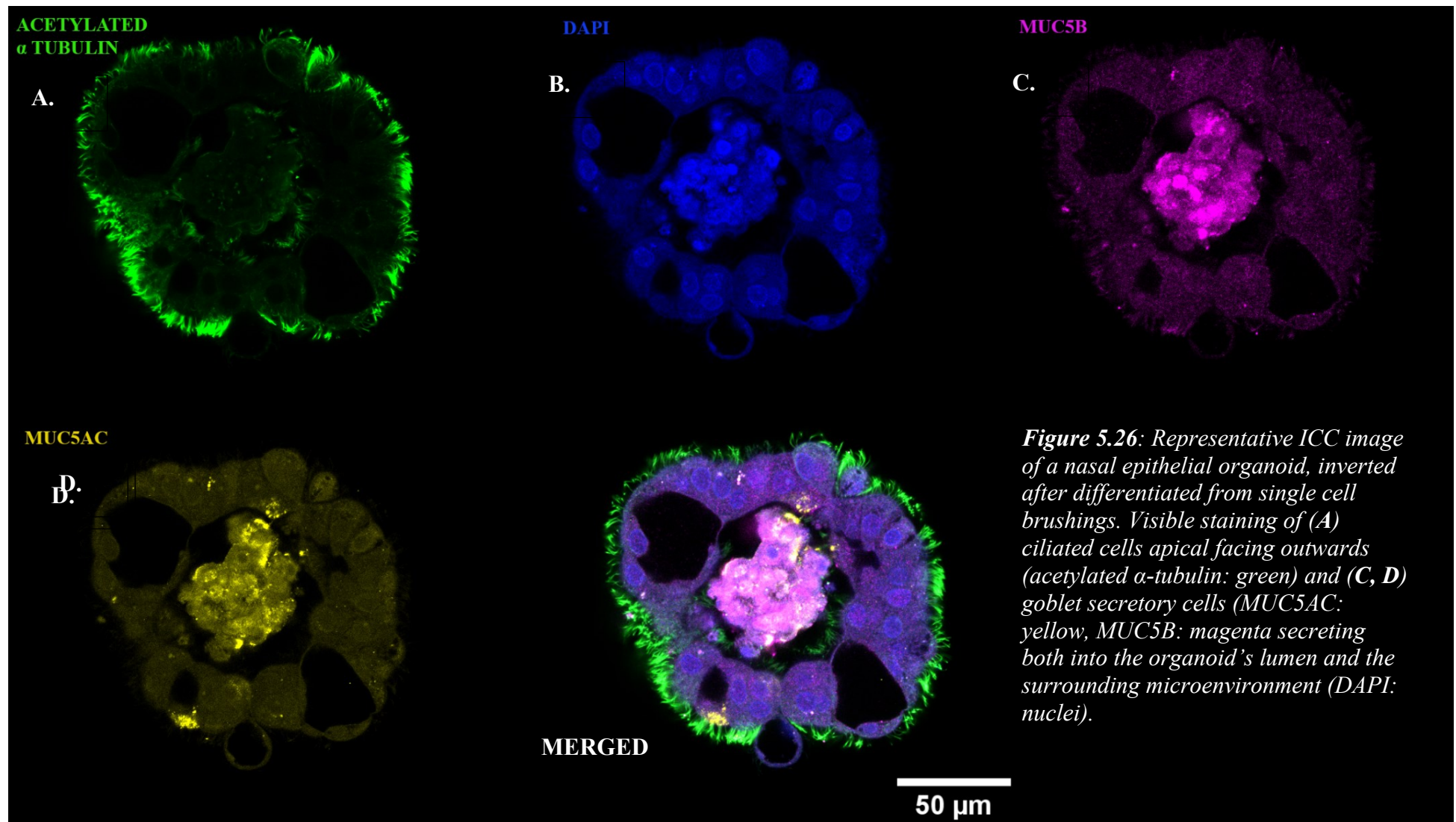


Figure 5.26: Representative ICC image of a nasal epithelial organoid, inverted after differentiated from single cell brushings. Visible staining of (A) ciliated cells apical facing outwards (acetylated α -tubulin: green) and (C, D) goblet secretory cells (MUC5AC: yellow, MUC5B: magenta secreting both into the organoid's lumen and the surrounding microenvironment (DAPI: nuclei).

5.6 Summary

As a conclusion, the single cell data derived from healthy and COPD bronchospheres display an altered genomic phenotype, influenced not only by changing the surrounding matrix stiffness but the amount of the time exposed within the new microenvironment. Subpopulations appeared to be present with bimodal expressions of housekeeping genes, noted in **EEF1A1**, **RPLP0** and **RPL13A**, perhaps coinciding with different rates of proliferation as markers **Ki67** and **SOX2**. This trend is even more apparent in the CHBEs, with photocuring stimuli increasing expression in mechanotransduction-associated markers e.g., **CTNNB1** and **YAP1**, highlighting their sensitivity to stronger mechanical cues. These last two markers also correspond with the upregulation seen in COPD donors (goblet, club, ciliated, AT1, AT2 and aberrant basaloid) via the single cell atlas platform *copdcellatlas.com* (Sauler et al., 2022). However, multiple replicates (samples and donors) are required to gather a fuller picture, with optimisation as a prerequisite to ensure more accurate readout per gene investigated and a detailed insight as to which cell type (and subtype) is contributing to this change. Single cell RNA-seq can then be considered as this will give a greater breadth of expression and allow for more cells to be detected. From there, more detailed analysis can be conducted, utilising tools like principal component analysis (PCA) and t-distributed stochastic neighbour embedding (tSNE) plots to interpret the meaningfulness of large data (and any population clustering) through dimensionality-reduction methods.

The proteomics data from the Western blots performed focuses on the transcriptional coactivators YAP and TAZ and their involvement in mechanobiology of the bronchospheres. An increase in YAP1 expression indicating the cell's responsiveness to the extracellular environment is seen in both gel conditions, with the healthy bronchospheres sustaining YAP expression especially at the onset of stiffening the gel. Data from the COPD organoids complimented the pattern seen with single cell qPCR (initially at 0 hours) – the PMET gel photocured for 1 minute eliciting a greater response just after the matrix has been altered.

Whilst the ICC images would also require more refinement to properly distinguish where YAP is present (perhaps with the additional antibody marker of the opposing phosphorylated YAP to detect its' location), the zymography graphs depicted slight increased

activity of the CHBE bronchospheres when stimulated with green light after 5 minutes in PMET gel. It may be assumed that cells from the COPD donors are pre-programmed to survive a harsher, stiffer parenchyma and ECM- more adaptable to any diversions from the norm and maintain their own sense of homeostasis. The stiffness of the 5 minute PMET gel would be out of their remit using its YM value of 138 ± 23 kPa as a point of reference, pertaining to matrix stiffness found in more severe forms of fibrotic respiratory diseases such as IPF as exhibited by *Booth et al* (Booth et al., 2012, Jandl and Kwapiszewska, 2020) and therefore act as a stronger initiator for MMP release. However, the results from the performed zymograms are not as concrete as expected- this may be overcome by additional experimental assays (e.g. fluorescence resonance energy transfer (FRET) MMP activity kits) to see if supernatant results after matrix alteration match the elevated MMP levels seen in ALI cultures induced from CSE extract (Agraval and Yadav, 2019) or BAL fluids from COPD patients (Mercer et al., 2005), with additional measurements of contrasting TIMPs levels. Once analysed, the intended PEGDA-MMP linker ECM could be synthesised and used to monitor ECM degradation by the bronchospheres.

Mechanotransduction is a key player in how the way the cell interacts with the ECM and change their behaviour in accordance. With standard mechanical cues from fibrotic ECM acting as a factor of its own entity, it needs to be further addressed as drug therapies for treatment may not be as effective as anticipated. Other non-specific sensors e.g. Piezo ion channels (Murthy et al., 2017) and inhibition of YAP with the downstream effects on genes relating to fibrosis can be explored. There are many YAP inhibitors available for downstream analysis e.g., Verteporfin and Flufenamic acid but some caution should be taken since as previously mentioned, YAP has multiple functions which may also be affected (Pobbati et al., 2015, Wang et al., 2016, Pocaterra et al., 2020). This chapter creates a good starting point to explore these initial conclusions further, utilising newly published cell atlases as well as more complex methods like scRNA-seq to pinpoint the true drivers of this response mechanism and how to effectively counteract them.

Chapter 6: **Conclusion**

6.1 Introduction

The remit of my PhD project was to investigate the effects of altering the matrix i.e., increasing the stiffness of the microenvironment on differentiated lung organoids, bronchospheres. The encapsulation created a biomimic of the lung ECM, which influenced the 3D cell culture of the bronchospheres derived from healthy and COPD donor samples. Within this project, several aims were addressed. In the initial part of this chapter, I will summarise the key findings of each of these aims and then re-assess the hypothesis tested in light of this data. I will then highlight some of the strengths and limitations of my thesis and consider possible future directions for this research.

6.2 Gel matrix synthesis

The synthesis of the PMET gel in chapter 3, used in following assays needed to be adaptable in order to emulate the extracellular matrix found in fibrotic airways and lung tissue. This was achieved by using the interaction of green visible light, the photoinitiator Eosin Y and its co-initiator Triethanolamine, alongside Matrigel[®] and PEGDA to create the hydrogel. The bronchosphere created can also be encapsulated into the gel. With tunability, the PMET gel's YM can be altered, depending on the length of green light exposure. As no concrete values of a COPD whole lung could be found in recent or past literature (whole lung, not decellularized), the stiffness or YM for COPD lung was based on *Hinz* and colleagues, whereby the fibrotic lung YM can range from 20-100 kPa (Goffin et al., 2006, Hinz, 2009, Hinz, 2010, Hinz, 2012). The PMET gel after 1 minute of green light exposure generated a YM value of 37 ± 4 kPa at 5% strain using rheometric compression testing. After 5 minute photocure, the YM increased to 138 ± 23 kPa at 2% strain.

There are many alternative approaches to 3D *in vitro* modelling, other than culturing organoids. Usage of air-liquid-interface (ALI) cultures with (bronchial) epithelial cells are often employed, due to the ciliated apical side of the pseudostratified epithelium being exposed to air and can therefore be involved in a range of assays including toxicology studies whilst the basolateral side is kept submerged in culture (separated using transwell inserts) (Kapetanović, 2011, Francis et al., 2022). The configuration of the ALI cultures also allows for any secretions to be easily harvested and analysed; data from many studies referenced in Chapters 4 and 5 are based on these models as the exposed epithelium can readily mimic the airways. Other cell types can be used as the starting source for organoid generation, including

cells from the small airways and from the alveoli (Conlon et al., 2020). Manipulating the surrounding microenvironment can be further advanced depending on what biological or synthetic reagent is used. The selection of the matrix substrate is also important. Natural polymers e.g. collagen (Tirella et al., 2012) and agarose (Dang et al., 2004) are malleable for the user to apply but may pose a risk of being degraded by cells or organoids and potentially harder to produce functional polysaccharides for hydrogel formation (Gasperini et al., 2014, Yang et al., 2022). Conversely, this attribute is dependent on the research hypothesis investigated- in this case, stiffness is the variable in question and therefore is beneficial to keep constant whilst observing influence on cellular behaviour, particularly over the short duration of the experiments. Should the experiments be conducted over a longer timeframe, the ability of the cells to degrade the external matrix and generate their own may be advantageous. Although Matrigel® is often used as the *in-vitro* matrix, eliminating the use of animal-derived reagents completely can be achieved with decellularised tissue of the organ as the starting material. Many research groups have used this method, creating *in-vitro* models of the COPD and IPF lung (O'Neill et al., 2013, Wagner et al., 2014, De Hilster et al., 2020, Hedström et al., 2021), conserving a semblance of the native ECM components found *in vivo*, and also in disease states.

Other *in-vitro* modelling techniques of the lung include:

- Organ (lung)-on-a-chip; introducing additional and complimentary cell types (e.g., from the immune system) to explore cell-to-cell interaction, using methods in microfabrication and microfluidics to also consider the element of vascularisation. Mechanical forces such as shear force and stretch can then be included as well as mimicking *in-vivo* concentration gradients and physiological breathing (Huh et al., 2010, Benam et al., 2016, Felder et al., 2019)
- Precision-cut lung slices; maintaining the 3D native microstructure of the tissue and ECM, whilst still involving different cell types found in the lung (Van Dijk et al., 2016, Alsafadi et al., 2017, Liu et al., 2019)
- Induced pluripotent stem cells as the primary cell source; allowing for the controlled differentiation of adult somatic cells into progenitors of preferred cell lineages (Gotoh et al., 2014, Dye et al., 2015, Konishi et al., 2016)

Each have their own merit and allowing the researcher to investigate different aims. **Table 6.1** highlights a few of the advantage and disadvantages of each modelling technique.

Culture systems	Advantages	Disadvantages
Air-Liquid Interface	• Differentiated pseudostratified epithelium formed • High throughput • Long-term cell viability • Low cost	• TC plastic still used • Multicellular formed, not 3D • No physiological aspect
Organoids: Primary cells	• Phenotypical/physiological relevance • Mimics native 3D tissue architecture • Differentiated pseudostratified epithelium • Cell-cell and cell-ECM interaction can be explored • Multiple cell types can be added • Patient specific • Polarity can be reversed • Healthy/'disease' samples can be used	• Protocols need to still be optimised • Limited nutrient and waste systems • Size variation • Lack in vasculature • Reproducibility can be difficult
Organoids: Induced Pluripotent Stem cells	• Same as 'Primary cells' • Controlled differentiation on adult somatic cells for desired lineages	• Same as 'Primary cells' • Higher cell maintenance
Matrix: Biosynthetic	• Defined composition • Controlled biomechanics • Can be animal free • Can mimic healthy/'disease' states	• Cell adhesion motifs may be needed
Matrix: Decellularised	• Conserving composition (including ECM proteins) • Human derived • Can be from healthy or diseased donors	• Can be unintentionally degraded • May be difficult to obtain tissue • May be difficult to mimic same mechanical properties from <i>in vivo</i>
Matrix: Matrigel®	• Popular culture systems with standardised protocols	• Animal derived • Can be unintentionally degraded
Precision-cut Lung Slices	• Drug interaction • Models of different stages in disease • Multiple cell types • Metabolically active	• Short-term viability • Difficult to get samples
Lung-on-a-chip	• 3D tissue architecture • Immune cells can be added • Biomechanics can be stimulated-physiological breathing • Patient specific cells • Microfluidics can be implemented: perfusion	• Limits in scalability • No standard protocols • Complex culture

Table 6.1: Comparisons (non-exhaustive) between different modelling methods of the lung. Advantages and disadvantages generated from extensive reviews (Francis et al., 2022) and (Nikolic and Rawlins, 2017) and other references (Liu et al., 2019, Vazquez-Armendariz et al., 2022).

6.3 *Effects of stiffness on epithelial mRNA from healthy and COPD donors*

Chapter 4 focused on the impact of stiffness on genomic expression, on epithelial cells. Phenotypic changes were expected to be present in both healthy and COPD bronchospheres, with an increase in mRNA of mucins (namely MUC5AC) from goblet cells in the COPD bronchospheres with the NHBEs following suit, reflecting the hypersecretion and cell hyperplasia seen in disease together with markers associated with cell proliferation (Whitsett, 2018, Okuda et al., 2019). The data obtained from the RT-qPCR assay showed the expected enhanced MUC5AC expression in CHBE bronchospheres, indicating either goblet cell hypersecretion or increased numbers of secretory cells. There were also increased expression of the proliferative marker SOX2, the ciliated cell marker FOXJ1 and, to a lesser extent, the basal cell marker TP63. Together, the data indicates reproduction of key features of COPD such as goblet cell proliferation and suggestion of epithelial -to-mesenchymal cell transition (Shaykhiev et al., 2013, Jonsdottir et al., 2015). The increase in FOXJ1 mRNA expression may also be an indicator of early cell transdifferentiation, with ciliated cells acting as progenitors for goblet cells (Turner et al., 2011). Studies have shown an inverse relationship in ciliated cell differentiation in COPD; favouring an increase in goblet or basal cell phenotype from the presence of cytokine IL-13 (Turner et al., 2011). Conversely, epigenetic modifications (due to e.g. continuous exposure to cigarette smoke) in the epidermal growth factor receptor signalling pathway have also been reported to favour ciliated cell hyperplasia instead (Tyner et al., 2006, Liu et al., 2010a, Gohy et al., 2019), leading to dysregulation like reduced length and cilia beat frequency (Thomas et al., 2021)- a profile expected from the COPD-derived ciliated cells. Additionally, any intermediary cell subtype would also express both markers, influencing the change in expression (Ruiz García et al., 2019, Davis and Wypych, 2022). The duration of the (bench) RT-qPCR assay following encapsulation was 72 hours and it would be interesting to see if change in expression was maintained after this timepoint.

However, Chapter 4's original assumption is partially valid as upregulation in MUC5B mRNA expression was seen notably with the NHBE bronchospheres, which was augmented when encapsulated in the stiffer 5 minute photocured PMET gel. ICC images corroborated this and showed increased secretion of both MUC5AC and MUC5B when the matrix is stiffened, although this is more evident for MUC5B with NHBEs and for MUC5AC

with CHBEs, respectively. Increased MUC5B plugs, formed from excessive secretion from the submucosal glands lean more towards hallmarks of a severe form of lung fibrosis instead of COPD (Conti et al., 2016a, Nakano et al., 2016, Okuda et al., 2019). The IPF- like stiffer matrix, which can vary in severity and simulated in this project by the 5 minutes photocure, may have modulated MUC5B mRNA expression to a greater extent than anticipated.

6.4 *Mechanisms of mechanotransduction via single cell (sc) analysis*

The aim of completing sc RT-qPCR in Chapter 5, was to explore further in detail whether upregulation would be apparent in gene markers concerning mechanotransduction pathways as well as proliferation and mucus secretion. The dataset from single cell analysis provided evidence of subpopulations amongst the individual cells screened once the matrix was altered. The bimodal violin plots of housekeeping genes, when photocuring took place, also supported the unstable Ct values seen in the same housekeeping genes during bench RT-qPCR, suggesting that cell subpopulations had differential growth rates as proliferative markers like SOX2 were upregulated following changes in stiffness. Future studies using scRNA-seq analysis of single cell suspensions from the photocured bronchospheres will provide much greater detail and understanding of individual cell types to changes in stiffness. However, a large number of cells are required as well as multiple biological donors to validate any trends seen. Some increase in the genes involved in mechanosensitive pathways were noted e.g., YAP1 and CTNNB1 which were more prominent in the bronchospheres derived from COPD donors and embedded in the 1 minute photocured matrix at baseline and also at 24 hours after the 5 minute photocure. But in part, the aim could not be fully validated due to the lack of data seen with MUC5AC and MUC5B, perhaps due to the mechanical and enzymatic nature of gel and organoid disruption. Due to time restrictions, I was unable to use YAP-TAZ pathway inhibitors in these experiments. It is assumed that the gels were homogeneously made, due to using the same reagents, protocol, and equipment. However, there may be some heterogeneity due to increased crosslinking within the gel moulds (e.g., in the 5 minute photocure) affecting the cells. Moving further with this assay, YM measurements can be performed to determine whether this is the case.

Supporting assays used protein characterisation and immunocytochemistry to investigate YAP1 from the hippo signalling pathway. If this mechanosensitive pathway was activated by matrix stiffening, YAP1 cytoplasmic-to-nuclear translocation would occur with subsequent phosphorylation by the dedicated kinases LATS1/2 directing degradation. Protein chemiblots from NHBE bronchospheres showed a dramatic spike in YAP1 expression immediately following encapsulation in the modified/stiffer matrix, which decreased over time in both stiffness conditions. YAP1's presence appeared more homogenous in CHBE bronchospheres, although a delayed increase in protein expression as determined by Western

blotting was seen 24 hours after mechanical stress is applied. However, this expression was lost (more rapidly) over time with following induction of the highest stiffness. Interestingly, it was the 1 minute photocure that induced the highest response from both bronchospheres. There may be a link in certain stiffness thresholds and YAP as well as any epigenetic regulation in mechanotransduction from the CHBEs in response to stiffness and YAP activation, but this would need to be explored further. Additional lengths of photocure can be utilised to produce a larger gradient of stiffnesses to investigate with PMET gel. In contrast, the normal lung stiffness range of 1-7.5 kPa* (*approximation), may explain why NHBES 'react' from 0 hours onwards, in photocured PMET gels (Booth et al., 2012). The increase of YAP expression is also similar to cells when cultured on TC plastic- particularly due to very stiff nature of the plastic substrate (2-4 GPa)- however, no direct comparisons have been made. Data on human mesenchymal stem cells (hMSCs) show an increase in YAP (regarding nuclear to cytoplasm ratio) when cultured on TC plastic in comparison to softer hydrogel substrates and therefore influencing cell type differentiation (Yang and Weinberg, 2008, Zhang et al., 2021). Further investigations are continuously emerging from different groups, exploring cellular biomechanics that occur and the important of substrate stiffnesses (Krishnan et al., 2016).

ICC images of YAP1 expression were obtained to support the above data. However, not enough discrimination between nuclear and cytoplasmic compartments was possible. Certain caveats have already been addressed which could help to strengthen the argument, including the use of phosphorylated YAP1 to discover if any YAP1 had been targeted by the Hippo signalling pathway and whether there is indeed an imbalance due to the matrix-induced stiffness or that the signalling pathway is already trying to compensate for the YAP1 activation. Using different anti-YAP1 antibodies may also improve the resolution, together with utilising other more sensitive imaging techniques e.g., light sheet fluorescence microscopy. The latter case uses part illumination of the bronchosphere to focus on a specific region of interest instead of the whole organoid, with increased image acquisition speed than seen with confocal methods (Power and Huisken, 2017, Dekkers et al., 2019). The increased production of matrix metalloprotease, particularly MMP2 and 9, is associated with enhanced tissue remodelling due to external cues sensed by the cell(s). Zymography was performed, in order to investigate the production of MMP2 and 9 and their downstream effects on the bronchospheres. In partnership with this technique, an MMP-targeted linker was synthesised on the PEGDA monomer to study the modulation of protease degradation *in vitro*, but

unfortunately, I was not able to carry out the planned assay due to COVID-19-related lab lockdown. Zymography of CHBE bronchospheres showed slight increased activity in ProMMP9 production in comparison to the NHBs, leading to the notion that CHBEs may already be primed to remodel their microenvironment from epigenetic changes (Liu et al., 2010a). However, since it was the precursor MMP produced (only pro-MMP9, not pro-MMP 2), rather than the active form, there may be issues regarding the fabricated scaffold being non-degradable and therefore favouring the non-active form of the enzyme, as well as the possible lack of additional components or factors required in this model assay to cleave the pro-domain on the zymogen (Löffek et al., 2011). The differentiation medium used was serum free, with Matrigel® spiked in its composition. Matrigel® only contains trace MMPs and is therefore considered to be non-contributing (Corning, 2021).

6.5 Hypothesis validation and summary

With all the evidence now addressed, the original hypothesis can now be reviewed. *Does airway stiffness regulate epithelial cell function and impact disease processes?* Yes, to a certain degree. In relevance to hallmarks of the relevant respiratory pathologies COPD and IPF, the biosynthetic PMET gel alone is able to induce increased mucus secretion as well as increased proliferation when the stiffness was modified (increased). Bronchospheres consisting of specialised epithelial cell subtypes like goblet and ciliated cells were impacted by the change in stiffness of matrix used, with NHBEs differentially affected in comparison to CHBEs. Evidence mainly from RT-qPCR, ICC and scRT-qPCR help to support this theory, with emphasis on mechanotransduction-associated proteins such as YAP, investigated using Western blots and ICC. The created *in-vitro* model however leans more towards an IPF model, whereby the stiffness created, especially at 5 minutes photocuring, is more reflective of that seen in severe IPF (Booth et al., 2012). It is possible that the source of epithelial cells within the airway, bronchial rather than small airways, used in the bronchosphere culture may affect the results. Using small airway epithelial cells or alveolar epithelial cells may better reflect COPD and IPF as this is where the diseases are located. Cells were also sourced from cell banks housed at the NHLI. Whilst they were from previous donors (frozen down for future use) and could have led to favouring set progenitor populations in culture, storage and viability were constantly maintained and checked. This was especially true when initially grown in co-culture fashion as well as when compared to newer vials sourced from Lonza.

There is some scope to optimise the matrix further, by creating a model for the healthy lung matrix but the adaptation would be a complex composition. Stiffness throughout the normal lung is not uniform, but instead the ECM is dynamically different along the airways and lung depending on the cell type, the ECM proteins involved and location of measurement. The same can be said for disease states such as COPD, where ECM changes according to severity. The hyperproliferation of airway smooth muscle cells in the small airways would generate a higher measure of stiffness than the vacuole -emphysematous nature of the alveolar tissue as well as different stages in GOLD diagnosis cohort. This project enforces the idea that the matrix itself can dictate cellular behaviour. It is well established that cells are able to receive cues due to perturbations, and therefore biochemical cues from mechanotransduction signalling at the cell-to-matrix interface too (Piersma et al., 2015, Haller and Dudley, 2022).

Cells traditionally interact with the matrix via integrins in focal adhesions and membrane bound associated proteins to alter phenotypes but the matrix can also exert influence on cells via different mechanisms, exhibiting bidirectional communication (Haller and Dudley, 2022). These mechanisms include force-sensing mechanisms relayed to the cell e.g. remodelling of collagen fibres, release of latent proteins e.g. TGF- β 1 or the exposure of cryptic integrin binding sites, all when mechanical stress is applied (Kubow et al., 2015, Sengle and Sakai, 2015, Haller and Dudley, 2022). Combined with the non-degradability of the gel, the bronchospheres experience a compounding effect from mechanical stress when the matrix is stiffened, which translates to intracellular biochemical cues. In comparison to existing studies, models that include stiffness as being an important variable, have employed decellularized tissue from COPD and IPF donors to mimic the YM of fibrotic tissues using hydrogels (De Hilster et al., 2020), photocrosslinking of hydrogels to recapitulate fibrotic influence on cellular (fibroblasts) behaviour (Nizamoglu et al., 2022) and have incorporated additional cell types (epithelial, endothelial and fibroblasts) to investigate the effects of a profibrotic stimulus like TGF- β 1 (Tan et al., 2017). However, the encapsulation of the lung organoid within a defined matrix like PMET gel permits for the model to be better suited in studies regarding remodelling mechanics seen in fibrosis; with bidirectional communication considered (cell-to-cell, cell-to-ECM, and ECM-to-cell). Additionally, the self-assembled differentiated bronchosphere, as a whole structure experiences the applied mechanical force, not just a typical cultured monolayer. Such physiological features are hard to replicate in other models e.g., ALI cell cultures and lung-on-a-chip models.

Conversely, there were many limitations to this project, particularly when applying standard downstream assays to the 3D model, mentioned in the previous chapters. These include the small number of donor samples (biological replicates), the physiological complexity of the PMET gel, which also resulted in the need to adapt ICC protocols and the limited amount of RNA and protein extracted. Furthermore, the current system is only based on epithelial cells and bears no exposed apical interface. However, in comparison to traditional 2D cell culture, where tissue culture plastic GPa stiffness creates another issue in biological relevance, and perhaps other 3D modelling options, this model has the capability of extending such research on mechanotransduction. Considerations of its pitfalls, suggestions on how they can be overcome and translation into alternate mechanobiological applications for future work are to be discussed further on, in the following section.

6.6 *Outlook of future work*

6.6.1 **Bronchosphere-PMET matrix model**

As highlighted in the previous result chapters, an increase in donors would aid in supporting the trends seen in reported data. With enough replicates, analysis would determine whether trends seen are indeed valid, independent of donor variability, with statistical testing being utilised in addition. Increase in donors need not be expensive (purchasing from certified sources like Lonza or relying on past cell banks as previous). Nasal epithelial cells could be used as an alternative as they have the same cell lineage as the bronchial epithelial cells and organoids derived from nasal cells are commonly used in research for Cystic Fibrosis (Liu et al., 2020). However, recent evidence using scRNA-seq suggests that there are distinct cell types and states existing between nasal and bronchial epithelial cells (Vieira Braga et al., 2019, Deprez et al., 2020) and it is important to determine whether nasal spheroids recapitulate the phenotype of the nasal or bronchial epithelium in health and disease. Bronchospheres cultured from p63⁺ cells which undergo differentiation, can be used to expand or enhance the limited numbers of primary epithelial cell numbers before they are used in ALI cultures or inverted to allow viral responses to be determined (Co et al., 2019, Eenjes et al., 2021b). Inversion of the bronchosphere can then be used to study viral infection of mucociliary cells or in research into COVID-19. I show representative examples of this latter approach in Chapter 5 as well as in attached supplementary folders (labelled NHBE bronchospheres).

Another section to optimise further would be the methods used to encapsulate the bronchospheres from the beginning. The current protocol (Chapter 2) required single cells to be seeded in 25% Matrigel to develop into an organoid, before being removed and placed into PMET gel for photocuring. Previous assays were attempted to grow the organoid directly in uncured PMET gel suspension before altering the stiffness, but this was unsuccessful, most likely due to the bioinert nature of the PEGDA, which is incidentally one of the chosen qualities for the matrix scaffold, as well as the lack of cell adhesion to the present matrix. Manipulating the hydrogel precursor chemistry further with complimentary peptide affinity binding sites e.g. introducing Arginine-Glycine-Aspartic acid (RGD) domains in the PEGDA monomer sequence may allow for the bronchial epithelial cells to successfully attach and proliferate (Ruoslahti, 1996). Additional cell types could then also be introduced, modelling the interaction synergy seen between epithelial cells, airway smooth muscle cells as well as

fibroblasts within the airways (Güney et al., 2021), an advantage that is present in precision-cut lung slice and lung-on-a-chip modelling (Nikolic and Rawlins, 2017, Vazquez-Armendariz et al., 2022).

Although unable to complete the planned work package, scRNA-seq analysis would be beneficial to undertake in the future as well as the additional optimisation of the various assays used. scRNA-sequencing would allow for full expression profiles of each individual cell, looking at heterogeneity instead of averaging across many cells. Optimisation would also include specialised dissociation techniques without impacting the cell's viability such as Laser Capture Microscopy/Microdissection (LCM), coupled with Smart-seq 2 for sc transcriptomics (Kolodziejczyk and Lönnberg, 2018, Chen et al., 2019). Unlike the method used for scRT-qPCR in this thesis (Chapter 2), LCM would allow for the careful handling of organoids, as they are not as dense in cell number compared to a typical monolayer or a section of tissue.

The use of bioinformatic tools with pre-existing single cell databases can be further utilised. Novel tools like Bisque can be used to deconvolute bulk RNA-seq datasets, using existing scRNA-seq data sets as reference if the yield from RNA extraction, time and funding is restrictive (Jew et al., 2020, Stegmayr et al., 2021). Also, when analysing the matrix itself after performing assays, new ImageJ plug-ins like TWOMBLI (The Workflow Of Matrix Biology Informatics) can be used to produce quantitative data of the remodelled matrix (Wershof et al., 2021). This includes generating data from investigating fibre alignment in the matrix to focusing on cellular cytoskeletons in accordance to the influence of the surrounding matrix's behaviour (Wershof et al., 2021). Visualisation of ECM modification through changes in the matrix can also be qualitatively analysed through the use of second harmonic generation microscopy, focusing on the non-centrosymmetric organisation of proteins found in the ECM e.g. collagen (Campagnola, 2011). Additionally, 3D traction force microscopy could be used to explore time-dependent interactions of the cells to their environment e.g. migration via focal adhesions (Barrasa-Fano et al., 2021).

The influence of the matrix does not just alter the biomechanics of the cell, but potentially the therapeutic action of drugs on cellular behaviour. Mechanopharmacology can also be addressed, using models such as the bronchosphere/PMET gel model to explore any conformational changes during protein-drug crosstalk from fibrosis and the resulting impact.

An extensive review by *Krishnan* and colleagues delves into this topic further, outlining the potential reasonings of pharmacological responsiveness during cells and tissue within disease states (Krishnan et al., 2016). Equally, recent publications of studies have utilised organoid work further, in terms of precision medicine and drug screening; with organoids derived from patients who suffer from fibrotic respiratory diseases in order to keep aspects of the disease pathophysiology (Wilkinson et al., 2017, Strikoudis et al., 2019, Saygili et al., 2022). Inversion of the bronchosphere, similar to methods used in barrier integrity studies in enteroid organoids (Co et al., 2019), would expose the apical surface so mucin secretions e.g. MUC5AC and MUC5B in response to drug interaction and efficacy can act as a potential drug screening tool for COPD and IPF.

This thesis highlights the importance of mechanobiology within respiratory disorders; helping to not only develop a deeper understanding of such mechanisms that occur, but to also aid in producing effective future therapies.

Chapter 7:

References

7.1 References

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