

Primary culture of human limbal-  
corneal epithelial cells under  
different conditions

Kishore Reddy Katikireddy

Ph.D. Thesis

2011

**A thesis submitted for the degree of Ph.D.**

By

**Kishore Reddy Katikireddy M.Sc.**

The research work described in this thesis was performed under the supervision of

**Dr. Finbarr O' Sullivan**

And

**Prof. Martin Clynes**

National Institute for Cellular Biotechnology

Dublin City University

2011

*I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D. is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.*

**Signed: Kishore Reddy Katikireddy\_(Candidate) ID No.: \_\_\_56113153\_\_\_**

**Date: \_\_\_\_\_**

## ABBREVIATIONS

%	-	Percent
μM	-	Micro molar
ABCG2	-	ATP-binding cassette sub-family G member 2
AMT	-	Amniotic Membrane Transplantation
ATCC	-	American Tissue Culture Collection
BSA	-	Bovine Serum Albumin
cDNA	-	Complementary DNA
CK	-	Cytokeratin
CLAU	-	Conjunctival Limbal Autografts
CLET	-	Cultivated Limbal Epithelial Transplantation
dHAM	-	Denuded Human Amniotic Membrane
DMEM	-	Dublecco's Minimum Essential Medium
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxynucleotide Triphosphate (N = A, C, T, G)
DTT	-	Dithiothreitol
ECM	-	Extracellular matrix
EDTA	-	Ethylene Diamine Tetraacetic Acid
EgtA	-	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ESc	-	Embryonic Stem cells
FCS	-	Fetal Calf Serum
FITC	-	Fluorescein isothiocyanate

FN	-	Fibronectin
GAPDH	-	Glyceraldehyde-6-Phosphate Dehydrogenase
HAM	-	Human Amniotic Membrane
HAM'S F12	-	Ham's Nutrient Mixtures
HCEC	-	Human Corneal Epithelial Cell line
HCEM	-	Human Corneal Epithelial Medium
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IMS	-	Industrial Methylated Spirits
Ir-3T3	-	Irradiated 3T3
Kbp	-	Kilo Base Pair
KDa	-	Kilo Daltons
LESC	-	Limbal Epithelial Stem Cells
LFLc	-	Limbal Fibroblast-like cells
LRCs	-	label-retaining cells
LSCD	-	Limbal Stem Cell Deficiency
Min	-	Minutes
mM	-	Milli Molar
MMLV-RT	-	Moloney Murine Leukaemia Virus- Reverse Transcriptase
mRNA	-	Messenger RNA
MUPP-1	-	Multi-PDZ Domain Protein 1
NEB	-	National Eye Bank
NICB	-	National Institute for Cellular Biotechnology
nM	-	Nano Molar
qRT-PCR	-	Quantitative real time PCR
rhEGF	-	Recombinant Human Epidermal Growth Factor
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
RNasin	-	Ribonuclease Inhibitor

RT-PCR	-	Reverse Transcriptase PCR
SC	-	Stem cells
SD	-	Standard Deviation
SDS	-	Sodium Doedecyl Sulphate
SE	-	Standard Error
SFM	-	Serum-Free Medium
TAC	-	Transit-Amplifying Cells
TAE	-	Tris-acetic acid-EDTA buffer
TBS	-	Tris-Buffered Saline
TDC	-	Terminally Differentiated Cells
TE	-	Tris-EDTA
TEMED	-	N, N, N', N'-Tetramethyl-Ethylenediamine
Tris	-	Tris (hydroxymethyl) Aminomethane
TRITC	-	Tetramethyl Rhodamine Isothiocyanate
TV	-	0.25% Trypsin/ 0.01% EDTA Solution in PBS
UHP	-	Ultra High Pure Water
UV	-	Ultraviolet
v/v	-	Volume/Volume
w/v	-	Weight per Volume
ZO-1	-	Zona Occludens

# Table of Contents

<b>ACKNOWLEDGMENTS</b>	<b>XViii</b>
<b>ABSTRACT</b>	<b>xxi</b>
<b>Section 1.0 Introduction</b>	<b>1</b>
1.0 Introduction to human Eye	2
1.1 Anatomy and physiology of ocular surface	3
1.1.1 Preocular tear film	3
1.1.2 The cornea	4
1.1.3 The corneal epithelium and physiology	4
1.1.4 The corneal stroma and physiology	6
1.1.5 The corneal endothelium	8
1.1.6 Corneal endothelial stem cells	8
1.1.7 Stem cells of the trabecular meshwork	9
1.1.8 The conjunctival epithelium and physiology	10
1.1.9 Conjunctival stem cells	12
1.1.10 Stromal stem cells	13
1.2 The limbus	15
1.2.1 The limbal epithelial stem cells	15
1.3 Corneal/limbal epithelial stem cell markers	19
1.3.1 Cytokeratins	21
1.3.2 Cytosolic proteins	22
1.3.3 Nuclear proteins	24
1.3.4 Cell surface proteins	25
1.3.4.1 Cell-cell, cell-matrix interaction molecule	25
1.3.4.2 Growth factor receptors	27
1.3.4.3 Transporter molecules	28
1.3.4.4 Cell surface glycoconjugates	28
1.3.5 Neuronal markers	29

1.3.6	Hematopoietic markers	29
1.4	Diseases of the ocular surface	31
1.5	Clinical presentation and diagnosis of limbal stem cell deficiency	32
1.6	Treatment for limbal stem cell deficiency	33
1.6.1	Amniotic membrane grafting	34
1.6.2	Direct limbal transplantation	35
1.6.3	Cultivated limbal epithelial transplantation	36
1.7	Culture methods	38
1.7.1	Explants culture technique	38
1.7.2	Cell suspension cultures	39
1.7.3	The feeder cell system	41
1.8	Various substrates used in limbal stem cell cultures	42
1.8.1	Biopolymers	43
1.8.2	Collagen-based substrates	44
1.9	Culture medium	47
1.9.1	Serum-free media and autologous serum-media	48
1.10	Alternate cell sources for limbal stem cell deficiency	49
1.10.1	Conjunctival cells	49
1.10.2	Oral mucosal epithelium	50
1.10.3	Embryonic stem cells	50
1.11	Regulatory issues/implications for regulations	51
<b>1.12</b>	<b>Aims of thesis</b>	<b>54</b>
<b>Section</b>	<b>2.0 Materials and methods</b>	<b>56</b>
<b>2.1</b>	<b>Cell culture methods</b>	<b>57</b>
2.1.1	Water	57
2.1.2	Treatment of glassware	57

2.1.3	Sterilisation	57
2.1.4	Media preparation	58
2.1.5	Preparation of medium supplements	59
2.1.5.1	Human corneal epithelial medium	60
<b>2.2</b>	<b>Mycoplasma analysis</b>	<b>61</b>
2.2.1	Indirect staining procedure	61
<b>2.3</b>	<b>Maintenance of cell lines</b>	<b>62</b>
2.3.1	Safety precautions	62
2.3.2	Culture of adherent cell lines	63
2.3.3	Subculture of adherent cell lines	64
2.3.4	Cell counting	64
2.3.5	Cell freezing	65
2.3.6	Cell thawing	65
2.3.7	Sterility checks	66
2.3.8	Irradiation of 3T3 fibroblast cells (Ir-3T3)	66
2.3.9	Collection of conditioned media from Ir-3T3 cells	67
<b>2.4</b>	<b><i>In vitro</i> Expansion of limbal-corneal epithelial cells</b>	<b>68</b>
2.4.1	Enzymatic digestion of limbal tissue and growing cells on Ir-3T3 feeder cells	68
2.4.2	Limbal explants culture on tissue culture plates	68
2.4.3	Limbal explants culture on tissue culture plastic with Ir-3T3 feeder layer	69
2.4.4	Limbal explants culture on gelatine coated plate with Ir-3T3 cells feeder layer	70
2.4.5	Limbal explants culture on trans-well membrane with no 3T3 cells (Condition A No-3T3)	71
2.4.6	Limbal explants culture on trans-well membrane with co-culture Ir-3T3 cells (Condition B Co-3T3)	72
2.4.7	Limbal explants culture on trans-well membrane	

	with Ir-3T3 separated (Condition C Bot-3T3)	73
<b>2.5</b>	<b>Limbal explants cultures on human amniotic membrane</b>	<b>74</b>
2.5.1	Preparation of denuded human amniotic membrane	74
2.5.2	<i>In Vitro</i> expansion of limbal explants on dHAM	75
2.5.2.1	Limbal explants culture on dHAM with no 3T3 cells (Condition D dHAM No-3T3)	75
2.5.2.2	Limbal explants culture on dHAM with Co- 3T3 cells (Condition E dHAM Co-3T3)	76
2.5.2.3	Limbal explants culture on dHAM with Bot- 3T3 cells (Condition F dHAM Bot-3T3)	77
<b>2.6</b>	<b>Characterization of cultured limbal-corneal epithelial cells</b>	<b>78</b>
2.6.1	Cell outgrowth, and ability to generate cell sheets	78
2.6.2	Cell sheet morphology	78
<b>2.7</b>	<b>Western blot analysis</b>	<b>79</b>
2.7.1	Lysis of cell pellet	79
2.7.2	Sample preparation	80
2.7.3	Quantification of protein	80
2.7.4	SDS acrylamide gel electrophoresis	81
2.7.5	Running buffer	82
2.7.6	TBS buffer (wash buffer)	83
2.7.7	Protein transfer and western blotting	83
2.7.8	Enhanced chemiluminescence detection	86
<b>2.8</b>	<b>Immunofluorescence staining (Immunophenotyping)</b>	<b>87</b>
2.8.1	Limbal-corneal epithelial cells –confocal microscopy	89
<b>2.9</b>	<b>Flow cytometry analysis</b>	<b>91</b>
2.9.1	Principles of flow cytometry analysis	91
2.9.2	Flow cytometry analysis of limbal-corneal epithelial cells	92
<b>2.10</b>	<b>Colony Forming Efficacy assay (CFE)</b>	<b>96</b>
<b>2.11</b>	<b>Culture of limbal fibroblast-like cells (LFLc)</b>	<b>97</b>

2.11.1 Proliferation assay for LFL cells	98
<b>2.12 Invasion assays</b>	<b>98</b>
2.12.1 Preparation of invasion chambers	98
2.12.2 <i>In vitro</i> invasion assays	98
<b>2.13 Motility/migration assay</b>	<b>100</b>
<b>2.14 Senescence-Associated <math>\beta</math>-Galactosidase assay</b>	<b>101</b>
<b>2.15 Extra cellular matrix adherence assay for LFL cells</b>	<b>103</b>
2.15.1 Reconstitution of ECM proteins	103
2.15.2 <i>In vitro</i> cell adhesion assay	103
<b>2.16 <i>In Vitro</i> differentiation of LFL cells</b>	<b>104</b>
2.16.1 Differentiation of LFLc to adipocytes	104
2.16.2 Preparation of adipogenic differentiation medium	104
2.16.3 Preparation of culture plates and culture protocol	105
2.16.4 Fixation and staining- adipocytes for immunofluorescence	105
2.16.5 Fixation and staining -Oil Red O stain	106
<b>2.17 Differentiation of LFLc to osteocytes</b>	<b>109</b>
2.17.1 Preparation of osteogenic differentiation medium	109
2.17.2 Preparation of culture plates and culture protocol	109
2.17.3 Fixation and staining osteocytes for immunofluorescence	110
2.17.4 Fixation and staining- Alizarin Red S	111
<b>2.18 Molecular biology techniques</b>	<b>112</b>
2.18.1 RNA isolation from cells by TriReagent	112
2.18.2 RNA quantification using NanoDrop	113
<b>2.19 Reverse-transcription polymerase chain reaction</b>	<b>114</b>
2.19.1 Reverse Transcription of RNA (cDNA Synthesis)	114
2.19.2 Polymerase chain reaction	116
2.19.3 Primers for limbal-corneal epithelial cells	117
2.19.4 Gel electrophoresis of PCR products	118
<b>2.20 Quantitative real-time polymerase chain reaction (qRT-PCR)</b>	<b>119</b>

2.20.1	Preparation of total RNA from cells using RNeasy Mini Prep Kit	120
2.20.2	High capacity RNA-to-cDNA	121
2.20.3	Evaluation of cDNA	122
2.20.4	Preparation of PCR reaction mix	122
2.20.5	Experiment parameters for TaqMan® Gene Expression Assay	123
2.20.6	qPCR-data analysis	124
2.20.7	Statistical analysis	125
<b>Section 3.0</b>	<b>Results</b>	<b>126</b>
<b>3.1</b>	<b>Standardisation of primary culture of human</b>	
	<b>limbal-corneal epithelial cells</b>	<b>127</b>
3.1.1	Assessment of cell culture morphology and marker expression	129
3.1.2	Explants culture on trans-well cell culture inserts with 3T3 feeder cells	140
<b>3.2</b>	<b>The influence of culture environment on primary</b>	
	<b>limbal-corneal epithelial cultures</b>	<b>143</b>
3.2.1	Outgrowth of limbal-corneal epithelial cells	151
3.2.2	Morphology of limbal-corneal epithelial cells	154
<b>3.3</b>	<b>Migration/Motility assay of limbal-corneal epithelial cells</b>	<b>161</b>
<b>3.4</b>	<b>Invasion assay of limbal-corneal epithelial cells</b>	<b>163</b>
<b>3.5</b>	<b>Characterisation of limbal-corneal epithelial cells grown on</b>	
	<b>cell culture inserts and dHAM</b>	<b>165</b>
3.5.1	Expression of limbal-corneal epithelial differentiation cytokeratin 3 (CK3)	167
3.5.1.1	Immunofluorescence analysis of CK3 expression	167
3.5.1.2	Western blot analysis of CK3	171
3.5.1.3	RT-PCR analysis for CK3	173
3.5.2	Expression of limbal-corneal epithelial differentiation marker cytokeratin 12 (CK12)	174

3.5.2.1	Immunofluorescence analysis of CK12 expression	174
3.5.2.2	Flow cytometry analysis of CK12	178
3.5.2.3	Western blot analysis of CK12	180
3.5.2.4	RT-PCR analysis of CK12	182
3.5.3	Expression of connexin 43 (Cx43)	184
3.5.3.1	Western blot analysis of connexin 43 (Cx43)	184
3.5.4	Expression of E-cadherin	186
3.5.5	Expression of occludin	187
3.5.6	Expression of ZO1	188
<b>3.6</b>	<b>Expression of limbal-corneal epithelial stem cell markers in cultured limbal-corneal epithelial cells</b>	<b>189</b>
3.6.1	Expression of CK19	190
3.6.1.1	Immunofluorescence analysis of CK19 expression	190
3.6.1.2	Western blot analysis of CK19	193
3.6.2	Expression of CK14	195
3.6.2.1	Immunofluorescence analysis of CK14 expression	195
3.6.2.2	Western blot analysis of CK14	198
3.6.3	Expression of stem cell marker $\Delta$ Np63 $\alpha$	200
3.6.3.1	Immunofluorescence expression of $\Delta$ Np63 $\alpha$	200
3.6.3.2	Flow cytometry analysis of $\Delta$ Np63 $\alpha$	201
3.6.3.3	Western blot of $\Delta$ Np63 $\alpha$ and p63	201
3.6.3.4	RT-PCR analysis of $\Delta$ Np63 $\alpha$	202
3.6.4	Expression of stem cell marker ABCG2	215
3.6.4.1	Immunofluorescence analysis of ABCG2	215
3.6.4.2	Flow cytometry analysis of ABCG2	227
3.6.4.3	Western blot analysis of ABCG2	227
3.6.4.4	RT-PCR analysis of ABCG2	229

3.6.5	Expression of $\alpha$ -2 integrin	231
3.6.5.1	Immunofluorescence analysis of $\alpha$ -2 integrin	231
3.6.5.2	Western blot analysis of $\alpha$ -2 integrin	234
3.6.6	Expression of $\beta$ 1 Integrin	236
3.6.6.1	Immunofluorescence analysis of $\beta$ 1 Integrin	236
3.6.6.2	Western blot analysis of $\beta$ 1 Integrin	239
3.7	qRT-PCR analysis of limbal-corneal epithelial cells	241
3.8	Colony Forming Efficiency (CFE) of limbal-corneal epithelial cells	243
3.9	The effect of conditioned media from 3T3 cells on limbal-corneal epithelial growth	249
<b>3.10</b>	<b>Limbal-corneal epithelial cultures translation to clinic</b>	<b>254</b>
3.10.1	Acelagraft™	255
3.10.1.1	limbal-corneal epithelial cells grown on Acelagraft™	256
3.10.2	AmbioDry2™	259
3.10.2.1	limbal-corneal epithelial cells grown on AmbioDry2™	262
3.10.3	Ambio5™	264
3.10.3.1	limbal-corneal epithelial cells grown on Ambio5™	265
3.10.4	EpiFix™	269
3.10.4.1	limbal-corneal epithelial cells grown on EpiFix™	270
<b>3.11</b>	<b>Characterization of limbal fibroblast-like Cells (LFLc)</b>	<b>276</b>
3.11.1	Morphology of LFL cells	276
<b>3.12</b>	<b>Proliferation of LFL cells</b>	<b>278</b>
<b>3.13</b>	<b>Limbal fibroblast-like cells (LFLc) motility assay</b>	<b>279</b>
<b>3.14</b>	<b>Limbal fibroblast-like cells (LFLc) invasion assay</b>	<b>280</b>

<b>3.15</b>	<b>Senescence-associated <math>\beta</math>-Galactosidase assay</b>	<b>282</b>
<b>3.16</b>	<b>Extra cellular matrix adherence assay for LFL cells</b>	<b>284</b>
<b>3.17</b>	<b>Cellular characterisation of LFL cells</b>	<b>286</b>
	3.17.1 Immunofluorescence of LFL cells for specific markers	286
<b>3.18</b>	<b><i>In Vitro</i> differentiation potential of LFL cells</b>	<b>288</b>
	3.18.1 Differentiation of LFL cells to adipocytes	288
	3.18.2 Differentiation of LFL cells to osteocytes	291
<b>Section</b>	<b>4.0 Discussions</b>	<b>294</b>
<b>4.1</b>	<b><i>In vitro</i> cultivation methods for limbal-corneal epithelial cells</b>	<b>295</b>
<b>4.2</b>	<b>Initial assessment of methods to generate limbal-corneal epithelial cell cultures</b>	<b>296</b>
	4.2.1 Enzymatic cell suspension culture of limbal-corneal epithelial cells with a 3T3 feeder layer	297
	4.2.2 Limbal explants on tissue culture plates	299
	4.2.3 Limbal explants on gelatine coating plates	301
	4.2.4 Limbal explants on tissue culture plastic with Ir-3T3 feeder layer	302
	4.2.5 Limbal-corneal epithelial cultures on cell culture inserts with Ir-3T3 feeder layers	304
<b>4.3</b>	<b>Investigation of the role of 3T3 feeder cells in limbal-corneal epithelial cell proliferation and differentiation</b>	<b>305</b>
	4.3.1 General discussion	305
	4.3.2 Effects of organ culture, optisol-GS storage and tissue preservation time on limbal-corneal epithelial cells	309
	4.3.3 Cell outgrowth, and ability to generate cell sheets	311
	4.3.4 Explants culture system: cell sheet morphology	313

4.3.5	Limbal-corneal epithelial cells motility/migration and invasion assay	314
<b>4.4</b>	<b>Characterisation of limbal-corneal epithelial culture with specific markers</b>	<b>316</b>
4.4.1	Introduction	316
<b>4.5</b>	<b>Expression of differentiation markers in cultured limbal-corneal epithelial cells</b>	<b>321</b>
4.5.1	Cytoskeletal proteins	321
4.5.1.1	Expression of CK3	322
4.5.1.2	Expression of CK12	325
4.5.2	Introduction to cell-cell and cell-matrix interaction molecules	328
4.5.2.1	Expression of Connexin43	330
4.5.2.3	Expression of E-cadherin	332
4.5.2.4	Expression of ZO-1 and occluding	333
<b>4.6</b>	<b>Expression of stem cell markers in cultured limbal-corneal epithelial cells</b>	<b>334</b>
4.6.1	Expression of CK19	334
4.6.2	Expression of CK14	336
4.6.3	p63 and $\Delta$ Np63 $\alpha$ (nuclear proteins)	338
4.6.3.1	p63	338
4.6.3.2	$\Delta$ Np63 $\alpha$	339
4.6.4	Expression of transporter molecule ABCG2	345
4.6.5	Integrins in corneal epithelial cells	349
4.6.5.1	Expression of $\alpha$ -2 integrin	350
4.6.5.2	Expression of $\beta$ 1 integrin	352
<b>4.7</b>	<b>qRT-PCR analysis of limbal-corneal epithelial cells</b>	<b>355</b>
<b>4.8</b>	<b>Colony Forming Efficacy assay (CFE) of limbal-corneal epithelial cells</b>	<b>357</b>
<b>4.9</b>	<b>Explants culture system in cell culture inserts with 3T3 conditioned medium</b>	<b>360</b>
4.10	Summary of immunofluorescence for differentiation markers	362
4.10.1	Summary of immunofluorescence for stem cell markers	362
4.10.2	Summary of western blot analysis for differentiation markers	363
4.10.3	Summary of western blot analysis for stem cell markers	363
4.10.4	Summary of CFE assay for limbal-corneal epithelial cells	363
4.10.5	Concluding remarks	364

<b>4.11</b>	<b>Limbal-corneal epithelial culturest translation to clinic</b>	<b>370</b>
4.11.1	Acelagraft™	372
4.11.2	AmbioDry2™	374
4.11.3	AmbioDry5™	375
4.11.4	EpiFix™	377
<b>4.12</b>	<b>Introduction to limbal ‘fibroblast-like’ cells (LFLc)</b>	<b>380</b>
4.12.1	Isolation and characterisation of LFL cells	382
4.12.2	Proliferation of LFL cells	383
4.12.3	Limbal fibroblast-like cells migration/motility assay	384
4.12.4	Limbal fibroblast-like cells invasion assay	385
4.12.5	Senescence-associated $\beta$ -galactosidase (SA- $\beta$ -gal) assay	387
4.12.6	Extra cellular matrix (ECM) adherence assay	389
4.12.7	Cellular characterisation of LFL cells	391
<b>4.13</b>	<b><i>In Vitro</i> differentiation potential of LFL cells</b>	<b>395</b>
4.13.1	Differentiation of LFLc to adipocytes	396
4.13.2	Differentiation of LFLc to osteocytes	396
<b>Section 5.0</b>	<b>Summary and conclusions</b>	<b>399</b>
5.1	Limbal-corneal epithelial cells	400
5.2	Limbal-corneal epithelial cells for clinical application	403
5.3	Limbal fibroblast-like cells (LFLc)	405
<b>Section 6.0</b>	<b>Future work</b>	<b>407</b>
6.1	Limbal-corneal epithelial cultures	408
6.2	Limbal-corneal cpithelial cultures translation to clinic	409
6.3	Limbal fibroblast-like cells (LFLc)	409
<b>Section 7.0</b>	<b>References</b>	<b>411</b>
<b>Section 8.0</b>	<b>Appendices</b>	<b>444</b>
	Appendices 1-7 qRT-PCR raw data values and caliculations	445-449
	Appendices 8 CFE	450-451

## ACKNOWLEDGEMENT

First and foremost I would like to express my gratitude and thank Director of NICB and my supervisor Prof. Martin Clynes and co-supervisor Dr. Finbarr O’Sullivan for giving me the opportunity to do a PhD, a dream project in my life, for always being there and for all the insight, support and encouragement over the past four years, especially towards the end. Their wide knowledge and guidance has always been a great value for me throughout my research work.

I would like to thank Dr. Sinead Adherne, Dr. Niall Barron, for their advice and input for in molecular biology experiments. Thanks to Dr. Clair Gallagher for her assistance and guidance with flow cytometry.

A special Thanks to Mr. Michael Henry, Mr. Joe Carey and Ms. Gillian Smith for all their help in the prep room and day to day deliveries that are essential to carry on the research project.

I am very grateful thanks for the opportunity I was given to work with hospital surgeons from Royal Victoria Eye and Ear hospital, Mr. William Power and Dr. Andra Bobart, and tissue bank facility at Irish Blood Transfusion Board (IBTS) to Dr. Sandra Shaw for providing the samples without which the research project would not have run smoothly.

A Special Thanks to Ms. Carol McNamara, Ms. Yvonne Reilly and Ms. Mairead Callan for all the help in administrative and finance support over the years. I would like to thank Mr. Julian McGovern for all the help and computers technical support.

I would also like to thank my lab colleagues and friends here in Ireland, great colleagues Erica Hennesy and her timely advices at work, Justine Meiller, her help in lab at day today work. Thank you very much to Dr. Sandra Roche for her timely advice and support in thesis write up. And my lovely friends Dr. Suresh and family, Ms. Bandita Bagchi, Dr. Jai and family, Dr. Ram, Dr. Mohan, Mr. Gurmeet and family, Mr. Suraj and family, Mr. Mubarak and family, who made my stay in Dublin memorable and their support all the time.

Most importantly would like to thank my parents and their moral support all the time. I would like to express my deepest gratitude and thanks to my wife, for her never-ending support, love, encouragement, and care all the time. A new addition to our life my lovely son Snithik born at the time of thesis submission is additional joyful dimension to our life mission.

At finally yet importantly, I thank to all the family members who generously donated tissue sample for research work, and my deep condolences to the family member who lost their loved ones. For all donors MAY GOD blesses them and rest in peace.

Thank you to everyone in the centre for all the help at various stages in some form.

# ABSTRACT

---

## ABSTRACT

The corneal epithelium is renewed by limbal stem cells (LSCs). Absence, damage or loss of the LSC population leads to the painful and blinding condition of LSC deficiency (LSCD). *In vitro* expansion of LSCs is an increasingly well recognized treatment modality for LSCD. The purpose of this study was to analyze the outgrowths from human cadaveric limbal explants cultured on cell culture inserts and denuded human amniotic membrane (dHAM) for properties associated with LSCs and to understand the human limbal stem-cell biology *in vitro* with and without 3T3 feeder cells. In particular, the expression of stem cell markers and colony-forming efficiency in different culture models were investigated.

The research can be divided into three sections a) development of tissue culture models, b) translation of limbal-corneal cells towards clinical trial. c) Culture, and characterisation of limbal fibroblast-like cells (LFLc),

The limbal explants were cultured, the outgrowths were measured, and colony-forming efficiencies (CFEs) of cells were calculated. In addition, the expression of LSC and corneal differentiation markers were assessed using immunofluorescence, western blot, and quantitative reverse transcription PCR (RT-PCR). Increased CFE in cells from explants grown on dHAM was observed. The expression of differentiation markers CK12, connexin 43 decreased in dHAM cultures, possibly indicating maintenance of the stem cell population.

We demonstrate the outgrowths from human cadaveric limbal explants, in six different culture systems, show a steady increase in a wide range of stem cell properties with dHAM and 3T3 feeder cells compared to no-3T3 condition. These findings support the importance of dHAM and 3T3 feeder cells in maintaining the undifferentiated state of culture limbal epithelial stem cells. These findings suggest potential modifications of existing techniques to ensure maximum number of LSCs following *in vitro* expansion protocols, which might contribute to even greater success of subsequent engraftment.

We developed a non-enzymatic method for isolation of LFLc, and demonstrated for the first time the invasion and motility of LFLc *in vitro*. These findings suggest an important role for LFLc in wound healing. LFLc have shown differentiation potential to adipo and osteogenic lineage.

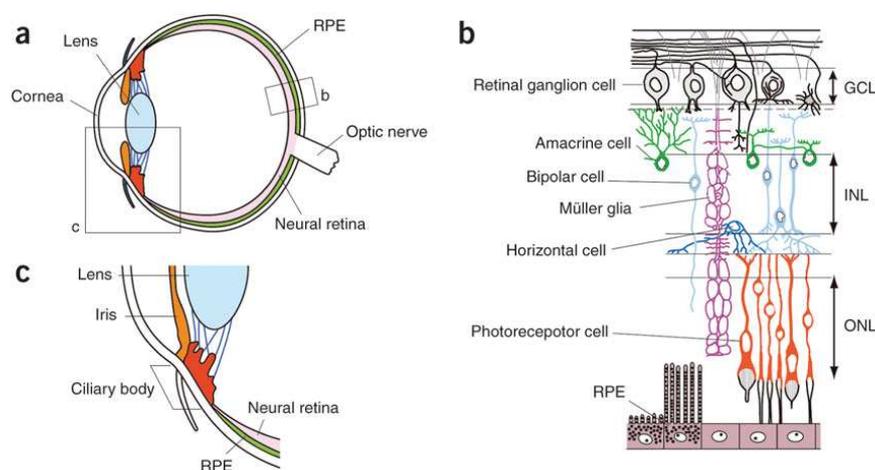
# 1.0 Section

---

# Introduction

## 1.0 Introduction to human Eye

The eyes are a pair of sensory organs - ball-like structures, which are placed on either side of the nose and seated in both cavities called orbit. These are the organs for providing sight in all organisms. The sensation of sight is based on a step-wise process in which light is focused on the retina, converted to electrical impulses that are conveyed through the visual pathways to the brain's visual cortex and interpreted as a visual message in related parts of the brain. The main structures of the eye are the cornea, sclera, anterior chamber, uveal tract, pupil, lens, vitreous, retina and optic disc/optic nerve (Adler's, 1992) **figure 1**. These important tissues have been reported to possess a variety of “adult stem cells” constantly performing their role in regeneration of various cell types within a normal/diseased eye.



**Figure 1: Schematic diagram of a cross section of an adult mammalian eye (a).** Magnified views of boxed region in panel a (b and c). Muller glia act as retinal progenitors in response to injury (b, purple). Retinal progenitors are present in the ciliary body (a,c: red). Iris-derived cells display neural stem/progenitor cell properties (a,c: orange). RPE, retinal pigment epithelium; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (**Exp. Med. 2006; 24, 256–262**)

## **1.1 Anatomy and physiology of ocular surface**

Functionally, ocular surface is a unit comprised of tear film, surface epithelium of the cornea, limbus, and conjunctiva as well as the lacrimal glands and eyelids. The ocular surface epithelium is a stratified, nonkeratinising epithelium that is connected to the underlying connective tissue stroma through adhesion complexes. From a functional standpoint, the ocular surface has critical protective and refractive roles. The blinking mechanism of the eyelids protects the eye from unwanted stimuli and serves to spread the tear meniscus over the ocular surface. The tear film provides lubrication to the ocular surface; it also permits proper focusing of light by providing a smooth refractive surface also, the tear film contains enzymes and peptides, which have important antimicrobial properties. The cornea contributes to more than two-thirds of the total refractive power of the eye and hence is critical for clear vision (Kazuo Tsubota, 2002).

### **1.1.1 Preocular tear film**

The preocular tear film lubricates the ocular surface and is composed of three layers—the innermost mucin layer secreted by the conjunctival goblet cells which are bonded to the glycocalyx of the surface epithelial cells. The lacrimal gland located in upper outer portion of each orbit secretes the middle aqueous layer. Moreover, it contains a wide range of biologically active substances such as histamine, prostaglandins, growth factors, and cytokines. The tear film thus serves not only as a lubricant and source of nutrients for the corneal epithelium but also as a source of regulatory factors required for epithelial maintenance and repair (Jay H. Krachmer, 2004).

### **1.1.2 The cornea**

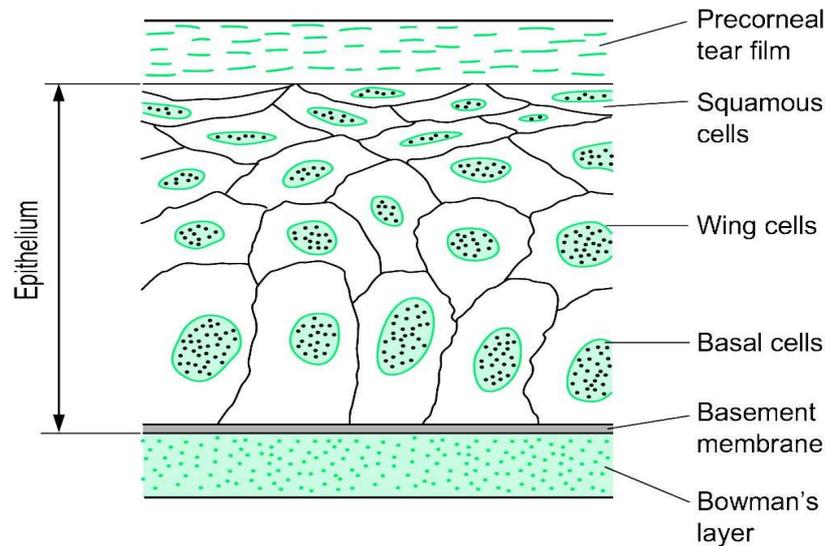
The cornea consists of three major parts, the epithelium, the stroma, and the endothelium. Its total thickness is about 0.52 mm and diameter is approximately 11 mm, together with the sclera. It forms the outer spherical rough coat of the eye. The anterior surface of the cornea is covered with the tear film, which is about 6- $\mu$ m thick and has a superficial oily layer, which limits evaporation from the cornea and provides a good optical surface.

### **1.1.3 The corneal epithelium and physiology**

The epithelium is the outermost layer of the corneal tissue, it accounts for 10% of the total corneal thickness. It rests on a basement membrane or basal-lamina, itself in close contact with Bowman's membrane, which separates it from the mesenchymal stroma **figure 1.1**. The epithelium is comprised of three groups of cells: (1) Squamous cells, a single highly organized row of basal cells localized next to the basement membrane; these cells are large and columnar. (2) Wing cells, a group of winged or polygonal cells forming an intermediate zone with thickness of two to three cells. These cells are no longer in contact with the basement membrane and no longer divide. An extensive cytoskeletal network consisting of intermediate filaments and a few microtubules characterise them ultra structurally. (3) Basal cells, two or three layers of flattened are plate like surface cells covering the intermediate layer (Ehlers, 1970).

The corneal epithelium turns over approximately every seven days by sloughing the outer surface cells in to the tear film (Hanna *et al.*, 1961). The outer two layers of cells at the

surface layer have few organelles. These cells have a limited life span due to terminal differentiation.



**Figure 1.1: Corneal epithelium:** The outermost layer of the cornea consisting of stratified epithelium mounted on a basement membrane with different layers of corneal epithelial cells. (*Millodot: Dictionary of Optometry and Visual Science, 7th edition*)

The epithelium must maintain the barrier function while cells are pushed anteriorly and eventually they are sloughed into the tear film. This is accomplished as the wing or middle cells form lateral junctions with the underlying, newly formed cells at basal layer, pushing the old wing cells towards the epithelial surface (Hazlett *et al.*, 1984, Pfister, 1973) .

Basal lamina is composed largely with type IV collagen and laminin. Type IV collagen has a primary structural chain of planar collagen molecules and is associated with all adult basal lamina (Madri *et al.*, 1984). The presence of the basal lamina between the basal epithelium and the underlying stroma fixes the polarity of epithelial cells. Furthermore, basal lamina

provides a matrix on which cells can migrate and is thought to be important for maintenance of the stratified and well-organized corneal epithelium.

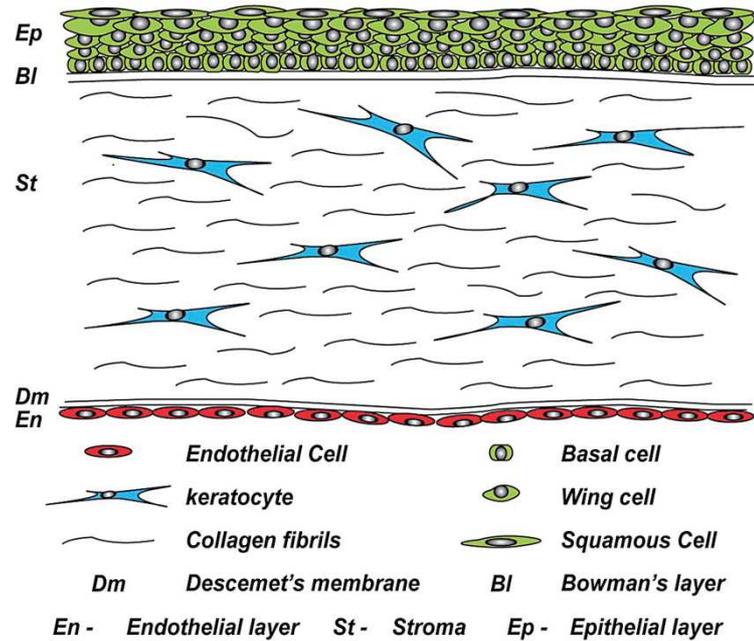
Bowman's layer, situated between the stromal and the cellular epithelium, is a 8 to 12 micron amorphous band of fibrillar material (Beuerman and Pedroza, 1996). This fibrillar material consists of short fibrils of type I collagen embedded in a proteoglycan matrix. Due to its unique embryologic nature, it does not regenerate following injury. It is often thought to be involved in re-epithelialisation of the cornea.

#### **1.1.4 The corneal stroma and physiology**

The corneal stroma is a mesenchymal tissue derived from the neural crest. The dense tissue of the stroma accounts 90% of the total corneal thickness and provides the mechanical strength required to withstand intraocular pressure and external trauma **figure 1.2**. The cellular content of the stroma consists of keratocytes (fibroblastic cells) neural tissue and the associated Schwann cells. Keratocytes occupy 3% to 5% of the stromal volume, and their function is to produce collagen and extracellular matrix of the corneal stroma.

The collagen fibrils that make up the stroma are packed in parallel arrays in some 300 to 500 lamellae (Hamada *et al.*, 1972). These lamellae extend from limbus to limbus and are angled at less than 90° in the anterior stroma, but nearly orthogonally in the posterior stroma (Maurice, 1972). The parallel arrangement of lamellae is formed from heterodimeric complexes of type I and type V collagen fibers to maintain transparency (Fini and Stramer, 2005). These collagen fibers are held in a uniform spacing pattern by

proteoglycans. Keratocytes (fibroblasts) are located between the lamellae (Hay *et al.*, 1979). These sparsely located keratocytes link to one another via dendritic processes (Muller *et al.*, 1995) and produce crystalline proteins to maintain corneal transparency (Jester *et al.*, 1999). Recent reports have described a keratocyte stem cell population in the anterior stroma (Funderburgh *et al.*, 2005) .



**Figure1.2: The human cornea in cross-section.** At the outer surface of the cornea, there is an epithelial layer, which sits on a basement membrane above Bowman's layer. The middle stromal layer, which is sparsely populated with keratocytes is surrounded by dense connective tissue. The final layer consists of a single sheet of endothelial cells, which sits on Descemet's membrane. (Secker GA, 2009).

### **1.1.5 The corneal endothelium**

The endothelial layer is a unique single layer comprising of approximately 400,000 cells that are 4 to 6 microns in thickness (Beuerman and Pedroza, 1996). These cells transport nutrients from the aqueous humour to the stroma and concurrently pump out excess water to prevent corneal edema (swelling) by maintaining optimal hydration. The endothelium sits on a thick basal lamina, which they secrete, known as descemet's membrane **figure 1.2**. This secretion begins approximately 4<sup>th</sup> month of gestation, and the banding appears in the anterior layer prior to birth. During life, this basal lamina increases in thickness, but the accumulation is limited to the posterior non-banded portion (Johnson *et al.*, 1982b, Johnson *et al.*, 1982a).

### **1.1.6 Corneal endothelial stem cells**

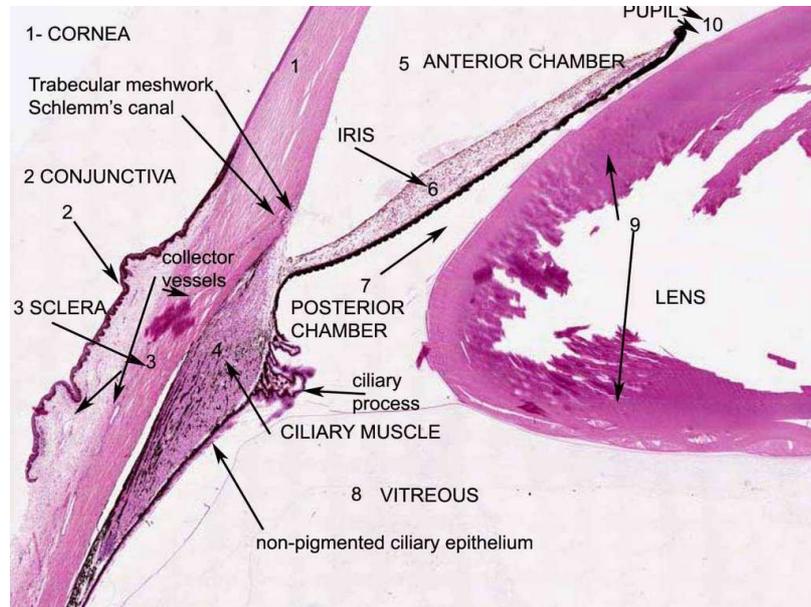
The corneal endothelial cell (CEC) is a single layer of flat hexagonal cells that lies on a basement membrane, descemet's membrane and forms a pure cell sheet without any other cell types (Arffa, 1991). The CEC is essential for maintaining corneal transparency (Waring *et al.*, 1982). In recent years, however, the studies have been published supporting evidence of stem cells in corneal endothelium. Whikehart *et al.*, observed that peripheral corneal endothelial cells incorporate BrdU indicating mitotic activity, moreover, the number of BrdU incorporating cells increased upon wounding (Whikehart *et al.*, 2005). Yokoo *et al.*, isolated endothelial cell colonies from human corneal endothelial cells that expressed neuronal and mesenchymal markers, and limited self-renewing capacity as indicated by failure to form spheres by the third passage (Yokoo *et al.*, 2005). In a similar sphere colony assay with human and rabbit endothelium by Amano *et al.*, a significantly higher sphere

formation was observed in peripheral corneal endothelium, compared to the central endothelium (Amano *et al.*, 2006). Mimura *et al.*, found that the corneal endothelial cells sphere-forming assay appears to enrich for precursors with longer telomeres, higher telomerase activity, and younger progeny than the original cells (Mimura *et al.*, 2010). These experiments strongly support the existence of endothelial stem cells, residing perhaps in the trabecular meshwork and the periphery of the corneal endothelium.

### **1.1.7 Stem cells of the trabecular meshwork**

The trabecular meshwork (TM) is an area of tissue in the eye located around the base of the cornea, near the ciliary body, and is responsible for draining the aqueous humour from the eye via the anterior chamber (the chamber on the front of the eye covered by the cornea) **figure 1.3**. In trabecular meshwork where the cell division is rare, a niche for trabecular meshwork stem cells exists. Stem cell work in trabecular meshwork was carried out in monkeys have shown that the stem cells possibly reside in the schwalbe line (Kaufman and Lutjen-Drecoll, 1975, Lutjen-Drecoll and Kaufman, 1986, Lutjen-Drecoll *et al.*, 1986). These cells appeared to be different from the trabecular meshwork cells, and seemed to migrate to the trabecular meshwork. Discovery of existence of such cells gives a ray of hope for glaucomatous eyes wherein transplantation of trabecular meshwork stem cells might improve aqueous outflow. The expression profile of trabecular meshwork cells, known as the *novel cells*, was compared with that of normal human TM cells (HTM), Schlemm's Canal (SC) cells, or fibroblasts by microarray suggest that age-related proteasome inhibition and cellular senescence could contribute to the pathophysiological alterations of the TM cells in glaucoma (Caballero *et al.*, 2004). Trabecular meshwork and peripheral endothelial

cells (transition zone) known to express nestin, telomerase and further stem cell and differentiation markers like Oct-3/4, Wnt-1, Pax-6, Sox-2 in wounded corneas (McGowan *et al.*, 2007).

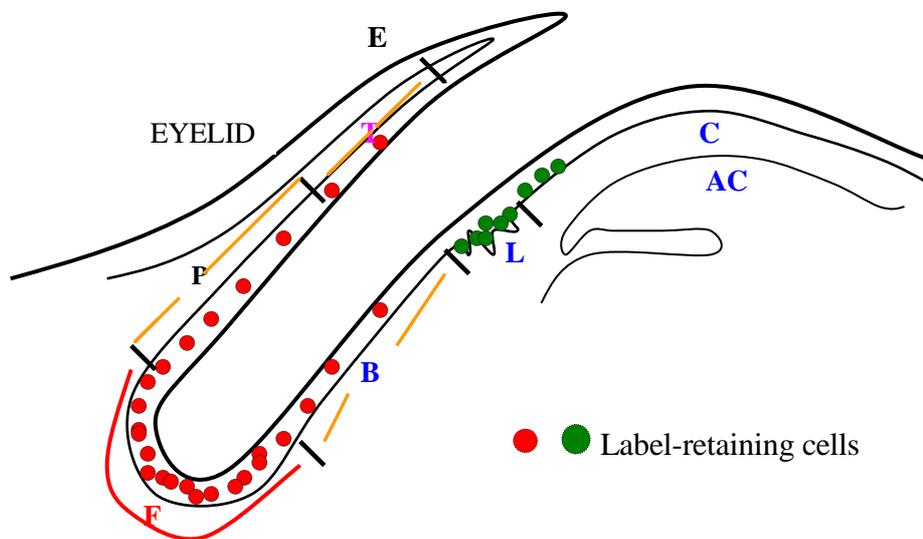


**Figure 1.3** : Cross-section of the corneoscleral transition. The corneal epithelium (1) is contiguous with the conjunctiva (2), the corneal stroma transits into the sclera. (<http://www.images.missionforvisionusa.org/anatomy/2006/02/eye-microscopic-section-labelled.html> )

### 1.1.8 The conjunctival epithelium and physiology

The conjunctiva is a thin translucent mucous membrane, which joins the eyeball to the lids, hence its name. It covers the lids posteriorly and reflects anteriorly to the sclera, becoming continuous with corneal epithelium. The surface layers actually vary gradually from columnar in the fornix and palpebral areas to cuboidal in the bulbar region, and squamous near the lid margins and over the lymphoid follicles. The conjunctival epithelium is generally three to six cells thick, with polygonal cells forming intermediate and basal layers. The distribution of goblet cells varies among species. The goblet cells are

chief source of tear mucin and are essential for moistening the ocular surface. Goblet cells probably arise from the basal layer of the epithelium and tend to retain attachment to its basement membrane. Round or oval in shape, 10-20 $\mu$ m wide with flat basal nuclei, the goblet cells become larger and more oval as they approach the surface where they develop a stroma and discharge mucin (Setzer *et al.*, 1987, Kessing, 1968). Goblet cells account for 5 to 10% of all ocular surface cells (Kessing, 1968).



**Figure 1.4:** Schematic diagram showing the relative densities of label-retaining cells in the palpebral, forniceal, and bulbar conjunctiva in the mice model. The highest concentration of stem cells noted in the forniceal conjunctiva, which is believed to be the site enriched in conjunctival stem cells. It also represents the site with the highest density of goblet cells. **E:** epidermis; **T:** transitional zone (muco-cutaneous junction); **P:** palpebra conjunctiva; **F:** fornix conjunctiva; **B:** bulbar conjunctiva; **L:** Limbus; **C:** Cornea. Figure modified from - (Ang and Tan, 2004)

### 1.1.9 Conjunctival stem cells

The conjunctiva maintains the homeostasis of the ocular surface. The goblet cells are crucial to produce mucin for the tear film and thus essential for ocular surface integrity. However, relatively less known about the nature and location of conjunctival stem cells. Wei *et al.*, showed the presence of slow cycling/label retaining (LRC) in the fornix. These appeared to have a greater proliferative capacity than either the bulbar or the palpebral (PB) conjunctival cells, which suggests that the fornix is the stem cell niche of the conjunctival epithelium (Wei *et al.*, 1995, Wei *et al.*, 1993) **figure 1.4**.

However, a number of studies have subsequently challenged these findings. The presence of LRCs was shown in the palpebral conjunctiva in rabbits and rats (Wirtschafter *et al.*, 1999, Chen *et al.*, 2003). In more recent study on movement of conjunctival epithelial cells in mice was monitored *in vivo* using GFP labelling and showed that bulbar conjunctival epithelial cells were mitotically active; indicating that they were capable of self-renewal *in situ*, that the LRCs were distributed uniformly in the bulbar conjunctiva, and no significant lateral cell migration occurred (Nagasaki and Zhao, 2005). Nagasaki and Zhao's conclusions are in agreement with the findings of Pellegrini *et al.*, who demonstrated a uniform distribution of cells with high proliferative capacity, which would include stem cells, in both the bulbar conjunctiva and fornix. Their results also indicated that conjunctival non-goblet and mucin-producing goblet cells are derived from a common bipotent progenitor, and that differentiation into goblet cells occurs relatively late, this indicates that goblet cells may be generated from transient amplifying cells (Pellegrini *et al.*, 1999a).

### 1.1.10 Stromal stem cells

As discussed in **section 1.1.4** the stroma in the cornea is composed of collagen bundles (85–90%), extracellular matrix and keratocytes. This constitutes 90% of the cornea's overall thickness and the gap junctions between normal corneal keratocytes and fibroblasts maintains the cellular interactions within the corneal stroma (Gilger, 2005). Corneal stroma consist of fibroblasts, keratocytes, and myofibroblasts, and these cell type can be isolated and cultured *in vitro* to understand physiology or its role in corneal wound healing models (Buss *et al.*, 2010). Various groups have been demonstrated isolation and culture of fibroblast-like cells from corneal stroma. One recent study suggested that numerous bone marrow-derived cells existed in mouse corneal stroma (Yamagami *et al.*, 2006). Funderburgh *et al.*, have shown that some cells in the adult murine and bovine corneal stroma express stem cell markers, and have the ability to generate adult keratocytes (Funderburgh *et al.*, 2005). A number of reports have also documented the plasticity of stromal cells which are isolated from corneal stroma by differentiating them to adipo, osteo, chondrocytes (Seigel *et al.*, 2003, Lu *et al.*, 2010, Dravida *et al.*, 2005).

In 2005, Du *et al.*, have demonstrated successful isolation of stromal stem cells from human cornea which express stem cell marker ABCG2 a breakthrough in identification of stem like cells in human corneal stroma (Du *et al.*, 2005). Dravida *et al.*, isolated multipotent fibroblast-like cells from human cornea/limbal tissue which could be differentiated *in vitro* in to epithelial cells of the cornea (Dravida *et al.*, 2005).

The multipotent fibroblast-like cells isolated by Dravida *et al.*, showed a unique marker profile (CD34<sup>-</sup>, CD45<sup>-</sup>, CD123<sup>-</sup>, Cd133<sup>-</sup>, CD14<sup>-</sup>, CD106<sup>-</sup>, HLA-DR<sup>-</sup>/CD31<sup>+</sup>, SSEA-4<sup>+</sup>, CD73<sup>+</sup>, CD105<sup>+</sup>), different from that of bone marrow mesenchymal by expressing embryonic stem cell markers (Oct-4<sup>+</sup>, Sox-2<sup>+</sup>, Tra1-60<sup>+</sup>, Tra1-81<sup>+</sup>).

Polisetty *et al.* were isolated limbal fibroblasts from the outgrowth of limbal explants. These fibroblast-like cells can form spheroids in culture, multipotent and exhibited a mesenchymal stem cell-like surface marker phenotype (CD105<sup>+</sup>, CD106<sup>+</sup>, CD54<sup>+</sup>, CD166<sup>+</sup>, CD90<sup>+</sup>, CD29<sup>+</sup>, CD71<sup>+</sup>, Pax6<sup>+</sup>/SSEA-1<sup>-</sup>, Tra1-81<sup>-</sup>, Tra1-61<sup>-</sup>, CD31<sup>-</sup>, CD45<sup>-</sup>, CD11a<sup>-</sup>, CD11c<sup>-</sup>, CD14<sup>-</sup>, CD138<sup>-</sup>, Flk1<sup>-</sup>, Flt1<sup>-</sup>, VE-cadherin<sup>-</sup>) (Polisetty *et al.*, 2008). Altogether, these results indicate that cornea/ limbal tissue have bone marrow derived cells which are easy to isolate and culture, and are multipotent.

However, there are still many problems concerning mesenchymal stem cell-like cells in human corneal/limbal stroma that need to be further explored, like the source of these cells is still an answered question in this area of limbal-corneal research. It could be that MSCs migrate into corneal/limbal stroma by humoral circulation or alternatively, MSCs as native cells may remain in corneal/limbal stroma.

## **1.2 The limbus**

The limbus forms the border between the transparent cornea and opaque sclera and it contains the pathway of aqueous humour outflow (Van Buskirk, 1989). The epithelium of the limbus consists of a multilayer of cells dispersed in the stroma, rich in minute vessels, with the presence of langerhans and melanocyte cells (which produce and secrete pigments) and a gradient of epithelial cells that includes progenitors of the corneal epithelium (Mann, 1944). The basal epithelium of the limbus has an undulated appearance called the palisades of Vogt a distinctive feature of the human corneo-scleral limbus, and this palisades of Vogt are radial in-folding located at the limbo-corneal junction, extending outwards for 1-2mm (towards cornea) (Van Buskirk, 1989).

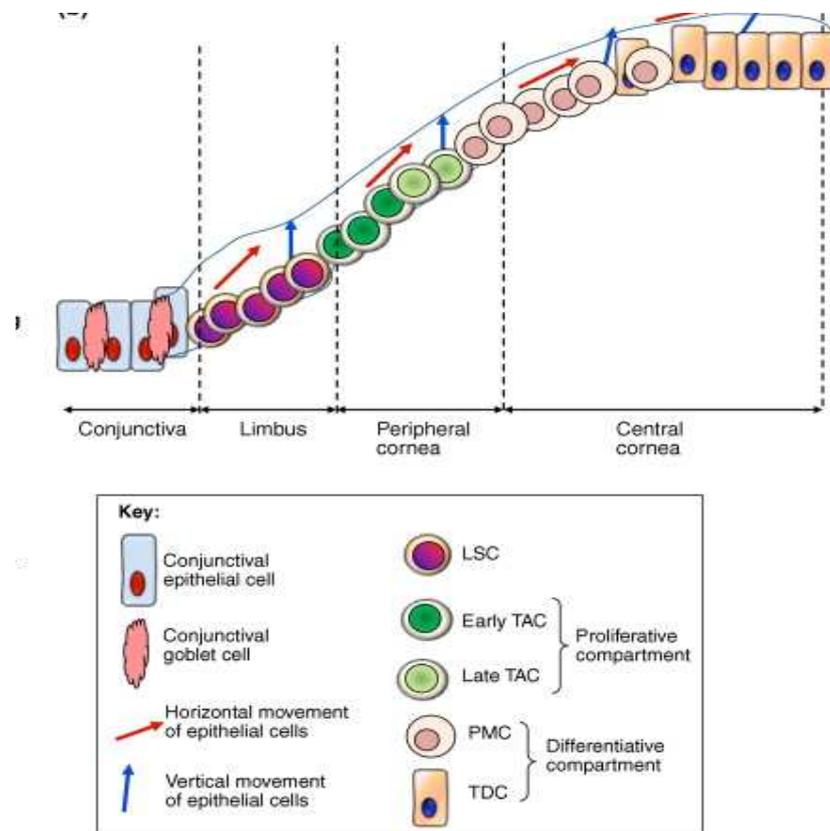
### **1.2.1 The limbal epithelial stem cells**

Limbal epithelium is located between the corneal and conjunctival epithelia and is about the thickness of the corneal epithelium. The limbus consists of thick epithelial cells at palisades of Vogt and limbal niche is vascularised and highly innervated (Lawrenson and Ruskell, 1991) unlike the avascular cornea and therefore is a potential source of nutrients and growth factors for LESC. Davanger and Evensen 1971 first proposed that the limbus had an important role in the renewal of corneal epithelium and published the first significant report regarding the palisades of Vogt. It is within the palisades of Vogt that the niche for limbal-corneal epithelial stem cells believed to exist (Davanger and Evensen, 1971, Bron, 1973).

Thoft and Friend developed the “X, Y, Z” hypothesis of corneal epithelial maintenance. The hypothesis proposed that addition of the proliferation of basal cells (X) and the centripetal migration of cells (Y) was equal to epithelial cell loss from the corneal surface. However, they were unable to rule out the involvement of the neighbouring bulbar conjunctiva (Thoft and Friend, 1983) **figure 1.5** .

As limbal stem cells proliferate and migrate centripetally across the limbal-corneal transition zone, their phenotypic characteristics might alter. The progeny of stem cells that exits the niche, the transient amplifying cells, have the potential to proliferate, migrate, and differentiate because of new local environmental influence at migration site. Maintenance of stemness, proliferation, and migration takes place in the basal layer of the epithelium, in contact with the basement membrane (BM) zone, and cells that have left the basal layer and BM contact undergo a terminal differentiation (Schlotzer-Schrehardt *et al.*, 2007).

*In vitro* studies have demonstrated that, human limbal explants cultures have greater proliferative potential when compared to central explants an indicative of high progenitor population in limbal tissue (Ebato *et al.*, 1988, Ebato *et al.*, 1987). Subsequently evidence for stem cell population was observed by retention of DNA labelling. Cotsarelis *et al.*, found slow cycling label retaining cells (LRCs) in the limbal basal epithelial region of the mouse cornea and postulated that, up to 10% of limbal basal cells were stem cells (Cotsarelis *et al.*, 1989).



**Figure 1.5: Schematic representation of limbal SCs and their differentiated products in relation to the ocular surface.** Limbal epithelial stem cells reside in the basal layer of the epithelium, which undulates at the limbus. Daughter transient amplifying cells (Early and late TACs) divide and migrate towards the central cornea (arrowed) to replenish the epithelium, with Premature cells (PMC)/terminally differentiated cells (TDC) (Ahmad *et al.*, 2010)

In the literature, support for the limbal location of corneal epithelial stem cells was derived from the following observations.

- (1) The centripetal migration of epithelial cells, during corneal epithelial wound healing (Mann, 1944, Buschke, 1949, Kinoshita *et al.*, 1981, Kaye, 1980, Buck, 1979, Buck, 1985, Dua and Forrester, 1987).
- (2) The circumferential migration of limbal epithelial cells during limbal wound healing (Dua and Forrester, 1990), and abnormal corneal epithelial wound healing when the limbal epithelium is partially remain (Chen and Tseng, 1990, Chen and Tseng, 1991) or completely removed (Kruse, 1994, Kruse *et al.*, 1990, Huang and Tseng, 1991). Also Ebato *et al.*, have shown that limbal basal cells have a higher proliferative potential in culture (Ebato *et al.*, 1988).

However, this theory (limbal stem cell concept) has recently been challenged with evidence from a study conducted on mice. The study suggests that cells from the central cornea but not the limbus are responsible for the maintenance of the corneal epithelium. To support the theory holoclone colonies were cultured from the central corneas of a number of mammalian species including two very young human donors (Sun *et al.*, , Majo *et al.*, 2008). This study has not yet been confirmed in the adult human cornea.

Recent studies have shown distribution of limbal epithelial cells by immunophenotyping, ultra structural features which emphasis on their size, morphology, intracellular connections and basal attachments (Shanmuganathan *et al.*, 2007). Meanwhile advanced techniques like confocal and scanning electron microscopy of whole mounted human corneas have given detail information on location and integrity of limbal stem cells within the palisades of Vogt or limbal crypts (Shanmuganathan *et al.*, 2007, Shortt *et al.*, 2007). These studies suggest that part of limbal tissue (12 O' clock or 6 O' clock) regions may yield greater numbers of stem cells.

### 1.3 Corneal/limbal epithelial stem cell markers

During the last 10 years, progress has been made towards development of molecular markers that may distinguish stem cells (SC) from transient amplifying cells (TAC) in situ. Although a variety of putative limbal SC markers have been proposed, their role for identification of limbal SC is still controversial. These uncertainties may be attributed to variations between species or different methodologies used as well as to uncertain specificities of the markers.

The expressions of combination of several features seem to allow greater specificity for the marker analysis of corneal/limbal stem cells due to lack of specific marker. The putative 'markers' can either be positive (present) or negative (absent). A large number of markers have been suggested for the identification of corneal/limbal epithelial cells *in vitro* and *in vivo*.

**Table 1.3** illustrates the list of markers used in identification of limbal-corneal epithelial cells *in vitro* and *in vivo* (Schlotzer-Schrehardt and Kruse, 2005, Zhou *et al.*, 2006, Chen *et al.*, 2004). These markers hold the key to providing an insight into the identification of limbal SC.

**Table- 1.3** Semiquantitative immunohistochemical localisation of putative stem cell markers in human ocular surface epithelia.

<b>Marker</b>	<b>Limbal Epithelium</b>		<b>Corneal Epithelium</b>	
	<i>basal</i>	<i>suprabasal</i>	<i>basal</i>	<i>suprabasal</i>
<b>CK 3/12</b>	-	+	++	++
<b>CK 5/14</b>	+	(+)	- or (+)	-
<b>CK 19</b>	++	-	-	-
<b>Vimentin</b>	++	(+)	-	-
<b><math>\alpha</math>-enolase</b>	++	(+)	(+)	-
<b>Metallothionein</b>	(+)	+	-	+
<b><math>\Delta</math>NP63 <math>\alpha</math></b>	++	(+)	(+)	-
<b>Connexin 43</b>	-	+	++	+
<b>Connexin 50</b>	-	NA	+	NA
<b>Nestin</b>	-	- or ++	++	++
<b>Involucrin</b>	-	++	+	++
<b>E-Cadherin</b>	(+)	++	++	++
<b>Integrin <math>\alpha</math>-2</b>	- or ++	+	++	+
<b>Integrin <math>\alpha</math>3</b>	- or ++	+	++	+
<b>Integrin <math>\alpha</math>6</b>	- or ++	+ or ++	++	+
<b>Integrin <math>\alpha</math>9</b>	++	(+)	-	-
<b>Integrin <math>\alpha</math>v</b>	++	+	++	+
<b>Integrin <math>\beta</math>1</b>	++	+	++	+
<b>Integrin <math>\beta</math>2</b>	+	+	+	+
<b>Integrin <math>\beta</math>4</b>	- or ++	+	++	+
<b>Integrin <math>\beta</math>5</b>	+	(+)	+	-
<b>EGF-R</b>	++	+	++	+
<b>KGF-R</b>	(+)	-	-	-
<b>HGF-R</b>	(+)	-	(+)	-
<b>NGF-R TrkA</b>	+	-	+	(+)
<b>Transferrin-R CD71</b>	- or +	+	- or +	+
<b>TGF-<math>\beta</math>-RI</b>	++	+	++	+
<b>TGF-<math>\beta</math>-RII</b>	++	+	++	+
<b>ABC-G2</b>	++	-	-	-
<b>Glucose transporter I</b>	+	NA	-	NA
<b>Epiregulin</b>	++	NA	(+)	NA
<i>dachshund</i>	++	NA	(+)	NA
<b>Sry</b>	++	NA	(+)	NA
<b>Diablo</b>	(+)	NA	++	NA
<b>Cyclin M2</b>	(+)	NA	++	NA
<b>Multiple PDZ</b>	(+)	NA	++	NA

- Negative, (+) weakly positive, + moderate positive, ++ strongly positive, NA- Data not available.

### 1.3.1 Cytokeratins

Cytokeratins are the principal component of the complex network of intermediate filaments of all epithelial cells. Keratins are divided into two subgroups, namely acidic type and family of neutral-basic type (Fuchs *et al.*, 1981, Moll *et al.*, 1982, Kivela and Uusitalo, 1998). Among these cytokeratins CK3 and CK12 are specifically expressed in corneal epithelial cells and are considered to be markers for corneal epithelial cell differentiation. Epithelial cells in the basal layer of the limbus, the proposed niche of limbal stem cells are devoid of these two cytokeratins pointing towards their undifferentiated nature (Schermer *et al.*, 1986, Liu *et al.*, 1993). Since then, numerous studies have confirmed the undifferentiated phenotype of limbal basal cells by using CK3 and CK12 (Liu *et al.*, 1993, Kiritoshi *et al.*, 1991, Matic *et al.*, 1997, Kurpakus *et al.*, 1994). Similarly, the positive expression for cytokeratin 19 (CK19) of limbal basal cells is considered further indicator of a possible stem cell or transient amplifying cell phenotype (Kasper *et al.*, 1988). In contrary to Kasper *et al.*, observation, a other groups have observed expression of CK19 in supra basal and apical layers of conjunctiva (Papini *et al.*, 2005, Dua *et al.*, 2005). CK19 along with another intermediate filament vimentin has been found to locate in the basal cells of human and murine limbal epithelium (Michel *et al.*, 1996).

Cytokeratin 14 has been proposed as a marker for proliferating keratinocytes in skin and has been used to isolate epidermal stem cells (Bickenbach, 2005). Like most of the undifferentiated cells in stratified epithelia, basal cells of both corneal and limbal epithelia have been shown to express the keratin pair CK5/CK14 (Hsueh *et al.*, 2004). Thus, the variation in marker expression could vary in relation to species. In conclusion, CK3 and

CK12 are specific markers for differentiated limbal-corneal epithelial cells but CK5/14 and CK19 are not specific for limbal stem cells, whereas vimentin seemed to localize specifically to basal cells along the corneal–limbal borderline *in vivo*.

### **1.3.2 Cytosolic proteins**

The expression of several proteins involved in cellular metabolic functions has been identified in basal cells of the limbal epithelium. These include a number of enzymes, such as cytochrome oxidase, Na/K-ATPase and carbonic anhydrase (Hayashi *et al.*, 1978, Lutjen-Drecoll *et al.*, 1986, Steuhl and Thiel, 1987). The high expression of these enzymes in the limbal epithelium has been associated with increased metabolic activity. In contrast, aldehyde dehydrogenase (ALDH) and transketolase (TKT) have been identified as major cytosolic proteins in the corneal epithelium, but are completely absent or only minimally expressed in murine limbus.

The expression of glycolytic enzyme  $\alpha$ -enolase has been reported in mitotically active, growing cells, but remains almost at an undetectable level in quiescent cells. The  $\alpha$ -enolase was originally reported as proposed marker for limbal stem cells, localising in the rat, rabbit and human basal limbal cells. Expression of  $\alpha$ -enolase has also been reported in corneal epithelial cell migration from limbal basal cells following epithelial debridement. However, a more recent study in human corneas showed that  $\alpha$ -enolase is not only expressed by the limbal basal cells but also by the limbal suprabasal and corneal basal cells (Chen *et al.*, 2004).

Other cytosolic proteins associate with cell-cycle, such as cyclins D, E, and A have been identified as proteins preferentially localized to limbal basal epithelial cells (Tseng *et al.*,

1996). In this study, Tseng *et al.*, demonstrated that majority of limbal basal cells with positive cytoplasmic staining rather than nuclear staining characteristic of actively cycling cells. This observation has been interpreted as storage of cell cycle proteins within the cytoplasm of quiescent cells awaiting translocation into the nucleus upon stimulation. In accordance with slow cycling cells observation Ki-67, which acts as a marker for actively cycling cells, was only observed in few basal cells at the limbus.

In 1996, Joyce *et al.*, demonstrated the presence of metallothioneins, cysteine rich, metal-binding intracellular proteins, which are link to cell proliferation, have been shown strongly expression by basal limbal cells of human corneas (Joyce *et al.*, 1996b, Joyce *et al.*, 1996a).

Involucrin, a structural protein found in the cytosol of differentiated human keratinocytes, was observed in superficial epithelial cells in human cornea and limbus, but not in the basal layer of the limbal epithelium (Chen *et al.*, 2004). Recently a calcium-linked protein associated, with early epithelial differentiation, and protein S100A12, which is involved in  $Ca^{2+}$  dependent signal transduction process in differentiated cells, was observed in corneal epithelial basal cells but not in limbal basal cells *in vivo* (Sun *et al.*, 2000, Lauweryns *et al.*, 1993). Whereas, these markers were not extensively used in identification of limbal-corneal epithelial cells *in vitro* studies.

### 1.3.3 Nuclear proteins

The nuclear transcription factor p63 has been frequently suggested in recent years as a potential marker to identify limbal stem cells in the human cornea (Pellegrini *et al.*, 2001). p63 is homolog of p53 and p73, and contains multiple functional domains, which are isotype specific, allowing for both p53 trans-activating and dominant negative signalling events (Yang *et al.*, 1998). However, Moore *et al.*, found a uniform expression of p63 in murine cornea (Moore *et al.*, 2002). Following this study localisation of p63 in corneal epithelial cells *in vitro* or *in vivo* condition was questionable.

In 2005 Di Iorio *et al.*, came up with explanation for p63 as stem cell marker. According to the authors the p63 gene generates transactivating and N-terminally truncated transcripts ( $\Delta$ Np63) initiated by different promoters. Alternative splicing gives rise to three different C termini, designated alpha, beta, and gamma. In the ocular epithelium, the corneal stem cells, which are segregated in the basal layer of the limbus, contain the alpha isoform but not beta or gamma. Holoclones derived from the limbus are known to express higher alpha isoform, whereas meroclones contain little, and paraclones contain none (Di Iorio *et al.*, 2005). These finding suggests that  $\Delta$ Np63 $\alpha$  is the most dominant isoform within human ocular surface epithelia and may contribute, at least in part, to the maintenance of cell proliferative capacity within the ocular surface epithelia (Kawasaki *et al.*, 2006).

*In vivo* wound healing experiments suggests that,  $\Delta$ Np63 $\alpha$  positive cells migrate toward the central cornea, and basal layer. Beta and gamma isoforms of  $\Delta$ Np63 appear to be in the supra basal layers of the cornea and limbus in response to wounding which are indicative of more differentiated cell type (Daniels *et al.*, 2006a, Daniels *et al.*, 2006b).

Despite the growing evidence that  $\Delta$ Np63 isoforms are present in both the limbal and corneal epithelium, the role of these proteins in cell cycle regulation, differentiation, and survival is still largely unknown. Furthermore, the level of expression of these isoforms in specific cell types may modulate signalling pathways important in effecting these processes.

#### **1.3.4 Cell surface proteins**

Cell surface proteins are useful in isolation, enrichment, and molecular characterization of viable stem cells. These include cell-cell, cell-matrix adhesion molecules, cell surface receptors, transporter proteins etc.

##### **1.3.4.1 Cell-cell, cell-matrix interaction molecules**

Communicating cell-cell junctions called gap junction, consist of six trans-membrane proteins called connexions. Gap junctions enable diffusion of ions, low molecular weight metabolites, and second messengers. Connexin 43 (Cx43), Cx50 are abundantly expressed throughout all layers and Cx43 being mainly confined to the basal cell layer (Dong *et al.*,

1994). In contrast both connexins have been reported to be absent from the basal layer of the human, mouse, chicken and neonatal rabbit limbal epithelium. Limbal basal cells that are completely devoid of Cx43 are thought to be stem cells, whereas those that stain weakly may represent early progenitor cells.

Cadherins are a family of  $\text{Ca}^{2+}$  dependent trans-membrane receptors that mediate cell-cell adhesion. E-cadherin mediated cell-cell contact result in cell activation and an increase in key signalling molecules that are involved in cell proliferation and survival. Recently Chen *et al.*, study has shown that E-cadherin and connexin 43 are expressed in superficial corneal and limbal epithelia. These findings suggest that connexin 43 and E-cadherin are expressed by differentiated epithelial cells, and the absence of these intercellular communication molecules in the basal limbus may be an inherent feature of SCs (Chen et al 2004).

$\beta$ -Catenin is a central component of the cadherin cell adhesion complex and, as an essential molecule in Wnt signalling pathway—it is a key regulator to epithelial differentiation and proliferation. It is shown to be essential in maintenance of keratinocyte stem cells (Huelsken *et al.*, 2001). In corneas of rat and rabbits,  $\beta$ -catenin was strongly positive in basal layers of limbus and weak in corneal basal layer.

Integrins are a large family of heterodimeric trans-membrane glycoproteins consisting of  $\alpha$  and  $\beta$  subunit, which attach cells to extracellular matrix proteins or to ligands on other cells. Some integrins have been suggested to be markers for SC, such as  $\beta 1$  and  $\alpha 6$  (Li *et al.*, 1998). Integrin  $\beta 1$  is highly expressed in limbal and corneal epithelia with much higher expression in limbal basal layer (Chen *et al.*, 2004). The differential expression of integrins in the

cornea and limbus could be due to variation in composition of basal membrane (Revoltella *et al.*, 2007).

Together, these observations made till date suggest that basal cells deficient in Cx43, P-cadherin, and integrins  $\alpha$ -2,  $\alpha$  3,  $\alpha$  6,  $\beta$ 4 are thought to represent corneal stem cells (Schlotzer-Schrehardt and Kruse, 2005).

#### **1.3.4.2 Growth factor receptors**

Growth factor receptors have been identified as proteins that preferentially localise to cell membranes of limbal basal cells. Undifferentiated cells in the basal limbal epithelium have been reported to contain higher levels of epidermal growth factor receptor (EGF-R) than suprabasal cells in the rat cornea, however, no difference was noted in the staining of corneal and conjunctival basal cells (Zieske and Wasson, 1993).

Keratinocyte growth factor receptor (KGF-R) and TrkA (receptor for NGF) have been shown to localise at the limbal basal cells (Touhami *et al.*, 2002). Hepatocyte growth factor and its receptor c-met were preferentially expressed by corneal keratocytes and epithelial cells respectively (Lauweryns *et al.*, 1993). Differential expression of transferrin receptor CD71 was observed in limbal and corneal epithelia. Both transforming growth factor (TGF- $\beta$ ) receptors II & I were reported to be weakly expressed in central corneal epithelium of human and rat eyes, but were present at much higher levels in limbus (Zieske *et al.*, 2001) .

In general, the presence of high levels of growth factor receptors may allow limbal basal cells to be differentially stimulated by fibroblast or blood derived growth factors to maintain their undifferentiated nature or to undergo proliferation upon wounding.

#### **1.3.4.3      Transporter molecules**

Stem cells from bone marrow, skeletal muscle, and other tissue can be isolated based on their ability to exclude the vital dye Hoechst 33342 (bisbenzimidazole) which defines a 'side-population' (SP) phenotype. Hoechst is able to enter live cells; it is also actively pumped out of cell by ABC (ATP-Binding Cassette) transporters, which include p-glycoprotein and ABCG2 in human cells. ABCG2, a member of the ATP binding cassette (ABC) transporters, which localises predominantly to the plasma membrane and actively involved in Hoechst efflux has been proposed as a universal and conserved marker for stem cells from wide variety of tissues (Kim *et al.*, 2002). Recent studies have provided convincing evidence that a subset of limbal epithelial cells belongs to the SP phenotype expressing ABCG2 protein (Watanabe *et al.*, 2004). About 0.3-0.5% cells of total limbal population exhibit the SP phenotype, whereas no SP cells were seen in corneal epithelium. SP cells isolated from limbal epithelium expressed ABCG2 and possessed colony forming efficiency *in vitro* suggesting ABCG2 as a limbal stem cell marker (de Paiva *et al.*, 2005).

#### **1.3.4.4      Cell surface glycoconjugates**

Limbal epithelial cells possess unsialylated galactose residues that are recognized by peanut lectin (PNA) and that lack any sialic acid bound through  $\alpha$ -2, 3 bonds. Differentiation of the cells causes sialylation of these residues and the appearance of  $\alpha$ -2,3 sialic acid residues, suggesting the expression or activation of  $\alpha$ -2-3-sialyltransferase (Wolosin *et al.*, 2000).

### **1.3.5 Neuronal markers**

It has been hypothesised that human corneal stem cells may exhibit neuronal properties, characteristic of their neuroectodermal origin. In rat basal cells of limbal epithelium have been reported to express transcription factor Pax-6 is present in developing central nervous system and play a central role in development of the eye (Chanas *et al.*, 2009, Collinson *et al.*, 2004),

Subpopulations of human limbal/corneal epithelial cells are known to exhibit neuronal properties *in vitro*. This was confirmed by immunoreactivity to nestin, GABA receptor, glycine receptor, and serotonin receptor, as well as functional neurophysiological responses to GABA and kainic acid. Thus, human corneal stem cells may represent a potential source of non-embryonic, autologous, surgically accessible graft material with neuronal potential (Seigel *et al.*, 2003).

### **1.3.6 Hematopoietic markers**

CD34, a cell surface glycoprotein and functions as a cell-cell adhesion factor, and CD133, a trans-membrane glycoprotein are known hematopoietic markers (Silvestri *et al.*, 1993, Silvestri *et al.*, 1992, Pessina *et al.*, 2010). Dua *et al.*, found that CD34 is strongly expressed by human corneal keratocytes, but not corneal epithelium, and CD133 was found along the cell membranes in all layers of the limbal and corneal epithelium of human cornea (Dua *et al.*, 2003).

Data reported in the literature regarding limbal-corneal epithelial markers suggest that the basal epithelial cells of human limbus are positive for ABCG2, CK19, Vimentin, KGF-R, metallothionein, and Integrin  $\alpha$ -9, but negative for CK3/CK12, Cx-43, P-cadherin, involucrin, Nestin. It has to be considered that most putative markers for limbal stem cells are expressed by majority of limbal basal cells, and occasionally also by suprabasal cells, suggesting that the bulk of positively staining cells are transient amplifying cells (TAC) and that some of these markers are present both on stem cells and TAC as well. ABCG2 immunopositivity is strictly confined to small clusters of basal cells in the limbal epithelium and therefore, appears to be at present the most useful cell surface marker. However, cultured limbal-corneal epithelial cells are known to express ABCG2 in basal and suprabasal layer of cells (Notara *et al.*, 2007). This observation questions the importance of ABCG2 for the identification of limbal epithelial stem cells. Thus, the validity of ABCG2 as a marker of limbal stem cells remains to be clarified.

Although none of the above molecules appear to be definitive markers for corneal stem cells, it remains possible that using a combination of the positive and negative markers, as discussed above and listed in **table 1.3**, the corneal epithelial stem cells could be isolated/characterised.

#### 1.4 Diseases of the ocular surface

Two major types of ocular surface failure have been identified by impression cytology based upon the resultant epithelial phenotype (Kessing, 1968). The first type of surface failure shows pathologic transition of normal non-keratinized ocular surface epithelia into keratinized epithelia in a process termed squamous metaplasia (Sonnenberg *et al.*, 1991). In conjunctiva, squamous metaplasia appears by a loss of goblet cells and mucin expression in epidermal keratins. Such phenotypic changes represent altered epithelial differentiation rendering ocular surface epithelia non-wettable. Squamous metaplasia is considered as a common phenotype of unstable tear film, which is the hallmark of various dry eye disorders (Tseng, 1989). The second type of ocular surface failure is characterised by the replacement of the normal corneal epithelial phenotype with an invaded conjunctival epithelium in a process termed limbal stem cell deficiency (LSCD) (Tsai *et al.*, 1990).

A number of human corneal diseases exist with LSCD, such as Stevens-Johnson syndrome, chemical and thermal burns, immunologic conditions, radiation injury, inherited syndromes (such as aniridia), and ocular pemphigoid. These can severely compromise the ocular surface and cause catastrophic visual loss in otherwise healthy eyes. The common pathogenic feature of this seemingly diverse group of diseases is the depletion of the stem cell population from corneal limbus. Damage or depletion of the corneal stem cells results in "conjunctivalisation" or in-growth of conjunctival elements on the surface of the cornea. This is associated with profound visual loss. **Table 1.4** summarises the corneal diseases arising out of abnormality of the limbal niche or deficiency of LSCs.

Destructive loss of stem cells	Dysfunction of limbal niche
Chemical or thermal burns	Aniridia/Iris coloboma
Steven Johnson syndrome/TEN	Multiple endocrine deficiency
multiple surgeries or Cryotherapies to limbus (Iatrogenic)	Chronic limbitis or peripheral Inflammatory/ulcerative disorders
Contact lens-induced keratopathy	Neuronal or ischemic neurotrophic keratopathy
Severe microbial keratitis	Pterygium or pseudopterygium
Anti-metabolite (5FU, MMC)	Chronic bullous keratopathy
Radiation	Idiopathic

**Table 1.4: Pathological classification of corneal diseases (Sangwan *et al.*, 2007)**

### 1.5 Clinical presentation and diagnosis of limbal stem cell deficiency

Limbal epithelial stem cell (LESC) deficiency can occur because of primary or acquired injuries. Partial or full LESL deficiency leads to deleterious effects on corneal wound healing and surface integrity (Chen and Tseng, 1991, Dua *et al.*, 2003). LESL deficiency leads to conjunctivalisation, neovascularisation, chronic inflammation, recurrent erosions, ulceration and stromal scarring, causing painful vision loss (Holland and Schwartz, 1996, Kenyon and Tseng, 1989, Puangsricharn and Tseng, 1995).

Limbal stem cell deficiency can be best confirmed histologically by the use of impression cytology (IC) (Egbert *et al.*, 1977), which can detect goblet cells containing conjunctival epithelium on the corneal surface. Immunohistochemically, the absence of a cornea-type

differentiation (such as the absence of keratin CK3), and the presence of mucin in goblet cells, has been shown by monoclonal antibodies. The weakest point of impression cytology (IC) based technique is the choice of inappropriate markers, thus leading to errors in diagnosis. Despite initial reports identifying CK3/CK19 pair as potential putative markers for corneal and conjunctival epithelia, (Donisi *et al.*, 2003) these findings were no longer confirmed/ detected by other groups in their studies (Yoshida *et al.*, 2006, Chen *et al.*, 1994).

A study by Barbaro *et al.* is further evidence of the inadequacy of CK3 and CK19 markers, since neither CK3 was not specific for the cornea nor was CK19 for the conjunctiva. CK12 and MUC1 were found to be more suitable for the evaluation of IC specimens as overlapping was minimal and expression was more specific (Barbaro *et al.*, 2009) .

## **1.6 Treatment for limbal stem cell deficiency**

In the event of partial limbal stem cell deficiency, the conventional treatment is repeated debridement (removal of overgrown/dead tissue) of migrating conjunctival epithelium in the acute phase following injury. This can reduce or prevent conjunctival ingrowths (Dua, 1998). The other conservation options available are intensive non-preserved lubrication, bandage contact lens and autologous serum eye drops (Geerling *et al.*, 2004, Poon *et al.*, 2001, Young *et al.*, 2004). Other surgical methods for treatment of ocular surface disorders include amniotic membrane grafting, direct limbal transplantation, and advanced cultured limbal/corneal cells techniques.

### 1.6.1 Amniotic membrane grafting (AMG)

Human amniotic membrane (HAM) has been reported to have anti-inflammatory properties due to down-regulation of key proinflammatory cytokines such as IL-1 $\alpha$  and IL-1 $\beta$  and TGF- $\beta$  (Solomon *et al.*, 2001). Shimmura *et al.* reported the anti-inflammatory effects of amniotic membrane transplantation in ocular surface disorders (Shimmura *et al.*, 2001). Amniotic membrane transplantation is useful for the first-stage procedure to reduce inflammation and scarring before the limbal allograft transplantation in patients with total LSCD (Tseng, 2000).

The biological properties of amnion in ocular surface rehabilitation are thought to be primarily related to the amnion matrix and basement membrane substrate. In addition, for the treatment of severe disorders such as Stevens Johnson syndrome, ocular cicatricial pemphigoid, recurrent pterygia, persistent epithelial defects (PEDs), acute chemical and thermal injuries (Sridhar *et al.*, 2000), shield ulcer of Vernal keratoconjunctivitis (VKC), (Sridhar *et al.*, 2001) neurotrophic keratitis, and partial stem cell deficiency. HAM is believed to maintain epithelial progenitor cells within the limbal stem cell niche and facilitate ocular surface epithelial renewal. These observations, together with HAM's are potential to provide a scaffold in tissue engineering. Niknejad *et al.* 2008 has supported its popularity as a carrier for *in vitro* expanded limbal epithelial stem cells for clinical use in the management of LSCD syndromes (Niknejad *et al.*, 2008).

### 1.6.2 Direct limbal transplantation

Kenyon and Tseng (1989) pioneered the clinical application of the limbal stem cell theory with impressive results by transplanting sectoral grafts of bulbar conjunctiva and limbus called conjunctival limbal autografts (CLAU) harvested from the normal eye, to manage a series of cases with unilateral limbal stem cell deficiency (LSCD) (Kenyon and Tseng, 1989). In case of bilateral LSCD, allogenic limbal stem cell transplantation is employed, where sectors of limbal tissue are sourced from a living relative donor with healthy eyes (a procedure known as living-related conjunctival limbal allograft lr-CLAL), or deceased donor where the tissue is obtained either from a corneo-scleral disc or whole eye (keratolimbal allograft transplantation: KLAL). All these procedures aim to transplant a new source of epithelium for a diseased ocular surface and removal of the host's altered corneal epithelium and pannus.

Although all techniques used in stem cell transplantation are similar in principle, the source of donor stem cells can vary. Donor tissue can be obtained from the fellow eye (limbal autograft) in cases of unilateral disease, from a living related donor (usually gives a better tissue match), or from a cadaver donor (limbal allograft) when both eyes are affected. Limbal transplantation procedures also vary depending up on the carrier tissue used for transfer of the limbal stem cells. Carrier tissue is needed in limbal transplants because it is not possible to transfer limbal stem cells alone. Limbal transplant procedures have used either conjunctiva (conjunctival limbal graft) or cornea (keratolimbal graft) as a carrier tissue for limbal stem cells (Holland and Schwartz, 1996, Tsubota *et al.*, 1995). Tseng *et al.*, used amniotic membrane transplantation (AMT) associated with limbal transplantation in cases with total LSCD (Kim and Tseng, 1995). In the past decade, some authors have used

conjunctival transplantation to treat corneal stem cell deficiency. This practice was (and perhaps is) supported by the belief that conjunctival epithelium "trans-differentiates" in to cornea-like epithelium (Tseng *et al.*, 1984, Herman *et al.*, 1983).

Successful limbal transplantation can achieve rapid surface healing, stable ocular surface without recurrent erosions or persistent epithelial defects, regression of corneal vascularisation and restoration of a smooth and optically improved ocular surface, resulting in improved visual acuity and, probably, increased success for subsequent keratoplasty. However, direct limbal transplantation procedure carries a risk of complication such as damage to healthy eye by removal of autologous tissue for transplantation or side effects from long-term immunosuppressant with allogenic tissue (Jenkins *et al.*, 1993).

### **1.6.3 Cultivated limbal epithelial transplantation (CLET)**

More recently, bioengineered grafts made by limbal/corneal epithelial sheets *in vitro*, and subsequent transplantation onto the recipient cornea, have become the subject of intense research and clinical activity. In case of bilateral total limbal stem cell deficiency, allogeneic limbal epithelium is harvested either from living related donors or from fresh cadaveric tissue. Pellegrini *et al.* 1997 who used a culture system of LSCs grown on mouse J2-3T3 fibroblasts with FCS supplemented media, published the first example of corneal restoration by means of autologous cultures of corneal stem cells (Pellegrini *et al.*, 1997). Subsequently Schwab and co-workers reported a successful outcome with cultivated limbal stem cells (autologous procedures) (Schwab *et al.*, 2000b, Schwab *et al.*, 2000a). The success was defined as restoration or improvement of vision, along with maintenance of corneal re-epithelialisation and absence or recurrence of surface disease. Recently, the

cultured limbal stem cell therapy has become a routine treatment in several clinical centres and has produced benefits for several types of limbal stem cell deficiencies (Schwab *et al.*, 2000b, Tsai *et al.*, 2000a, Sangwan *et al.*, 2003a). Theoretical advantages of *in vitro* cultured limbal epithelial stem cells are manifold. These include:

- (1) A minimised risk of precipitating stem cell failure in the donor eye,
- (2) Potential for taking a further biopsy if required, and
- (3) A reduced risk of allograft rejection due to the absence of antigen-presenting langerhans cells in the expanded/grafted cells.

## **1.7 Culture methods**

### **1.7.1 Explants culture technique**

Explants cultures involve plating the whole piece of limbal tissue or limbal explants approximately 1 mm by 1 mm in size, on a tissue culture surface and allowing them cells to grown out. (Nakamura *et al.*, 2003, Koizumi *et al.*, 2001b, Grueterich *et al.*, 2002a, Sangwan *et al.*, 2003b). A range of various substrates have been used for explants culture system are fibrin glue (Rama *et al.*, 2001), fibrin gel (Talbot *et al.*, 2006, Han *et al.*, 2002) and temperature responsive polymer (Nishida *et al.*, 2004b).

In explants culture system, an additional process termed as airlifting was used in some studies. In which, the level of culture medium in the dish has to be lowered to the level of the surface of the epithelium (Ban *et al.*, 2003, Cooper *et al.*, 2004, Koizumi *et al.*, 2001b, Koizumi *et al.*, 2001a, Nakamura *et al.*, 2003, Nakamura *et al.*, 2004b). However, this promotes stratification and differentiation of the epithelium, which may mean loss of limbal stem cells. If limbal stem cells are required for transplantation in limbal stem-cell deficiency, airlifting may not be appropriate, but this technique may be ideal for toxicity assays as it mimics the *in vivo* corneal epithelium. The use of irradiated or mitomycin-C treated 3T3 feeder cells in explants co-culture system is a variation of explants technique (Koizumi *et al.*, 2001b, Koizumi *et al.*, 2001a, Nakamura *et al.*, 2004b, Nakamura *et al.*, 2003, Inatomi *et al.*, 2006). Both amniotic membrane and growth arrested 3T3 fibroblasts inhibit the differentiation of corneal epithelial cells *in vitro*, which allows the expansion of the population of LESC. Vemuganti *et al.* shown the *in vitro* growth potential of fresh

limbal tissue is better than cadaveric limbal tissue explants (Vemuganti *et al.*, 2004) suggesting that, for cultivated limbal transplant choosing living related tissue is better than cadaveric tissue. In explants culture system, the limbal explants positioned epithelial side down may give rise to cultured epithelia with high expression of p63 and  $\Delta Np63\alpha$  (Raeder *et al.*, 2007). Recently Kolli *et al.* demonstrated that outgrowths from human limbal explants, showed a steady decline in a wide range of stem cell properties with distance from the central explants, which suggests the importance of proximity of stem cells to their niche environment in maintaining their stem cell niche (Kolli *et al.*, 2008).

### **1.7.2 Cell suspension cultures**

The enzymatic isolation of limbal epithelial cells from limbal tissue is a cell suspension culture. Enzymatic isolation can be performed either by using dispase to separate the limbal epithelium from the stroma and then trypsinising the limbal epithelium to isolate single limbal epithelial cells (Liu *et al.*, 2006a), or by trypsinising the limbal epithelium to single limbal epithelial cells (Pellegrini *et al.*, 1997, Koizumi *et al.*, 2002). The incubation time with enzymes (Dispase/Trypsin) vary. However, minimum 1-hour incubation with dispase followed by trypsin treatment is common methodology for limbal cell suspension culture (Liu *et al.*, 2006a). Longer incubations up to 18 hours have been conducted to obtain intact limbal epithelial sheets (Espana *et al.*, 2003b). However, increase incubation with dispase II can result in extensive bleeding of corneal basal epithelial cells (Spurr and Gipson, 1985).

The single cell suspensions can be seeded either onto HAM (Koizumi *et al.*, 2001a), or onto plastic tissue culture dishes (Lindberg *et al.*, 1993). Koizumi *et al.* hypothesized that

the suspension cultured limbal epithelium was significantly superior to the explants cultures and should ideally include stem cells (Koizumi *et al.*, 2002). Subsequently Zhang *et al.* experimental results showed that, a cell-suspension culture technique was significantly superior to explants culture technique in terms of stem cells content (Zhang *et al.*, 2005). Whereas, Kim *et al.* shown no significant differences in the limbal epithelia cultured both by explants and cell suspension methods, where the phenotypes of corneal epithelial cells, ranging from basal cells to superficial differentiated cells, are known to see well maintained in both culture systems (Kim *et al.*, 2004).

Varghese *et al.* compared migration of limbal cells from dispase treated and non-treated limbal explants, and observed that higher migration rates in dispase treated limbal tissue cell outgrowth than non-treated explants. However, there is loss of stem cells properties in dispase treated cultures. Therefore, these results suggest that enzyme treatment is not necessary for migration of limbal cells from explants in culture (Varghese *et al.*, 2010). Also enzyme treatment may damage stem cells, as stem cells of corneal epithelium reside in the basal layer of the limbus (Tan *et al.*, 2004).

### 1.7.3 The feeder cell system

The *in vitro* culture of stem cells often requires the addition of reproductively inactive cells, known as feeder cells to stimulate the stem cell microenvironment by secreting extracellular metabolites that maintain the stemness of stem cells. Puck *et al.*, first reported the use of feeder cells in cell culture (Puck *et al.*, 1956). Feeder cells are especially effective for the support of growth of cells that are difficult to culture. Feeder cells also provide a suitable environment in the co-culture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interaction, (Ehmann *et al.*, 1998). Production of soluble growth factors and removal of toxicants from the culture medium (Burroughs *et al.*, 1994). Both growth promoting and anti-apoptotic activities are present in fibroblast-derived serum-free conditioned media (Meller *et al.*, 2002, Grueterich *et al.*, 2003b, Tseng *et al.*, 1996). Several studies have shown favourable clinical outcomes, adopting different culturing techniques for *in vitro* expansion, especially regarding the preparation of amniotic membrane and the inclusion of 3T3 fibroblast feeder layers (Lindberg *et al.*, 1993, Germain *et al.*, 1999, Rama *et al.*, 2001).

Many studies have shown that long-term survival and propagation of limbal epithelial cells is possible if co-cultured with fibroblast feeder layers like 3T3 or limbal fibroblasts (LFs) (Grueterich *et al.*, 2003b, Kim *et al.*, 2004, Kim *et al.*, 2008, Pellegrini *et al.*, 1999a, Tseng *et al.*, 1996). Recently, Miyashita *et al.*, have described the importance in location of feeder cells at culture system; they suggest that feeder cells in duplex systems are important to have the soluble factors (by distance feeder cells) and adhesion molecules (when in contact

with feeder cells) in maintenance the limbal stem cell niche (Miyashita *et al.*, 2008). The reason for the maintenance of stem cell markers in the cultured cells under the influence of feeder layer is not clearly understood. This process is multi-factorial includes possible release of diffusible factors or cytokines released by the 3T3 fibroblast system, or presence of anti apoptotic survival factor in 3T3 fibroblast conditioned medium (Tseng *et al.*, 1996).

## **1.8 Various substrates used in limbal stem cell cultures**

Among various substrates used for limbal epithelial cell cultures, HAM has proved to be the most common, for both the culturing of cell *in vitro*, and as vehicle to transfer the expanded cells to the ocular surface during transplantation. Wang *et al.*, have identified the role of human amniotic membrane (HAM) in maintaining the stemness of stem cells that probably provide the right kind of "niche" which act as *in vivo* stroma (Wang *et al.*, 2003). Human amniotic membrane has been used safely and successfully on the ocular surface for many years. Over the past 9 years, HAM is used as substrate to expand the limbal epithelium (Grueterich *et al.*, 2002b, Koizumi *et al.*, 2001b, Koizumi *et al.*, 2001a, Nakamura *et al.*, 2003, Nakamura *et al.*, 2004b, Sangwan *et al.*, 2003b, Sangwan *et al.*, 2003a, Sangwan *et al.*, 2005, Sangwan *et al.*, 2006, Tsai *et al.*, 2000a, Shimazaki *et al.*, 2002). However, HAM does require the use of donor tissue; even when tissue is sourced from accredited tissue banks there is always a small concern regarding viral disease transmission. Apart from HAM there are many other substrates used for culture of limbal stem cells *in vitro*. Fibrin is another such material, which was used by Pellegrini *et al.*, to grow and transplant cultures into patients. The same group has made use of soft contact lenses as a substrate for cells during transplantation (Pellegrini *et al.*, 1997). Various basement membranes like paraffin gauze (Pellegrini *et al.*, 1997), proteins such as collagen

shields (Schwab *et al.*, 2000b), or fibrin gel (Rama *et al.*, 2001) have been used to grow limbal epithelial cells. Rama *et al.*, demonstrated that the use of fibrin as a carrier has supported the maintenance of stem cells. Espana *et al.*, grew limbal epithelial cells on limbal/corneal stroma, to provide the evidence of role of stromal niche in controlling the plasticity of limbal and corneal epithelial differentiation in rabbits (Espana *et al.*, 2003b).

### **1.8.1 Biopolymers**

To avoid cell damage from trypsin or Dispase II while detaching cells, Nishida *et al.*, used temperature-responsive polymer poly N-isopropylacrylamide. This gel reversibly alters its hydration properties with temperature. A chemically immobilized thin film with cell culture surface facilitates cell adhesion and growth of oral mucosal cells in normal culture conditions at 37°C. The reduction of the culture temperature below 30°C causes the surface to hydrate and swell rapidly; this promotes complete detachment of adherent cells which can be transplanted without suture to the ocular surface of patients. The authors concluded that this type of ocular surface substrate might be an alternative for allogenic limbal transplantation to patients with severe bilateral disease (Nishida *et al.*, 2004b, Nishida *et al.*, 2004a). The results of rabbit autologous corneal epithelial cells expanded on a thermoreversible polymer (Mebiol Gel) for the management of unilateral limbal stem cell deficiency (LSCD) suggest that transplantation of autologous limbal epithelial cells may restore a nearly normal ocular epithelial surface in eyes with unilateral LSCD (Sitalakshmi *et al.*, 2009).

In order to exclude animal products/ xenogeneic material (bovine fetal calf serum, FCS) or cells (murine feeder cells) and to have convenient substrate for transferring cells to cornea, multiple novel substrates have been examined. These have focused on the growth and delivery of corneal epithelial cells. Substrates like hydroxyl-terminated dendronized (perfectly branched polymers) have been used to grow human corneal epithelial cell line (HCEC) and mouse 3T3 fibroblasts (M-3T3) where cells showed greater affinity for the dendritically modified surface (Benhabbour *et al.*, 2008). To culture limbal epithelial cells Deshpande *et al.*, adopted new a culture technique, where cells were grown on acrylic acid plasma polymerization coating on the inner surface of a bandage contact lens. Thus, this methodology provides immobilization and protection of the cells on the eye (Deshpande *et al.*, 2009). However, another research group was able to successfully culture human epithelial cells from a tissue explants on non-coated contact lens and then transferred the cells to the eye of three patients with LESC deficiency to restore a transparent corneal epithelium (Di Girolamo *et al.*, 2009).

### **1.8.2 Collagen-based substrates**

A collagen type III scaffold was cross-linked using a water-soluble carbodiimide (A carbodiimide is a functional group consisting of the formula  $RN=C=NR$ ) that facilitates cross-linking of the collagen with refractive index, transmission and backscatter properties. Which are similar to that of native cornea, were able to stratify limbal corneal epithelial stem cells and express putative stem cell and differentiated cell type markers in a similar fashion to the cells on HAM (Dravida *et al.*, 2008).

A collagen vitrigel membrane is a type of substrate that has superior optical properties compared to all collagen substrate subtypes. A vitrigel is a gel in a stable state produced via a three-stage sequence namely gelation, vitrification and rehydration. This process creates a transparent, rigid, glass like material from an opaque, soft gel after low-temperature evaporation of fluid (McIntosh Ambrose *et al.*, 2009) which is suitable for culture of human limbal epithelial, bovine fibroblasts and rabbit endothelial cells on the surface of the vitrified membranes. Limbal epithelial cells grown on vitrigel substrate expressed markers of both differentiated corneal epithelial cells (CK3, CK12 and connexin-43) and putative stem cells (P63 and ABCG2) suggesting a mixed population of cells (McIntosh Ambrose *et al.*, 2009). Although vitrigel membranes do boost many of the required properties for replacement of HAM, there is one major limitation in this procedure; cells cannot be seeded within the gel due to the lengthy (two weeks) dehydration process. This would eliminate the possibility of close replication of the native cornea by seeding epithelial cells on top of a fibroblast containing collagen stroma.

Karamichos *et al.*, demonstrated that corneal fibroblasts align and compact collagen parallel to the axis of the greatest extracellular matrix stiffness (Karamichos *et al.*, 2007). In this study, the cellular collagen matrices were fully or partially constrained by attaching them to immobilised plastic bars. Moreover, after 24 hours, it was noted that cells were aligned parallel to the long axis in the anisotropic region of constrained matrices whereas unconstrained (isotropic) matrices cells were randomly aligned.

Epithelial cell sheets cultured on fibrin glue (Higa *et al.*, 2007) or temperature-sensitive polymers (Nishida *et al.*, 2004b, Nishida *et al.*, 2004a) have advantages over HAM sheets.

These advantages include higher transparency and rapid attachment to the ocular surface without sutures. One possible disadvantage of such carrier-free sheets is advanced differentiation, shown by the up regulation of K3 (Higa *et al.*, 2007). **Table- 1.8** shows summary of various substrates used for *in vitro* proliferation of limbal stem cells.

S.No.	Substrate used	Group /Reference	Application
1	Human Amniotic Membrane	(Schwab, 1999)	Clinical
		(Tsai <i>et al.</i> , 2000a)	Clinical
		(Sangwan <i>et al.</i> , 2003b)	Clinical
		(Wang <i>et al.</i> , 2003)	Research
2	Fibrin	(Pellegrini <i>et al.</i> , 1999a)	Clinical
3	Corneal stroma	(España <i>et al.</i> , 2003b)	Research
4	Soft Contact Lenses	(Pellegrini <i>et al.</i> , 1997)	Clinical
5	Culture Inserts	(Koizumi <i>et al.</i> , 2002)	Research
6	Matrix coated Plates	(Nakagawa <i>et al.</i> , 1990)	Research
		(Schwab, 1999)	Clinical
7	Temperature Responsive Gels	(Nishida <i>et al.</i> , 2004a)	Research
8	Synthetic polymer Mebiol gel	(Sitalakshmi <i>et al.</i> , 2009)	Research
9	Myogel and Matrigel	(Francis <i>et al.</i> , 2009)	Research
11	collagen-based corneal substitutes	(Dravida <i>et al.</i> , 2008)	Research
13	Collagen Vitrigel (CV) membrane	(McIntosh Ambrose <i>et al.</i> , 2009)	Research
14	Plasma polymer coated contact lens	(Deshpande <i>et al.</i> , 2009)	Research

**Table 1.8:** Various substrates used for *in vitro* proliferation of limbal stem cells.

## **1.9 Culture medium**

Limbal-corneal cells are often cultivated in a medium of minimal essential medium (MEM) and Ham's F-12 (3:1) supplement with hydrocortisone, insulin, tri-iodothyronine, adenine, cholera toxin and EGF; this is based on the original works of Rheinwald and Green in the 1970s (Rheinwald and Green, 1975). The addition of hydrocortisone improves the growth and morphology of epithelial cells in culture (Rheinwald and Green, 1975). The addition of insulin reduces the serum requirement of the culture medium (Hayashi *et al.*, 1978, Maciag *et al.*, 1981). The addition of both insulin and tri-iodothyronine reduces the requirement of foetal calf serum in epithelial culture medium from 20 to 10% (Forbes, 2002, Ibaraki, 2002, Yamaya *et al.*, 2002). The addition of adenine to the culture medium improves the ability of epithelial cells to form colonies (Allen-Hoffmann and Rheinwald, 1984). Cholera toxin stimulates DNA synthesis by increasing cellular cAMP levels (Marcelo, 1979). The addition of cholera toxin to epithelial cell cultures promotes cell proliferation (Rheinwald and Green, 1977). Addition of cholera toxin opposes terminal differentiation of the epithelial cells (Sun and Green, 1977). The addition of EGF promotes the migration of growing epithelial cells and also prevents crowding at the centre of colonies (Green *et al.*, 1977). In addition, EGF antagonizes the differentiation of cultured epithelial cells and therefore promotes the undifferentiated cell state, which is particularly important when preserving limbal stem cells in the culture.

### **1.9.1 Serum-free media and autologous serum-media**

Maciag *et al.*, developed the first serum-free media for skin epithelial cultures but as substitute for serum, bovine pituitary extract was used in the medium (Maciag *et al.*, 1981). Since then the serum-free medium have been modified to exclude bovine pituitary extract by addition of defined growth-promoting additives. Such defined serum-free media has been used to successfully culture both corneal and limbal epithelial cells for *in vitro* cytokine studies (Kruse and Tseng, 1991). The addition of B-27 serum free supplement growth-factor mix to basal limbal epithelial medium can eliminate the requirement of serum (Yokoo *et al.*, 2008). Notara *et al.*, used xenobiotic-free culture system with MRC-5 human embryonic fibroblasts that could be potentially used for clinical corneal regeneration applications, and maintenance of stem cell niche by xenobiotic-free condition (Notara *et al.*, 2007). A serum- and feeder-free culture system using Epilife medium was supplemented with bovine pituitary extract, purified growth factors and hormones, could be an alternative to grow human corneal epithelial equivalents, which minimizes the risk of contamination from serum and feeder cells, during culture. The technique may also be useful for the clinical application of limbal stem cell culture (Lekhanont *et al.*, 2009).

## **1.10            Alternate cell sources for limbal stem cell deficiency**

The need for alternative cell sources for limbal stem cell deficiency (LSCD) is important in severe bilateral limbal stem cell deficiency, in order to avoid allogenic graft. The use of allogenic limbal tissue leads to long-term use of systemic immunosuppressant's in order to avoid graft rejection (Tsubota *et al.*, 1999, Daya and Ilari, 2001). Immunosuppressant's have several side effects that would affect the quality of the patient's life and are expensive. Immunosuppression does involve the risk of rejection and graft failure. The problem of allograft rejection prompted the development of autologous epithelial transplantation of cells from other ocular epithelium and non-ocular origin for severe cases of LSCD.

### **1.10.1          Conjunctival cells**

Some investigators have explored the potential of conjunctival epithelium as an alternative for cultured limbal cells. These studies suggest that transplantation of cultured conjunctival cells are a better option than amniotic membrane graft alone, if transplantation of cultured limbal cells is not possible (Ang *et al.*, 2004, Tanioka *et al.*, 2006).

### **1.10.2 Oral mucosal epithelium**

Extensive studies have been performed to check the feasibility of using oral mucosal epithelium. The use of autologous buccal epithelium was first established in rabbits (Nakamura and Kinoshita, 2003, Kinoshita *et al.*, 2004, Kinoshita and Nakamura, 2004, Hayashida *et al.*, 2005), and later performed in humans with promising results (Nakamura *et al.*, 2004a, Inatomi *et al.*, 2005, Nishida *et al.*, 2004b).

To culture oral mucosa epithelial cells, a suspension culture method is widely adopted by growing cells on amniotic membrane with an inactivated 3T3 feeder layer (Nakamura *et al.*, 2006b, Kinoshita *et al.*, 2004, Kinoshita and Nakamura, 2004, Nakamura and Kinoshita, 2003) or on temperature-sensitive culture dishes (Hayashida *et al.*, 2005, Nakamura *et al.*, 2004a). The Madhira *et al.*, group used oral mucosal explants culture methods on amniotic membrane without 3T3 feeder layer cells and showed expression of corneal markers *in vitro* indicating a potential use of oral mucosal cells for ocular surface disorders (Madhira *et al.*, 2008). Clinical application of oral mucosal epithelial cells is yet to conform to pre-clinical trials with restoration of ocular surface.

### **1.10.3 Embryonic stem cells**

Embryonic stem cells (ESCs) are derived from blastocysts, generated through *in vitro* fertilization. Embryonic stem cells are pluripotent i.e. they can give rise to any cell type in the body. The differentiation of ESCs to a corneal epithelial lineage could be achieved by

replication of the LSC niche environment. This approach was first applied to the generation of corneal epithelium from mouse ESCs in preclinical studies (Homma *et al.*, 2004). The *in vitro* differentiation of human embryonic stem cells were differentiated in to corneal epithelial cells were successfully achieved by Ahmad *et al.*, by culturing hESCs on an extracellular matrix of collagen-IV and feeding with medium conditioned by limbal fibroblasts, where the LSC niche environment was achieved after differentiation (Ahmad *et al.*, 2007). Recently induced corneal epithelium-like cells from monkey ES cells were successfully transplanted to mice, where grafted cells were stained with anti-human nuclear protein antibody, which cross reacted with nuclei of monkey cells but not with those of mouse cells (Kumagai *et al.*, 2010).

### **1.11 Regulatory issues/implications for regulations**

Once the scientific hurdles have been cleared, and a therapy appears ready to move into the clinic, there are a series of regulatory hoops that need to be jumped through before phase I trials can even begin (Daniels *et al.*, 2006a, Daniels *et al.*, 2006b). In order to comply with the legislation, processing of cells and tissues will need to be carried out in a specialized clean-room environment, with monitored and regularly maintained equipment. Before any protocol can be put in place, or a significant change made to the tissue processing, the process must be properly validated. The documentation and quality management system needs to be thorough, and should be continuously updated. In addition to this, the maintenance of strict procurement and donor records required, which are traceable from donor to recipient, and vice versa.

Although the process of producing transplant grade cultured tissue is necessary it brings with it many problems. The regulatory process and the requirement of 'clean' laboratories are extremely costly. In addition, the requirement of expertise in culturing the limbal tissue is pivotal and is another rate-limiting step. These problems mean that the production and transplantation of cultured limbal epithelium are not viable in all ophthalmology centres and the process of culture in particular needs to be centralised. Such centralisation however means that effective transportation strategies for cultured limbal epithelial tissue to all ophthalmology centres are required. This also means that cultured tissue may need to cross international boundaries and transcend multiple regulatory authorities. All these issues remain to be addressed still.

The culture of limbal epithelium is essential for furthering our understanding of limbal stem-cell biology and for the purposes of transplantation. There are many variations for culturing limbal epithelial cells: different media and sera; culture with 3T3 fibroblasts or amniotic membrane, or both; removal of amniotic membrane epithelial cells from the membrane, or not; use of the explants method or suspension method; and airlifting the culture, or not.

Variations in the culture methods could potentially induce significant variation in the maintenance of stem cell population in culture system. In all the culture methods mentioned above, that a very few of them have analysis performed for the extent of differentiation within the culture. If it is limbal stem cells one want to culture, it is essential that we maintain cells in similar niche in culture, it is essential that such analysis be performed. In addition, it is important that the various culture methods are compared with each other analytically for stem cell population and differentiated cells in culture system.

Finally, this study, focused on understanding of importance of the role of niche in preventing the differentiation of limbal stem cells in culture system. To examine the niche maintenance we have designed different culture systems for our study, and the aims of the current research work are as follows.

## 1.12 Aims of thesis

The research work carried out in this thesis can be divided into three main sections,

### A) Investigation of cultured limbal-corneal epithelial stem cells.

Since the time *in vitro* culture of the limbal epithelial cells was initiated by Sun et al. 1977 there have been quite a few strategies followed to culture these cells *in vitro*. These strategies frequently used 3T3 feeder cells on their own, HAM on its own, or a combination of 3T3 feeder cells and HAM. However, there is lack of data on which method yields the most stem cells *in vitro*. Thus, this study aimed to investigate the role of 3T3 feeder cells *in vitro* culture of limbal-corneal epithelial cells with following strategies.

- Establishment of limbal-corneal epithelial stem cell cultures from cadaveric limbal tissue.
- Investigation of role of 3T3 feeder cells in maintenance of the stem cell population in cultured limbal-corneal epithelial cells in different culture systems.
- Identification of differentiated and stem cell population in cultured limbal-corneal epithelial cells grown on different culture system.

## **B) Limbal-corneal epithelial cultures translation to clinic**

- Assessment of limbal-corneal epithelial cultures, on hypodried and freeze dried commercially available human amniotic membranes, compared to fresh frozen HAM as a substrate carrier.
- Characterisation of cultured limbal-corneal epithelial cells for clinical use.

## **C) Isolation, characterisation and differentiation potential of limbal ‘fibroblast-like’ cells (LFLc):**

Mesenchymal stem cells can be isolated from various tissues besides bone marrow and can differentiate into cells of three germ layers. Recent studies indicate that some cells in corneal stroma express stem cell markers and can also differentiate into adipocytes and osteocytes. A short pilot study was conducted for

- Establishment of limbal fibroblast-like cells from limbal explants culture system.
- Characterisation and differentiation potentials of limbal fibroblast-like cells.

## 2.0 Section

---

# Materials and methods

## **2.1 Cell culture methods**

### **2.1.1 Water**

Ultra high pure water (UHP) was used in the preparation of all media and solutions. Pre-treatment of water, involving activated carbon, pre-filtration and anti-scaling was first carried out. This water was then purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP), which is low in organic salts, organic matter, colloids and bacteria with a standard of 12 - 18 M $\Omega$ /cm resistance.

### **2.1.2 Treatment of glassware**

All solutions used for cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles, lids and all other glassware used for any cell-related work were prepared as follows: - all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least one hour. This is a deproteinising agent, which removes proteineous material from the bottles. Glassware were scrubbed and rinsed several times under tap water; the bottles were then washed in a dishwasher using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with UHP water and sterilised by autoclaving.

### **2.1.3 Sterilisation**

Water, glassware and all thermo-stable solutions were sterilised by autoclaving at 121°C for 20 min under 15-p.s.i. pressure. Thermolabile solutions were filtered through a 0.22  $\mu$ m sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all

protein-containing solutions. Acrodisc (Pall Gelman Laboratory, C4187) 0.8/0.2  $\mu\text{m}$  filters were used for non-serum/protein solutions.

#### **2.1.4 Media preparation**

The basal media were purchased from SigmaPrior to use, 100ml aliquots were supplemented with 5% foetal calf serum and this was used as the routine culture medium. This was stored for up to 2 weeks at 4<sup>0</sup>C, after which time fresh culture media was prepared. For all DLKPmitox and A549 cell lines ATCC Hams F12/DMEM (1:1) supplemented with 5% FCS was routinely used. For BT20 cell line, Minimum Essential Medium (MEM) (Sigma Cat. No. M5650) supplemented with 5% FCS, and 1% L-glutamine (Cat. No. 21051-040) were added to the required basal medium.

### 2.1.5 Preparation of media supplements

The following media supplements were prepared in aseptic conditions in a laminar flow hood.

#### A) rhEGF (Recombinant epidermal growth factor) (Sigma- E9644)

rhEGF was reconstituted as per the manufacturer's instructions. 10mM Acetic Acid with 0.1% (W/V) BSA was prepared and filter sterilized with 0.22 $\mu$ M filter. 200 $\mu$ g of rhEGF was dissolved in 2ml of the above solution to yield a 100 $\mu$ g/ml solution. This 100- $\mu$ g ml<sup>-1</sup>rhEGF was stored at -20<sup>0</sup>C in 10 $\mu$ l aliquots.

#### B) Hydrocortisone (Sigma: H0135)

1mg of hydrocortisone was dissolved in 250 $\mu$ l of 95% ethanol (filter sterilized with 0.22 $\mu$ M filter). The above solution was diluted with 2.25ml of basal media (DMEM), to yield a stock solution of 8.3x10<sup>-7</sup> M. The solution was stored at -20<sup>0</sup>C in 100 $\mu$ l aliquots.

#### C) Triiodothyronine (Sigma: T6397)

1 mg Triiodothyronine was dissolved in 1ml of PBSA (sterile) to yield a 1.5x10<sup>-3</sup> M stock solution. 26.6 $\mu$ l of this stock solution was diluted in 19.973ml of PBS to yield a 2x10<sup>-6</sup> M working stock. The working stock was sterilized by passage through a 0.22 $\mu$ M filter, and stored at -20<sup>0</sup>C in 100 $\mu$ l aliquots.

#### D) Cholera toxin (Sigma: C8180)

Cholera toxin was reconstituted as per the manufacturer's instructions; 1.18ml of sterile UHP was added to 1mg of cholera toxin powder to yield a 1x10<sup>-5</sup>M solution. 100 $\mu$ l of this solution was then added to 10ml of basal medium (DMEM) to yield a 1x10<sup>-7</sup>M stock solution, which was sterilized by passage through a 0.22 $\mu$ M filter and stored at -20<sup>0</sup>C in 100 $\mu$ l aliquots.

### 2.1.5.1 Human corneal epithelial medium

Human corneal epithelial (HCE) medium **table 2.1** for culturing of limbal tissues was prepared as follows. 67.5ml of 1x Dulbecco's Modified Eagle Media, and 22.5ml of 1x Ham's F-12 Nutrient Media was aseptically added to a 100ml sterile plastic bottle. This created a 3:1 mix of Dulbecco's Modified Eagle Media:Ham's F-12 Nutrient Media. Addition of 10ml of Fetal Calf Serum (FCS) creates a 10% v/v concentration of FCS. 10µl of Epidermal Growth Factor stock solution was added to the basal media to result in a final concentration of 10ng/ml of Epidermal Growth Factor. 50µl of insulin stock solution was added to the basal media to give a final concentration of 5µg/ml of insulin. 100ul of Hydrocortisone stock solution was added to the basal media to give a final concentration of  $10 \times 10^{-10}$  M of Hydrocortisone. A 100 µl of triiodothyronine was added to basal media to give a final concentration of  $2 \times 10^{-9}$  ml<sup>-1</sup>. Finally, 100 µl of cholera toxin was added to the basal medium to give a final concentration of 100 ng/ml.

Reagents	Manufacturer	Catalogue No.	Final Concentration	For 100 ml
<b>DMEM+ Ham F12 (3:1) (1X), Liquid</b>	Gibco	11330	DMEM:F12 3:1	DMEM- 67.5ml + 22.5 F12
<b>FCS</b>	BioWhittaker	DE14-870F	10%	10 ml
<b>Insulin</b>	Sigma	I-9278	5 µg /ml	50 µ l
<b>rhEGF (100 µg/ml)</b>	Sigma	E-9644	10 ng/ml	10 µ l
<b>Cholera toxin A Subunit (100 µg/ml)</b>	Sigma	C-8180	100 ng/ml	100 µ l
<b>Hydrocortisone (50 µg/ml)</b>	Sigma	H-0135	0.4 µg /ml	100 µl
<b>Triiodothyronine</b>	Sigma	T-6397	$2 \times 10^{-9}$ ml <sup>-1</sup>	100 µl

**Table 2.1** -Composition of human corneal culture media

## **2.2 *Mycoplasma* analysis**

*Mycoplasma* examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. Mr. Michael Henry at NICB performed this analysis.

### **2.2.1 Indirect staining procedure**

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells and incubated with supernatant from test cell lines to test *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cells in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and occasionally outside the cells.

NRK cells were seeded onto sterile cover slips in sterile Petri dishes (Greiner, 633 185) at a cell density of  $2 \times 10^3$  cells per ml and were allowed to attach overnight at 37°C in a 5% CO<sub>2</sub>, humidified incubator. 1 ml of cell-free (cleared by centrifugation at 17.9 G for 5 min) supernatant from each test cell line was then inoculated onto a NRK cover slip and incubated as before until the cells reached 20 - 50% confluency (4-5 days). After this time, the waste medium was removed from the Petri dish and the cover slips (Chance Proper, 22 x 22 mm) were washed twice with sterile PBS, once with a cold PBS/Carnoy's (50/50) solution and fixed with 2 ml of Carnoy's solution (acetic acid: methanol - 1:3) for 10 min. The fixative was then removed and after air-drying, the cover slips were washed twice in deionised water and stained with 2 ml of Hoechst 33258 dye (BDH) (50 ng/ml) for 10 minutes.

From this point on, work proceeded in the dark to limit quenching of the fluorescent stain. The cover slips were rinsed three times in PBS. They were then mounted in 50% (v/v) glycerol in 0.05 M citric acid and 0.1 M disodium phosphate and examined using a fluorescence microscope with a UV (ultraviolet) filter.

Prior to removing a sample for *Mycoplasma* analysis, cells were to be passaged a minimum of 3 times after thawing to facilitate the detection of low-level infection. Optimum conditions for harvesting supernatant for analysis occur when the culture is in log-phase near confluency and the medium has not been renewed for 2-3 days, for limbal-corneal epithelial cells media is collected in the middle of culture time (between day-10 and 14 ).

## **2.3 Maintenance of cell lines**

### **2.3.1 Safety precautions**

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuair Biological Cabinet) and any work that involved toxic compounds was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all the items used in the cabinet. Each cell line (including low and high passage cells) was assigned specific media and waste bottles and only one cell line was used at a time in the cabinet, which was allowed to clear for 15 minutes between different cell lines. The cabinet and incubators were cleaned each week with the industrial detergent virkon (Antec International; TEGO, TH. Goldschmidt Ltd.). A separate laboratory coat was kept for aseptic work and gloves were worn at all times during cell work.

### 2.3.2 Culture of adherent cell lines

The cell lines used during the course of this study, their sources and their basal media requirements are listed in **table 2.3**. Cell lines were generally maintained in vented 25 cm<sup>2</sup> (Costar, 3056) and 75 cm<sup>2</sup> flasks (Costar, 3376) and fed every one to three days.

**Table 2.3: Cell lines used in this study**

Cell Line	Details-Histology	Source	Growth Media
<b>A549</b>	Established from a tumour explants removed from a 58year old male with a lung adenocarcinoma	ECCAC	DMEM/Ham's F-12, 5% FCS
<b>DLKPmitox</b>	Established at NCTCC (Geraldine Grant) from a lymph node biopsy of a 52year old male diagnosed with poorly differentiated squamous cell lung carcinoma, Drug resistance Cell line	Developed by Ms.Helena Joyce at NICB	DMEM/Ham's F12, 5% FCS
<b>MCF-7</b>	Pleural effusion from a 69year old female with breast carcinoma after radiation and hormone therapy	ATCC	RPMI 1640, 5-10% FCS
<b>BT-20</b>	Established from cells spilling out of the tumour which was cut into thin slices, tumour obtained from a 74year old female	ATCC	MEM, 1% NEAA, 2mM L-glutamine, 10% FCS
<b>hTCEpi</b>	Corneal epithelial cells infected with a retroviral vector encoding human telomerase reverse transcriptase (hTERT)	♣Prof.James V. Jester	KGM-2 (Lonza Cat.No. CC3103) supplements (CC-4152)
<b>3T3 Feeder cells</b>	Established from NIH Swiss mouse embryo cultures	♠James,S. Elizabeth	DMEM, with 10% FCS
<b>hMSC-BM</b>	Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM). (Catalogue number.C-12974)	<b>PromoCell</b>	Mesenchymal Stem Cell Growth Medium from PromoCell C-28010

♣ Kind gift from, Prof. James V. Jester; Department of Ophtalmology; University of Texas SW Medical Centre, Dallas.

♠ Kind gift from Dr. James, S. Elizabeth, University of Brighton. UK

**ECCAC**- European Collection of Cell Cultures.

**NICB** - National Institute for Cellular Biotechnology.

**ATCC**- American Type Culture Collection.

### **2.3.3 Subculture of adherent cell lines**

Prior to subculture, cells were always monitored for any contamination and were only sub-cultured when the cells were 70-80% confluent. During routine sub-culturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment.

Media were emptied from cell culture flasks and the cells were rinsed with a pre-warmed (37°C) trypsin/EDTA (Trypsin Versene - TV) solution [0.25% trypsin (Gibco, 25090-028), 0.01% (W/V) EDTA (Sigma, E-5134)] solution in PBS-A (Oxoid, BR14a). The purpose of this was to inhibit any naturally occurring trypsin inhibitors, which would be present in residual serum. Fresh TV was then placed on the cells (2ml/25cm<sup>2</sup> flask or 3ml/75cm<sup>2</sup> flask) and the flasks incubated at 37°C until the cells were detached (5-10 min). The flasks were struck once, roughly, to ensure total cell detachment. The trypsin was deactivated by addition of an equal volume of growth medium (*i.e.* containing 10% serum). The entire solution was transferred to a 20ml sterile universal tube (Greiner, 201151), and centrifuged at 17.9X G or 1000 rpm for 5 min. The resulting cell pellet was re-suspended in pre-warmed (37°C) fresh growth medium, counted (**Section 2.3.4**) and used to re-seed a flask at the required cell density or to set up an assay.

### **2.3.4 Cell counting**

Cell counting and viability determinations were carried out using a trypan blue (Gibco, 15250-012) dye exclusion technique. An aliquot of trypan blue was added to a sample from a single cell suspension at a ratio of 1:5. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted using

a microscope; an average per corner grid was calculated with the dilution factor being taken into account, and final cell number multiplied by  $10^4$  to determine the number of cells per ml. The volume occupied by the chamber is 0.1cm x 0.1cm x 0.1cm i.e.  $0.0001\text{cm}^3$ . Therefore cell number x  $10^4$  is equivalent to cells per ml. Non-viable cells were those that stained blue while viable cells excluded the trypan blue dye and remained unstained.

### **2.3.5 Cell freezing**

Cryoprotective medium or freezing medium was prepared in complete culture medium containing 10% dimethylsulfoxide (DMSO, Sigma- D5879) and filter sterilised using a 0.22  $\mu\text{m}$  filter and syringe. The appropriate number of cryogenic vials (Greiner, 122 278) were labelled with the cell line, passage number and date. Cells were trypsinised as outlined previously (**section 2.3.3**). The supernatant from the centrifuged cells was removed and the cell pellet was resuspended in 1 ml of fresh media. Freezing medium (10% DMSO) was slowly added drop wise to the cell suspension to give a final concentration of 5% of DMSO, and a final cell concentration of  $5 \times 10^6 - 1 \times 10^7$  cells/ml. This step was very important, as DMSO is toxic to cells. When added slowly, the cells had a period to adapt to the presence of the DMSO, otherwise cells may have lysed. A 1.5-1.8 ml of the DMSO-containing cell suspension was then added to each of the vials. The cryovials were then quickly placed at  $-80^\circ\text{C}$ . To allow long-term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below  $-180^\circ\text{C}$ .

### **2.3.6 Cell thawing**

Prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells. This is to reduce their exposure time to the DMSO freezing solution, which is toxic at room

temperature. The cryovial was removed and thawed quickly by rubbing by hand. When almost fully thawed, the DMSO-cell suspension was quickly transferred to the media-containing universal and centrifuged at 17.9xG or 1,000 rpm for 5 minutes. The DMSO-containing supernatant was removed and the pellet re-suspended in fresh growth medium. Thawed cells were then placed into 25cm<sup>2</sup> tissue culture flasks with 5ml of the appropriate type of medium and allowed to attach overnight. After 24hrs, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

### **2.3.7 Sterility checks**

Sterility checks were routinely carried out on all media, supplements and TV used for cell culture. Samples of basal media were inoculated into Columbia blood agar plates (Oxoid, CM331), Sabouraud dextrose (Oxoid, CM217) and Thioglycollate broth (Oxoid, CM173) which when combined detects most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum) was sterility checked at least 3 days prior to use by incubating samples at 37°C. These were subsequently examined for turbidity and other indications of contamination. Freshly thawed cells were also subjected to sterility checks.

### **2.3.8 Irradiation of 3T3 fibroblast cells**

3T3 fibroblasts were routinely maintained in DMEM supplemented with 5 % fetal calf serum (FCS) (Hyclone, Logan, UT). Cells were trypsinised at 60-70% confluency, washed and carried in HBSS buffer for irradiation. Inactivation of 3T3 fibroblasts was achieved by lethal irradiation 60 Gray. Irradiated 3T3 cells were stored in liquid nitrogen with freezing medium (10% DMSO). Irradiated 3T3 cells were used for culture of limbal-corneal cells, with density of  $2.4 \times 10^4$ /cm<sup>2</sup> cells per well/ insert. (Irradiated 3T3 feeder cells referred as Ir-3T3).

### **2.3.9 Collection of conditioned media from Ir-3T3 cells**

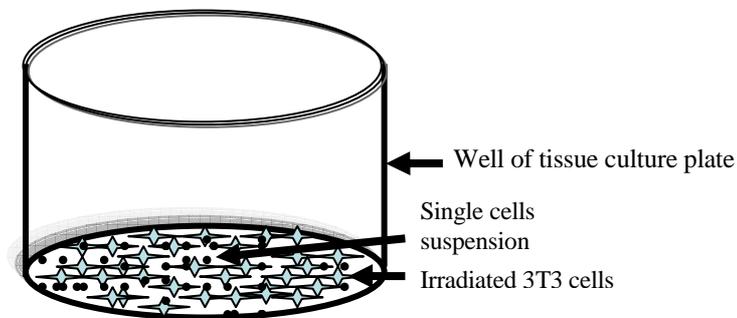
$2.4 \times 10^4/\text{cm}^2$  Irradiated 3T3 feeder cells were added to 6 well plates with 2 ml of human corneal epithelial (HCE) media, after 48 hours incubation at  $37^0\text{ C}$ ,  $\text{CO}_2$  incubator, conditioned media (CM) was collected, centrifuged for 5 min at 17.9X G or 1000 rpm, filtered through  $0.22\ \mu\text{m}$  filter and stored at  $-80^0\text{ C}$ . A fresh 2 ml HCE media added to Ir-3T3 cells for next collection after 48 hours. (Note- use 3T3 feeder cells only twice (total 96 hours) for CM collection)

## 2.4 *IN VITRO* EXPANSION OF LIMBAL-CORNEAL EPIHELIAL CELLS

To determine the best and reliable method to generate limbal-corneal cell cultures, four methods/conditions were assessed.

### 2.4.1 Enzymatic digestion of limbal tissue and growing cells on Ir-3T3 feeder layer

The limbal tissue was dissected from the corneo-scleral rim with surgical blade number 22, to remove excess of cornea and conjunctiva, the tissue was further dissected to bits 2-3 mm in size of , limbal bits were enzymatically digested with trypsin EDTA 0.25% (Gibco, 25090-028), 0.01% EDTA (Sigma, E-5134) for 1 hour at 37<sup>0</sup>C. The loosened epithelial sheets were removed and separated into single cells by pipetting, and cells were seeded on to Ir-3T3 feeder cells **figure 2.0**.



**Figure 2.0:** Limbal-corneal cell suspension culture with Ir-3T3 cells

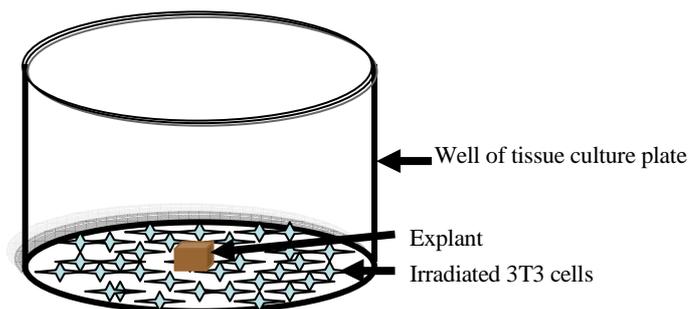
### 2.4.2 Limbal explants culture on tissue culture plates

Corneal epithelial cells were grown from limbal explants using a modification of a previously reported culture system by Koizumi *et al.*, the corneo-scleral tissue were rinsed in optisol

media (transport medium used for corneal tissue) and the corneo-scleral rim was dissected to remove excess corneal, sclera, iris, corneal endothelium, conjunctiva (Koizumi *et al.*, 2000). Each remaining limbal ring was then divided into  $1 \times 1 \text{ mm}^2$  segments, and pieces of the segments were placed epithelial side up at the centre of a well, and allowed to settle before adding growth media **figure 2.1** and **2.2**.

#### 2.4.3 Limbal explants culture on tissue culture plastic with Ir-3T3 cells

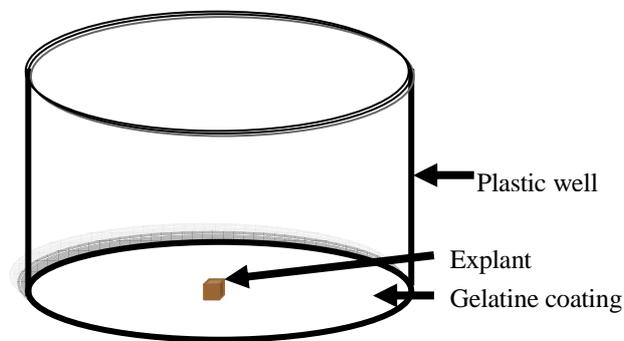
Limbal tissues were used from corneo-scleral rims, extra sclera was dissected from the limbal ring as described in section 2.4.2, limbal tissue was chopped into 2 to 3mm bits, and tissues were placed on to pre-seeded irradiated 3T3 feeder ( $2.4 \times 10^4$  cells per  $\text{cm}^2$ ) layers.



**Figure 2.1:** Limbal-corneal explants cultures on cell culture plate with Ir-3T3

#### 2.4.4 Limbal explants culture on gelatine coated tissue culture with Ir-3T3 cells

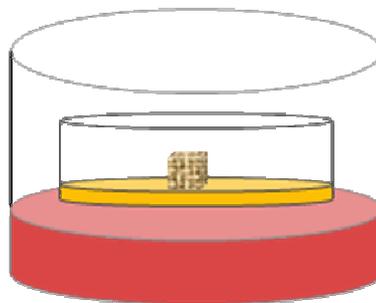
Limbal tissues were used from the corneo-scleral rims, extra sclera was dissected from the limbal ring, and limbal tissue was chopped into 2 to 3mm bits and tissues were placed on to pre-coated gelatine (0.025%) (Sigma 271616), and tissues were allowed to settle before adding growth media.



**Figure2.2:** Limbal-corneal explants culture on gelatine coated plate.

#### **2.4.5 Limbal explants culture on trans-well membrane with no 3T3 cells (Condition A no-3T3)**

Limbal tissue was dissected from the corneo-scleral rim as described in **section 2.4.2**. The tissue segments were then picked up with a 24-gauge sterile needle and explanted onto the trans-well inserts by placing epithelial side up. After 20 minutes of explantation, 1ml of culture medium was added into the well (not to the insert), and kept in the CO<sub>2</sub> incubator to allow the adherence of the tissue bits to the membrane overnight. The next day another 1 ml of media was added to the well and incubated over night. After 48 hours when explants were showing out growth, 1ml of media was added to the explants in the insert, and followed by every alternate change in media **figure 2.3**.

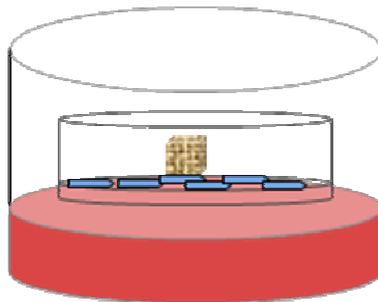


**Figure 2.3:** Limbal explants culture on trans-well membrane without Ir-3T3 cells (condition A No-3T3)

#### 2.4.6 Limbal explants culture on trans-well membrane co-culture with Ir-3T3 cells (Condition B Co-3T3)

Limbal tissue was dissected from the corneo-scleral rim as described in **section 2.4.2**. The tissue segments were picked with 24-gauge sterile needle, and explanted onto the trans-well inserts by placing epithelial side up. After 20 minutes of explantation 1ml of culture medium were added into the well (not to the insert), and kept in the CO<sub>2</sub> incubator for allowing the adherence of the tissue bits to the membrane for overnight.

Next day (approximately 24 hours), another 1 ml media was added to the well. When explants were showing outgrowth,  $2.4 \times 10^4$  cells per cm<sup>2</sup> irradiated 3T3 feeder cells were added to the insert i.e. in contact with limbal-corneal epithelial cells **figure 2.4**.

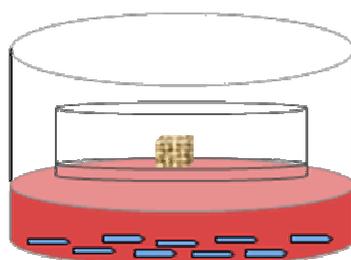


**Figure 2.4:** Limbal explants on trans-well membrane with Ir-3T3 cells in insert (condition B Co-3T3)

#### **2.4.7 Limbal explants culture on trans-well membrane with Ir-3T3 separated (Condition C Bot-3T3)**

Limbal tissue was dissected from the corneo-scleral rim as described in **section 2.4.2**. The tissue segments were picked with 24-gauge sterile needle, and explanted onto the trans-well inserts by placing epithelial side up. After 20 minutes of explantation 1ml of culture medium were added into the well (not to the insert), and kept in the CO<sub>2</sub> incubator for allowing the adherence of the tissue bits to the membrane for overnight.

Next day (approximately 24 hours), another 1 ml media was added to the well. When explants were showing outgrowth,  $2.4 \times 10^4$  cells per cm<sup>2</sup> irradiated 3T3 feeder cells were added to the well (bottom to the insert) **figure 2.5**.



**Figure 2.5:** Limbal explants on trans-well membrane with Ir-3T3 cells away from limbal-corneal cells (Condition C Bot-3T3)

## **2.5 LIMBAL EXPLANTS CULTURES ON HUMAN AMNIOTIC MEMBRANE**

### **2.5.1 Preparation of denuded human amniotic membrane (HAM)**

Fresh cryo-preserved human amniotic membrane (HAM) (Transplant Service Foundation, Spain; Code-30102-619) was pre-thawed from  $-80^{\circ}\text{C}$ , and carefully removed from freezing medium. The AM was carefully peeled from the nitrocellulose paper with sterile forceps on a sterile plastic culture dish. Next, the AM was stretched over and treated with 0.25% trypsin EDTA solution for 15-20 minutes. AM was gently scrubbed with a cell scraper (Costar 3010) to remove the amniotic epithelium without breaking the underlying basement membrane. The membrane was washed with PBS to remove scraped cells, this method allowed removal of 90-100% of epithelium. The membrane was turned over to the endothelial side and scraped with a cell scraper to remove mucus. The resulting membrane is termed as denuded human amniotic membrane (dHAM).

Keeping the de-epithelialised side facing upward, dHAM is stretched on a base-less cell culture insert and a sterile elastic band placed around HAM were completely attached to the base of insert all sides and stretched uniformly, and placed in a well of a 6-well plate and covered the HAM with 1 ml growth medium.

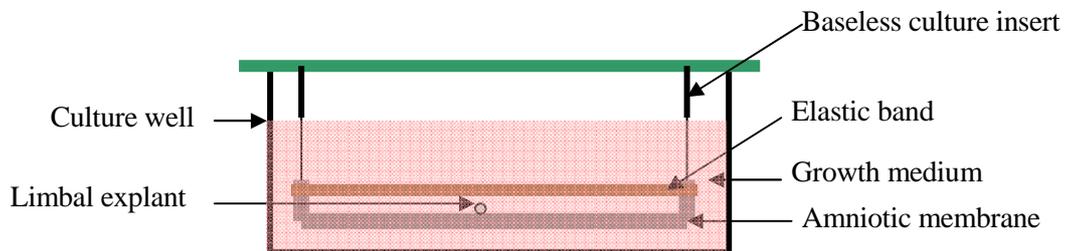
## 2.5.2 *In Vitro* expansion of limbal explants on dHAM

Limbal biopsy was dissected from the corneo-scleral rim as described in **section 2.4.2**. The epithelial medium from the previously plated HAM was removed from the culture well. The limbal biopsies (approximately 6-7) was placed centripetally on the centre of the HAM and gently pressed downward to promote attachment and 1 ml of epithelial medium was gently added to the culture well (next to the membrane) after 20 minutes. The culture was examined for explants adhesion to HAM and subsequently fed with 2 ml of growth medium, on alternate days up to 15-20 days.

The following are three different culture conditions with denuded Human Amniotic Membrane (dHAM).

### 2.5.2.1 Limbal explants culture on dHAM with no 3T3 cells (Condition D dHAM No-3T3)

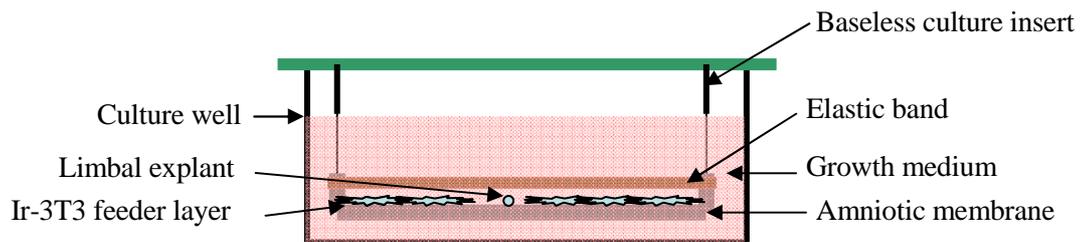
Preparation of dHAM and limbal biopsy was as described in sections **2.5.1** and **2.4.2** respectively **figure-2.6**



**Figure 2.5:** Explants on HAM with no 3T3 feeder cells (Condition D dHAM no-3T3)

### 2.5.2.2 Limbal explants on dHAM with co- 3T3 cells (Condition E dHAM Co-3T3)

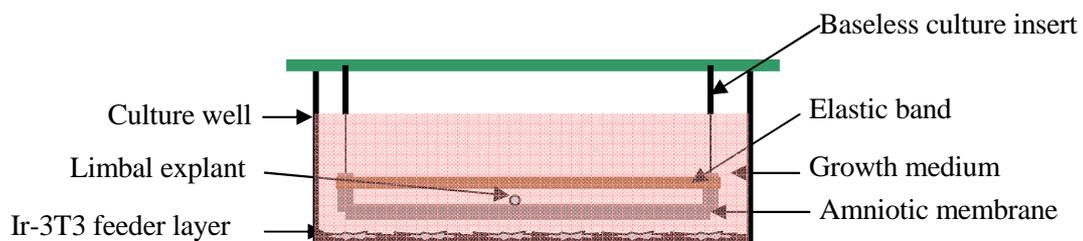
Preparation of dHAM and limbal biopsy was as described in sections 2.5.1 and 2.4.2 respectively. The culture was examined for attachment of explants to the basement membrane. The Ir-3T3 feeder cells were added to insert dHAM (dHAM Co-3T3), and subsequently in both chambers (inserts and well) fed with 2 ml of epithelial medium on alternate days up to 15-20 days **figure-2.7**.



**Figure 2.7:** Explants on dHAM with co- 3T3 feeder cells (Condition E dHAM Co-3T3).

### 2.5.2.3 Limbal explants on dHAM with Bot- 3T3 cells (Condition F dHAM Bot-3T3)

Preparation of HAM and limbal biopsy was as described in sections 2.5.1 and 2.4.2 respectively. The culture was examined for attachment of explants to basement membrane. The Ir-3T3 feeder cells were added to the well (not the insert chamber of dHAM) (dHAM Bot-3T3) and subsequently in both chambers (inserts and well) fed with 2 ml of epithelial medium on alternate days up to 15-20 days.



**Figure 2.8:** Explants on HAM with Bot- 3T3 feeder cells (Condition F dHAM Bot-3T3).

## **2.6 Characterization of cultured limbal-corneal epithelial cells**

### **2.6.1 Cell outgrowth, and ability to generate cell sheets**

The outgrowth of an area was measured over previously marked outgrowth areas on the underside of the tissue culture well. Quantitative analysis of the area of epithelial outgrowths indicated comparable exponential growth in each of the cultures at different time intervals during culture process and the ability of complete monolayer formation in 4.2 cm<sup>2</sup> cell culture trans-well membrane. The epithelial out growth from each explant (s) were measured in all six conditions at days 2, 5, 8, 12, 14 and 17 for cell culture inserts and until day 20 for dHAM cultures with repeating 6 biological samples, and a graph plotted with growth rate vs. number of days.

### **2.6.2 Cell sheet morphology**

Cell sheet morphology was assessed by phase contrast microscopy at days 2 and 8 and at termination day (day 16 ± 2).

## 2.7 Western blot analysis

### 2.7.1 Preparation of lysis buffer

A stock of lysis buffer was prepared using the reagents as shown in **table 2.7**. Working lysis buffer was prepared fresh every time before use as shown in **table 2.7.1**.

**Table 2.7: NP40 Lysis buffer stock**

Reagents	Volume
1M Tris pH 7.4	2 ml
3M NaCl	1.66 ml
1M NaF	5 ml
10% NP40	1 ml
H <sub>2</sub> O	90.34 ml
<b>Total</b>	<b>100 ml</b>

**Table 2.7.1: Working lysis buffer**

Reagents	Volume
100mM Sodium Orthovanidate	10 $\mu$ l
100 mM PMS	10 $\mu$ l
Protease Inhibitor Cocktail	40 $\mu$ l
NP40 Lysis Buffer	940 $\mu$ l
<b>Total</b>	<b>1000<math>\mu</math>l</b>

### **2.7.2 Sample preparation**

When limbal-corneal epithelial cells attain confluent termination day (between approximately days 15-18), prior to lysis, cell sheets were washed with cold PBS and drained of all supernatant. Cell sheets were separated from the insert by cutting the insert plastic with a surgical blade and plated on 6 well plates. Cold lysis buffer was added to the cells, dropping it evenly over the whole well/plate (20  $\mu$ l). The cells were scraped from the well and all the lysed/scraped cells were gathered in one corner of the well. The solution was pipetted up and down without frothing and placed in a pre-chilled eppendorf tube. The tube was vortexed for 30-60 sec until the solution was homogenised. The tubes were placed on ice for 20 minutes and the tube was then centrifuged at maximum speed in a microfuge for 15 min. The supernatant was transferred to a fresh chilled eppendorf tube and immediately quantified for the protein (**Section: 2.7.3**). The samples were prepared with 2-5X loading buffer (Sigma, S-3401) and water was added to maintain all the samples at the same concentration. Parafilm was wrapped around the lids of the eppendorfs (to avoid evaporation) and the samples were boiled for 3-5 minutes. If not used immediately, the samples were stored at  $-20^{\circ}\text{C}$  until needed.

### **2.7.3 Quantification of protein**

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad; 500- 0006) with a series of bovine serum albumin (BSA) (Sigma, A9543) solutions as standards. A stock solution of 2 mg/ml BSA (Bio-Rad) was used to make a standard curve. 10 $\mu$ l samples were diluted into tubes in a stepwise fashion from zero – 2 mg/ml BSA. The Biorad was first filtered through 3MM filter paper (Schleicher and Schuell, 311647) and then diluted 1/5 with UHP as it was supplied as a 5-fold concentrate. The diluted dye reagent (490  $\mu$ l) was added to each

standard and sample tube and the mixtures vortexed. The 500- $\mu$ l samples were diluted out in 100  $\mu$ l aliquots onto a 96- well plate (Costar, 3599). After a period of 5 min to 1h, the OD570 was measured, against a reagent blank with a (Bio-TEK<sup>®</sup>) plate reader. From the plot of the OD570 of BSA standards versus their concentrations, the concentration of protein in the test samples was determined. From this, a relative volume for each protein sample was determined for loading onto the gels. Usually 10-20  $\mu$ g protein per lane was loaded.

#### **2.7.4 SDS Polyacrylamide gel electrophoresis**

Proteins for western blot analysis were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Resolving and stacking gels were prepared as outlined in **table 2.7.2** and poured into clean 10 cm x 8 cm gel cassettes, separated by 0.75 cm plastic spacers. The plates had been cleaned by tap water, followed by UHP and after drying, the plates were wiped down in one direction using tissue paper soaked in 70% Industrial Methylated Spirits (IMS). Similarly, the spacers and comb used were also cleaned and air-dried. The resolving gel was poured first and allowed to set for 20 min at room temperature. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in wet tissue paper and stored at 4°C for 24hrs.

A 1 X running buffer was added to the running apparatus before samples were loaded. The samples were loaded onto the stacking gels in equal amounts relative to the protein concentration of the sample. The empty wells were loaded with loading buffer.

Components	Resolving gel (7.5%)	Resolving gel (12%)	Stacking gel
Acrylamide stock* (Sigma, A3574)	3.8 mls	5.25 mls	0.8 mls
Ultra high pure water	8.0 mls	6.45 mls	3.6 mls
1.5M-Tris/HCl, pH 8.8	3.0 mls	3.0 mls	-
1.25M-Tris/HCl, pH 6.8	-	-	0.5 mls
10% SDS (Sigma, L-4509)	150 $\mu$ l	150 $\mu$ l	50 $\mu$ l
10% Ammonium per sulphate (Sigma, A-1433)	75 $\mu$ l	75 $\mu$ l	40 $\mu$ l
TEMED (Sigma, T- 8133)	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l

**Table 2.7.2:** Preparation of electrophoresis gels

Note: \*Acrylamide stock solution consists of 29.1g acrylamide (Sigma, A8887) and 0.9g NN'-methylene bis-acrylamide (Sigma, 7256) dissolved in 60ml UHP water and made up to 100ml final volume. The solution was stored in the dark at 4°C for up to 1 month. All components were purchased from Sigma, SDS (L-4509), NH<sub>4</sub>-persulphate (A-1433) and TEMED, N,N,N,N'-tetramethylethylenediamine (T-8133).

### 2.7.5 Running buffer

BIO-RAD (cat. number- 107348/2009-03-06) 10x Tris/Glycine/SDS buffer was used as running buffer. To make 1 liter of 1x running buffer, 900ml of Ultra High Pure (UHP) water was added to 100 ml of 10x buffer, the final concentration of 1x solution contains 25mM Tris, 192 mM Glycine, and 0.1% (w/v) SDS, with pH 8.3.

### **2.7.6 TBS buffer (Wash buffer)**

A 10x TBS buffer was made by dissolving NaCl (Sigma S5886) 43.83g and 30.275g Tris (T87602) in 300ml UHP water initially. After dissolving the salts in solution, the pH was adjusted to 7.4 and the final volume was adjusted to 500ml with UHP water. To make 1x TBS, 50ml of 10x TBS added to 450ml of UHP water.

### **2.7.7 Protein transfer and western blotting**

The samples were loaded onto gels as described above including 7  $\mu$ l of molecular weight dual color precision plus protein standards marker (Bio-Rad, 161-0374). The gels were run at 200 V, 45 mA for approximately 1.5hrs. When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped.

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine (Bio-Rad- 161-0734) for 10 min. The protein in gels were transferred onto nitrocellulose membranes (Boehringer Mannheim, 1722026) by semi-dry electroblotting. Eight sheets of Whatman 3 mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus (Bio-Rad). Excess air was removed from between the filters by rolling a universal tube over the filter paper. A piece of nitrocellulose membrane, cut to the same size of the gel, was prepared for transfer by soaking in transfer buffer and was then placed over the filter paper, ensuring there were no air bubbles.

The acrylamide gel was placed over the nitrocellulose membrane and eight more sheets of pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling the

universal tube over the filter paper. The proteins were transferred from the gel to the nitrocellulose at a current of 34 mA at 15V for 24-25 min.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus (Stovall, Bellydancer) to ensure even exposure of the blot to all reagents. The nitrocellulose membranes were blocked for 2 hours at room temperature with fresh 5% non-fat dried milk (Cadburys, Marvel skimmed milk) in Tris-buffered saline (TBS) with 0.5% Tween-20 (Sigma, P-1379). After blocking, the membranes were washed 3 x 5 min using 1 X TBS. The membrane was then incubated with 5 to 10ml primary antibody (concentration of primary antibodies was used as in **table 2.7.3**) at 4<sup>0</sup>C overnight. The next day the membrane was again washed 3 x 5 min using 1 X TBS/PBS. It was then incubated in secondary antibody (the specific conditions for each antibody are outlined in **table 2.7.3**). Finally, the membrane was washed 3 x 5 min using 1 X TBS/PBS. Bound antibody was detected using enhanced chemiluminescence (ECL) (Amersham, RPN2109).

S. No	Antibody / Clone	Primary antibody Dilution	Size KDa	Company/ Cat No.	Secondary Antibody	Secondary antibody dilution
1	<b>CK3/AE5</b>	1:200	64	Chemicon/ CBL218	Anti-mouse	1:5000
2	<b>Cytokeratin12 /H-60</b>	1:200	54	Santa cruz/ sc-25722	Anti-Rabbit	1: 5000
3	<b>CK8/M20</b>	1:1000	40-68	Abcam/ ab9023	Anti-mouse	1: 5000
4	<b>CK19</b>	1:1000	40-68	Chemicon/ MAB3238	Anti-mouse	1: 5000
5	<b>ABCG2/BCRP1 /Bxp-21</b>	1:500	72.3	Alexis Biochemicals/ ALX-801-029	Anti-mouse	1: 5000
6	<b>Vimentin/V9</b>	1:5000	58	Sigma/ V6389	Anti-mouse	1: 5000
7	<b>p63 Ab-1 (Clone 4A4)</b>	1:200	63	Lab vision/MS-1081	Anti-mouse	1: 5000
8	<b>CK14 (RCK-107)</b>	1:1000	40-68	Chemicon/ MAB3232	Anti-mouse	1: 5000
9	<b>GAPDH</b>	1:5000	50	Abcam/	Anti-mouse	1: 5000
10	<b>Integrin <math>\beta</math>1/CD29/JB1A</b>	1:200	115 & 130	Chemicon/ MAB1965	Anti mouse	1: 5000
11	<b>Intergrin Alpha2/VLA2<math>\alpha</math>/CD49b</b>	1:500	150	BD bioscience / 611017	Anti-mouse	1: 5000
12	<b>ZO1</b>	1:80	194	Abcam/ ab 59724	Anti- guinea pig	1:1000
13	<b>Occludin</b>	1:30	65	BD bioscience / 611016	Anti-mouse	1: 5000
14	<b>Connexin43</b>	1:100	43	BD bioscience/610062	Anti-mouse	1:5000
15	<b>E-cadherin/ 36-E-cadherin</b>	1:2000	120	BD bioscience/610182	Anti-mouse	1: 5000
16	<b><math>\Delta</math>Np63<math>\alpha</math></b>	1:1000	51-57	Santa Cruz/sc-8344	Anti-rabbit polyclonal	1:2000

**Table 2.7.3:** List of primary and secondary antibodies used for western blot analysis

### **2.7.8 Enhanced chemiluminescence detection**

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions. The blot was moved to a darkroom for all subsequent manipulations. A sheet of parafilm / transparent sheet was flattened over a smooth surface, e.g. a glass plate, making sure all air bubbles were removed. The membrane was then placed on the parafilm/ transparent sheet, and excess fluid removed. A 1:1 ratio of ECL detection reagents 1 and 2 were mixed; and the mixture was poured so that it covered the membrane. The reagent was removed after one minute and the membrane was covered in cling film. The membrane was exposed to auto-radiographic film (Boehringer Mannheim, 1666916) in an auto radiographic cassette for various times, depending on the signal (30 – 15 min). The auto radiographic film was then developed.

The exposed film was developed for 5 min in developer (Kodak LX24 diluted 1:6.5 in water). The film was briefly immersed in water and then fixed (Kodak FX-40, diluted 1:5 in water), for 5 min. The film was transferred to water for five minutes and then air-dried.

## 2.8 Immunofluorescence staining (Immunophenotyping)

Immunofluorescence was performed on the limbal-corneal epithelial cells grown on cell culture inserts or HAM. The inserts/HAM were washed twice for 5 min with PBS to remove traces of growth media. Cells were fixed using ice-cold methanol or 1:1 ratio of acetone and methanol for 10 min or ice-cold acetone for 2 seconds.

**Table 2.8 PHEM buffer composition**

Component/Manufacture	Concentration	For 500ml
PIPES/Sigma P6757	60 mMol <sup>-1</sup>	9.072 gm
HEPES/Sigma H3375	25mMol <sup>-1</sup>	3.254 gm
EgtA/Sigma E4378	10 mMol <sup>-1</sup>	1.902 gm
Mgcl <sub>2</sub> /Sigma M0250	2 mMol <sup>-1</sup>	0.203 gm
Adjust pH to 6.9 with 10M KOH – 7ml		

Samples were blocked in relevant serum (normal goat serum 5% in PBS) for 1 hour at room temperature. The serum was removed and the slide was then washed using PHEM (**table 2.8**) with 0.25% Tween-20 once, and the primary antibody (in 1% normal goat serum) was applied (antibodies and dilutions are listed in **table 2.9**). This was incubated in suitable condition (refer **table 2.9**). The following day, the primary antibody was removed and the slides were washed three times in PHEM with 0.25% Tween 20 at an interval of 5 min per wash. The fluorescent secondary antibodies (light sensitive) were prepared in the dark room under dim conditions and were coated in foil. The cells were incubated with secondary antibodies diluted in 1% normal goat serum **table 2.9** for 60 minutes at room temperature in the dark. All work from this point onwards was carried out in the dark to prevent ‘quenching’ of the fluorescent signal. After 60 minutes incubation, the antibodies were removed and the slides were washed three times in PHEM with 0.25% Tween 20. The cells were then counterstained for nuclei with DAPI (4', 6-

diamidino-2-phenylindole) for 2 minutes, then washed twice with wash buffer, slides were mounted with SlowFade® Gold antifade reagent (Invitrogen- S36937) and covered with coverslips. Slides were examined with a Laser Scanning Confocal Microscope (Leica SP2 AOBS).

**Table 2.9:** List of primary and secondary antibodies used for Immunofluorescence analysis

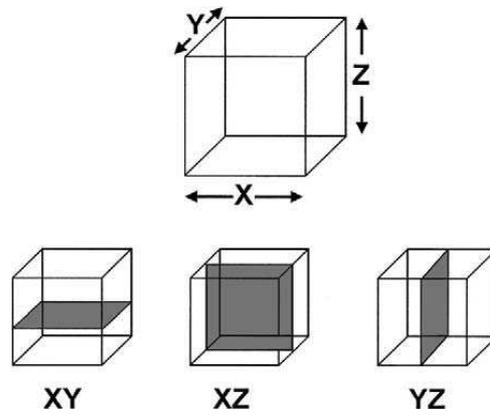
S. No	Antibody/ Clone	Company/ Cat No.	Fixative	Primary antibody Dilution	Incubation, Time/T emp	Secondary	2 <sup>0</sup> Ab dilution
1	<b>CK3/AE5</b>	Chemicon/ CBL218	Ice Cold Methanol	1:80	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
2	<b>Cytokeratin 12 /H-60</b>	Santa cruz/ sc-25722	Ice Cold Methanol	1:100	ON/4 <sup>0</sup> C	Anti-Rabbit Alexa fluor 647	1:1000
3	<b>CK8/M20</b>	Abcam/ ab9023	Ice Cold Methanol	1:200	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
4	<b>CK19</b>	Chemicon/ MAB3238	Ice Cold Methanol	1:100	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
5	<b>Vimentin/V 9</b>	Sigma/ V6389	Ice Cold Methanol	1:2000	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
6	<b>CK14 (RCK-107)</b>	Chemicon/ MAB3232	Ice Cold Methanol	1:800	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
7	<b>Integrin Alpha2/VL A2<math>\alpha</math>/ CD49b</b>	BD bioscience / 611017	Ice Cold Methanol	1:200	ON/4 <sup>0</sup> C	Anti-mouse Alexa fluor 488	1:1000
8	<b>E-cadherin/ 36-E-cadherin</b>	BD bioscience/610182	Ice cold Acetone: Methanol (1:1)	1:2000	1hr/37 <sup>0</sup> C	Anti-mouse Alexa 488	1:3000
9	<b><math>\Delta</math>Np63<math>\alpha</math></b>	Santa Cruz/sc-8344	Ice cold Acetone: Methanol (1:1)	1:150	ON/4 <sup>0</sup> C	Anti-rabbit Alexa fluor 647	1:1000
10	<b>ABCG2/BC RP1 /Bxp-21</b>	Alexis Biochemicals/ ALX-801-029	Ice cold Acetone: Methanol (1:1)	1:250	1hr/37 <sup>0</sup> C	Anti- Alexa fluor 488	1:1000

### **2.8.1 Limbal –corneal epithelial cells- Confocal microscopy**

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. The fluorescence produced by the interaction of the laser with the labelled parts of the specimen is then detected by highly sensitive electronics and a computer to generate high-resolution image sets called image “stacks” using the signal. Once collected, the stacks can be re-constructed to make highly detailed, accurate, three-dimensional renderings. In this study, limbal-corneal epithelial cultures are thick cell sheet on cell culture inserts and dHAM, to obtain image of corneal epithelial cells, the mounted cultured epithelial cell sheets were examined with confocal laser scanning.

The instrument photo multiplier tube (PMTs) and offsets were adjusted at the start of each sample/antibody to give an optimum image. There was no adjustment made to the instrument parameter settings within a sample. The emission of fluorophore used in this study are; (1) DAPI with maximum emission at 459nm. (2) Alexa Fluor 488, with maximum emission at 520nm and, (3) Alexa Flour 647 with emission at 671nm.

**Figure 2.9** represents the optical sectioning in the confocal microscope, where the optical sections can be generated in three different planes: in the xy or horizontal direction which is parallel to the microscope stage, and in the xz and yz axes, which are in the vertical direction. Sectioning in the xy plane is most common because the lateral spatial resolution (xy plane) is better than the axial spatial resolution (xz and yz planes).



**Figure 2.9:** Optical sectioning in the confocal microscope (Matsumoto, 2002).

Cultured limbal-corneal epithelial cell sheets were imaged with confocal microscopy by two methods. First X-Y optical sectioning (surface scan) across all layers of cells. This simple technique characterizes flat samples (flat image) by mapping a thin 2D layer on the sample surface which is called a single optical X-Y section, secondly X-Z cross-sectioning (depth profiling) to obtain information about a sample's 2D cross-section in X-Z direction. Once the image is captured in a series of sections, images were processed with Leica simulator software to create average projections.

## **2.9 Flow cytometry analysis**

### **2.9.1 Principles of flow cytometry analysis**

Flow cytometry is a powerful laser-based technology which can be used to measure the micro-particulate characteristics of inorganic microcapsules and biological samples (including whole cells, cellular organelles and epitopes) in the range of ~0.5-40 microns. Particles are presented for analysis in a liquid medium and during processing are hydro-dynamically focussed by a higher pressure sheath fluid which surrounds the sample; a process which allows particles to be interrogated individually within the flow cell. During interrogation particles are excited by one or more lasers and scatter and fluorescence data collected by photomultiplier tube (PMT) detectors for each particle.

This technique offers a number of advantageous features not facilitated by alternate analysis techniques such as western blotting and immunohistochemistry including very high throughput analysis rates of 50,000 – 70,000 per/sec, high sensitivity analysis of single epitopes, both quantitative and qualitative measurement of multiple parameters (a feature especially useful for identification of subpopulations) and for those cytometers equipped with a sort facility; isolation of rare, live subpopulations which may be sub-cultured and/or collected for further analysis.

Flow cytometric analysis of stem cell markers ABCG2 and  $\Delta$ Np63 and differentiation marker CK12 was performed on primary limbal corneal epithelium cultures condition A no-3T3,

condition B Co-3T3, and condition C Bot-3T3 to determine what if any effect these conditions had on cellular differentiation and or homogeneity.

S.No	Primary Ab	Dilution	Isotype	Dilution	Secondary Ab	Dilution
1	ABCG2	0.66µg/ml	IgG2a	0.66µg/ml	Anti Mouse 488	1:50
2	ΔNp63α	1.0 µg/ml	IgG	1.0 µg/ml	Anti Rabbit 647	1:100
3	CK12	20 µg/ml	IgG	20 µg/ml	Anti Rabbit 674	1:100

**Table 2.10:** Primary, Secondary antibody, and Isotype dilutions used for flow cytometry

### 2.9.2 Flow cytometry analysis of limbal-corneal epithelial cells

The cultured limbal epithelial cells were indirectly labelled using two dual stains ABCG2 (Alexis Biochemicals/ ALX-801-029) / ΔNp63α (Santa Cruz/sc-8344), and CK12 (Santa cruz/sc-25722) / ABCG2 and analysed via flow cytometry (BD FACS Aria) **table 2.10**.

Limbal epithelial cells were harvested using 0.025% trypsin, 0.53mM EDTA and incubation at 37°C for 5 min. Trypsin was subsequently inactivated using 10% FBS in DMEM, 200mM L-Glutamine and washed twice in PBS. Cell counting and viability determinations were carried out using a trypan blue (Gibco, 15250-012) dye exclusion technique. PBS was then removed and cells resuspended in an ice cold methanol:acetone solution (1:1 ratio) and stored at -20<sup>0</sup>C for a minimum of 48 hours.

In order to perform antibody staining 1x10<sup>6</sup> viable cells/well were aliquoted into a 96 well plate. 100ul of permeabilisation solution was added to a final concentration of 0.01% tween20 / PBS and cells incubated for 5 minutes at room temperature. Cells were spun down, supernatant

removed and washed twice with PBS (250µl per well). Cells were blocked with 0.5% goat serum by incubating at room temperature for 15 minutes, after incubation cells were washed with PBS once. Primary antibody and isotype antibody solutions were added at specified concentrations (**table 2.10**) to relevant sample, and incubated at 4<sup>0</sup>C with rotation for 1hour. Cells were washed twice in PBS and 100µl of secondary antibody added as described **table 2.10**. Samples were then incubated at 4<sup>0</sup>C in darkness for 30 minutes. Following three PBS washes, all samples were analysed via flow cytometry. For a full list of all samples and singleplex and multiplex controls used to perform gating during this experiment, please see **table 2.11** below.

**Table- 2.11:** Systematic representation of controls used in the study.

	Note	Primary antibody	Isotype	Secondary antibody
<b>1</b>	Untreated control	No	No	No
<b>2</b>	Primary antibody control	Yes (for all Abs)	No	No
<b>3</b>	Secondary antibody control	No	No	Yes (for all Abs)
<b>ABCG2</b>	ABCG2	Yes	No	Yes (AF 488)
	IgG2a Isotype	No	Yes	Yes (AF 488)
<b>CK12</b>	CK12	Yes	Yes	Yes (AF 647)
	IgG Isotype	No	Yes	Yes (AF 647)
<b>ΔNp63α</b>	ΔNp63α	Yes	Yes	Yes (AF 647)
	IgG Isotype	No	Yes	Yes (AF 647)
<b>Duplex</b>	ABCG2+ CK12 Both Abs	Yes (ABCG2+CK12)	No	Yes (AF 647+488)
	ABCG2+ CK12 Both Isotypes	No	Yes IgG2a+IgG	Yes (AF 647+488)
<b>Duplex</b>	ABCG2 Ab + CK12 Isotype	Yes (ABCG2)	Yes (IgG)	Yes (AF 647+488)
	CK12 Ab+ ABCG2 Isotype	Yes (CK12)	Yes ( IgG2a)	Yes (AF 647+488)

Note prior to analysis of primary cultured limbal-corneal epithelial cells; antibody titrations were performed on the human corneal epithelial cell line (hTCEpi) for each primary and secondary antibody to identify the antibody concentrations, which provide the largest isotype adjusted mean fluorescent intensity (or signal). Using the procedure as described above;

antibody concentrations (dilutions 1:50, 1:100, 1:150, 1:200, 1:250, 1:300) of 1:50, 1:250, 1:300 were used for CK12, ABCG2 and  $\Delta$ Np63 $\alpha$  respectively with isotype matched controls. Dilutions of 1:50, 1:100, 1:150, 1:200, 1:250, were used to identify optimal dilutions/concentrations for secondary antibodies. Once optimal dilutions/concentrations had been identified for all antibodies individually, singleplex and multiplex analysis of samples could begin.

## 2.10 Colony forming efficacy assay

Colony forming efficacy (CFE) was performed to determine the proliferative potential of cells in the cultured limbal-corneal cell sheets by prolonged culturing time and a lower seeding density, which could maintain SCs. Irradiated-3T3 fibroblasts (Ir-3T3) were seeded on to 6 well plates at a density of  $2 \times 10^6$  cells per well one day before plating primary limbal-corneal cells. The next day limbal-corneal cell sheets were trypsinised with 0.025% trypsin+ 0.1%EDTA and a viable cell count (**section 2.3.4**) was performed and cells were plated at a density of 200 cells per well. The medium was changed every other day, until day 14. The clonal growths were visualized by crystal violet staining (0.025%), colony-forming efficiency, colony size were analyzed in duplicates.

Images were captured with a Nikon D5000 by adjusting constant focus and distance between object and camera. Images were converted to JPEG format to use in ImageJ software for colony analysis. The required image in ImageJ was opened, and a binary/threshold made. (The terminology depends on the version you are running). Once the image was created in binary format, the *circle tool* or the *freehand tool* was used to outline the area of the selected colonies. An example of the binary/threshold images and original images are shown in appendices (page number 449-450). The colonies measuring 2 to 10 mm<sup>2</sup> diameter have been selected for counting, which is represented as yellow arrows in **figure 3.8.1** and **3.8.1b**. Similarly, the values for all colonies were measured and total number of colonies per plate was measured. The generated colonies measured as Colony Forming Efficacy (CFE), calculating percentage as by using the formula:

$$\text{Number of colonies formed} / \text{Number of cells plated} \times 100$$

## **2.11 Culture of limbal fibroblast-like cells (LFLc)**

To generate limbal fibroblast-like cells, a single cornea-scleral ring was used; in brief the epithelium and the endothelium were removed by scraping with a surgical blade. The corneal side tissue and excess scleral tissue was removed from limbal tissue, the leftover limbal tissue was chopped into fine pieces. Small pieces of limbal tissues were cultured in DMEM with 10% FBS and incubated at 37<sup>0</sup>C in 5% humidified CO<sub>2</sub>. The fine pieces were left for a week without medium changes. The number of adherent cells was observed at day 7-8, the small pieces of limbal tissue were removed and the culture was continued in the same culture medium until confluency. At confluence, cells were trypsinised with 0.25% trypsin-1% EDTA, plated on T25 flasks with 1:1 ratio.

### **2.11.1 Proliferation assay for LFL cells**

Fibroblast-like cells were taken at different passages from p2 to p15 stage, and cells were allowed to grow for 3, 5 and 7 days in 6 well plates. After the incubation period, cells were washed with PBS 3 times, 0.25% crystal violet was added and incubated at room temperature for 10 min. The dye was removed; rinsed thoroughly with tap water and allowed to air dry. When completely dried, 500µl of 33% glacial acetic acid was added to each well. The eluted dye was taken in to triplicate in 96 well plates; and absorbance was measured at 570nm wavelength which is specific for maximum absorbance of the crystal violet dye and at 620nm, which was a reference wavelength.

## **2.12 Invasion assays**

### **2.12.1 Preparation of invasion chambers**

Invasion assays were carried out using BD BioCoat™ Growth Factor Reduced (GFR) MATRIGEL™ Invasion Chambers (BD Biosciences). The insert and the plate were incubated for one hour at 37°C to allow the proteins to polymerize. Inserts were rehydrated as specified by the manufacturers' protocol.

### **2.12.2 *In vitro* invasion assays**

Cell suspensions were prepared in culture media containing 5% FCS at a concentration of  $1 \times 10^6$  cells/ml. 500 µl of media containing the same concentration of FCS was added to the well of the BD Falcon™ TC companion plate. 100µl of cell suspension was then added into the insert. The invasion assays were then incubated for 48 hours at 37° C, 5% CO<sub>2</sub> atmosphere. Limbal-fibroblast like cell suspensions were prepared at different passages in culture media without serum, and 500µl of media containing 5% FCS was added to the well of the BD Falcon™ TC companion plate. The invasion chambers were then incubated for 24 hours at 37° C, 5% CO<sub>2</sub> atmosphere.

After incubation, the non-invading cells were removed from the upper surface of the membrane. The inner side of the insert was wiped with a wet swab (PBS soaked not UHP) while the outer side of the insert was stained with 0.25% crystal violet for 10 minutes and then rinsed in UHP and allowed it to dry. Inserts were then viewed under the microscope.

Cell counting was facilitated by photographing the membrane using an inverted microscope. The cells were observed at 10X magnification. Cells in the central fields of duplicate numbers were counted and an average count calculated from 10 counts per chamber. Data was expressed as the number of cells invaded through the GFR Matrigel<sup>TM</sup> Matrix and membrane relative to the migration through the control membrane.

### **2.13 Motility/migration assay**

Membrane chambers with a pore size of 0.8  $\mu\text{m}$  (BD bioscience 353097) were used to assess motility. Limbal fibroblast-like cells were seeded into the upper chamber of the membranes at a density of 8000 per well in 100  $\mu\text{l}$  DMEM with 5% serum and allowed to attach for 4 hours. A volume of 600  $\mu\text{l}$  DMEM 5% FBS was added to the lower chambers (6 replicates). The cells were incubated for 24 hours at 37<sup>0</sup> C, 5% CO<sub>2</sub> atmosphere to permit motility.

After incubation, inserts were washed in PBS and the cells were carefully removed from the inner insert. The cells that are passed through pores were stained with 0.25% crystal violet stain for 10 min. The settled cells in the upper wells were removed using cotton swabs, and washed with PBS and air-dried. From stained cells, crystal violet is eluted with 33% glacial acetic acid and measured the stain at 570nm absorbance. Data was presented as absorbance rate compared with positive control cells BT20 and negative control MCF-7.

## 2.14 Senescence-associated $\beta$ -Galactosidase assay

The senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) assay is based on a senescence-induced increase in levels of lysosomal  $\beta$ -galactosidase (Dimri and Campisi, 1994, Dimri *et al.*, 1995). In nonsenescent cells, the lysosomal hydrolase  $\beta$ -galactosidase cleaves galactose from glycoproteins at an optimum pH of 4.0 to 4.5. Lysosomal  $\beta$ -galactosidase activity can be detected in most mammalian cells by performing a cytochemical assay at pH 4.0 in which cleavage of Xgal by  $\beta$ -galactosidase leads to the formation of a blue precipitate (Kurz *et al.*, 2000).

The number of senescent cells were assessed using a senescence  $\beta$ -galactosidase staining Kit (Cell Signalling #9860). Cells were seeded in 6 well plates and cultured for 48 hours. Growth media was removed from cultures and washed with 1 X PBS, then cells were fixed with 1 X fixative solution (20% formaldehyde, 2% glutaraldehyde) for 10-15 minutes at room temperature. While plates were in fixative solution, staining solution was prepared. Using a polypropylene plastic tube, 930  $\mu$ l of the staining solution, 10  $\mu$ l of staining supplement A and B, and 50  $\mu$ l of 20 mg/ml X-gal in DMF (N-N-dimethylformamide) were added. Cells were washed with 1 x PBS after fixation, and 1 ml of staining solution mix added and incubated at 37<sup>0</sup>C for overnight. The development of a perinuclear blue colour was indicative of senescent cells. Cell staining was observed using a standard light microscope, and counted total number of cells, stained cells in 6 fields.

The percentage of senescent cells was obtained by dividing the average number of senescent associated- $\beta$ -gal-positive cells per field by the average total number of cells per field and

multiplying the result by 100. This calculation yields the percentage of senescent cells in a given tissue sample. A graph was plotted with the number of cells, number of blue cells, and percentage of senescence cells in culture.

## **2.15 Extra cellular matrix adherence assay for limbal ‘fibroblast-like’ cells**

### **2.15.1 Reconstitution of ECM proteins**

Collagen IV (BD/ 354233) was diluted to a working stock of 0.63 mg/ml in 0.05N HCl, fibronectin (FN) (Sigma/F2006) was diluted to working stock of 500 $\mu$ g/ml in sterile PBS, and laminin (BD/ 354232) was diluted to working stock of 1.64 mg/ml in serum free basal medium (DMEM).

### **2.15.2 *In vitro* cell adhesion assay**

Limbal fibroblast-like cells were examined for different extracellular matrix, 60 minutes time with 10 $\mu$ g/ml, collagen IV, Fibronectin (FN), Laminin, and combinations of Collagen IV+ FN, Collagen IV+Laminin and Collagen IV+Laminin+ FN.

The protocol was adapted from Hague et al, (Hague and Jones, 2008). Different ECMs with combination of ECMs were placed on 96 well plate lids and incubated at room temperature for 1 hour and the plates washed briefly with PBS. The wells were blocked with 0.1% heat inactivated bovine serum albumin (BSA) for 2 hours at 37<sup>0</sup>C, in a humid chamber (Incubator) and blocked empty wells act as a control. Trypsinised cells are washed twice in serum free media and plate 3x10<sup>4</sup> viable cells per circle in 80 $\mu$ l. Cells were left to adhere for 60 minutes at 37<sup>0</sup>C in a humidified CO2 incubator. After 60 minutes incubation, the cells were washed with PBS. Cells were fixed with 4% Para formaldehyde for 20 minutes at room temperature. The cells were stained with 0.1% crystal violet overnight, excess stain was washed by dipping the plates in distilled water and the plates were then air-dried.

Stained cells are counted under the microscope for four fields going across the well in a methodical fashion- not randomly, because the seeding is never random- there is always edge effect. A graph was plotted with ECMs versus cell number.

## **2.16 *In Vitro* differentiation of limbal fibroblast-like cells (LFLc)**

To determine the potential of LFL cells to differentiate into adipogenic and osteogenic lineages in comparison to bone marrow mesenchymal cells, a human mesenchymal stem cell Functional Identification Kit (R&D systems: SC006) was used.

### **2.16.1 Differentiation of LFLc to adipocytes**

#### **2.16.2 Preparation of adipogenic differentiation medium**

The human mesenchymal stem cell functional identification Kit contained Adipogenic Supplement (Part # 390415) - 0.5 mL of a 100X concentrated solution containing hydrocortisone, isobutylmethylxanthine, and indomethacin in 95% ethanol. This supplement was prepared to 1 x concentration with  $\alpha$ -MEM Basal Medium with 10% FBS and stored at 2-8°C, up to one month.

### **2.16.1 Differentiation of LFLc to adipocytes**

#### **2.16.2 Preparation of adipogenic differentiation medium**

The human mesenchymal stem cell functional identification Kit contained adipogenic supplement (Part # 390415) - 0.5 ml of a 100X concentrated solution containing hydrocortisone, isobutylmethylxanthine, and indomethacin in 95% ethanol. This supplement was prepared to a 1 x concentration with  $\alpha$ -MEM Basal Medium with 10% FBS and stored at 2-8°C for up to one month.

### **2.16.3 Preparation of culture plates and culture protocol**

1. Coverslips were sterilised with hot air over 160<sup>0</sup>C for 4 hours. Sterile coverslips were placed into each well of a 24-well plate.
2. As each well is approximately 1.76 cm<sup>2</sup> a seeding density of 2.1x10<sup>4</sup> cells/cm<sup>2</sup> was required, therefore 3.7 x 10<sup>4</sup> cells in a 0.5 ml of  $\alpha$ -MEM with 10% FBS were added to each well. Cells were incubated overnight in a 37° C, 5% CO<sub>2</sub> incubator.

*Note: Cells should be 100% confluent after overnight incubation. If they are not confluent, replace medium every 2 - 3 days with  $\alpha$ -MEM Basal Medium until 100% confluency is reached*

3. When the cells were 100% confluent. The medium in each well was replaced with 0.5 ml of adipogenic differentiation medium to induce adipogenesis.
4. Adipogenic differentiation media was replaced (0.5 mL/well) every 3 - 4 days. After 5 - 7 days, lipid vacuoles started to appear in the induced cells.

*Note: The adipogenic cells are fragile; medium replacement should be performed gently so as not to disrupt the lipid vacuoles. The appearance of vacuoles can be monitored by microscopic examination. Cover slips may be removed for Oil Red staining*

5. After 7 - 21 days, induced cultures were fixed and saved for immunostaining

### **2.16.4 Fixation and staining adipocytes for immunofluorescence**

1. Wash the cells twice with 1 ml of PBS.
2. Fix the cells with 0.5 ml of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells three times with 0.5 ml of 1% BSA in PBS for 5 minutes.

4. Permeabilise and block cells with 0.5 ml of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, dilute the reconstituted goat anti-mouse FABP-4 antibody in PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 µg/mL.

*Note: A negative control should be performed using PBS containing 1% BSA and 10% normal donkey serum with no primary antibody.*

6. After blocking, incubate cells with 300 µl/well of goat anti-mouse FABP-4 antibody working solution overnight at 2 - 8° C.
7. Wash the cells three times with 0.5 ml of PBS containing 1% BSA for 5 minutes.
8. Dilute the secondary antibody according to the manufacturer's suggestion in PBS containing 1% BSA.
9. Incubate the cells with secondary antibody at 300 µl/well in the dark for 60 minutes at room temperature.
10. Wash the cells three times with 0.5 ml of 1% BSA in PBS for 5 minutes.
11. Carefully remove the cover slips with forceps and mount cell side down onto a drop of mounting medium on a glass slide.

#### **2.16.5 Fixation and staining procedure – Oil Red O stain**

##### **A) Reagents preparation**

1. Oil Red O Stock: (Sigma O-0625), FW 408.5. Weigh 0.35 g Oil Red O and put in 100 ml of isopropanol. Stir O/N, filter (0.2 µ) and store at RT.
2. Oil Red O Working Solution: Mix 6 ml of Oil Red O stock solution with 4 ml of UHP water. Store at room temperature for 20 min followed by filtering (0.2 µm).

3. 10% Formalin in PBS: dilute 27 ml of formalin stock solution (37%, Merck, Cat# K36658003) in 63 ml of UHP H<sub>2</sub>O and 10 ml of 10X PBS.
4. 100% Isopropanol (Merck, K36543834)
5. 60% Isopropanol: Mix 6 ml of 100% Isopropanol with 4 ml of UHP H<sub>2</sub>O.

#### **B) Fixation and staining of adipogenic cultures**

Remove cells from incubator and place in the fume hood. All procedures involving formalin must be performed in a fume hood.

1. Aspirate the media and gently rinse (twice) the well with 500 µl of PBS along the sides of each well so as not to disturb the monolayer.
2. Aspirate the PBS and add 500 µl of 10% formalin along the sides of each well of the plate, again being careful not to disrupt the cells, and incubate the plate for 30 at room temperature.
3. Slightly tilting the plate, remove the formalin from the sides of each well and discard the formalin into a designated formalin waste receptacle. Gently add 500 µl of PBS to each well to rinse once. Remove the PBS and discard in the formalin waste receptacle.
4. Add 500 µl of 60% isopropanol to cover the bottom of each well and let sit for 2-5 minutes.
5. Pour off the isopropanol and add 500 µl of the working solution of Oil Red O along the side of each well so that the cells are completely covered.
6. Slowly rotate the dish to spread Oil Red O evenly over the cells and then let stand for 15 minutes.
7. After incubation, remove Oil Red O stain and rinse with PBS by adding at the edge of each well until the PBS runs turn out clear (approximately 3 washes). Be sure not to disrupt the monolayer during wash step.

8. Add 500  $\mu$ l of the hematoxylin counterstain into each well so that the cells are completely covered and let stand for 1 minute. (This is optional step).
9. Remove the hematoxylin and rinse the well with PBS (twice) as in the above steps.
10. Remove cover slip from well and mount on slide with mounting media (1% glycerine in PBS)

## **2.17 Differentiation of LFLC to osteocytes**

### **2.17.1 Preparation of osteogenic differentiation medium**

The human mesenchymal functional identification kit contained an osteogenic supplement (Part # 390416) - 2.5 mL of a 20X concentrated solution containing dexamethasone, ascorbate-phosphate, and  $\beta$ -glycerolphosphate, this supplement is prepared to 1x concentration in  $\alpha$ -MEM Basal Medium with 10% FBS and stored at 2-8<sup>0</sup>C for up to 1 month.

### **2.17.2 Preparation of culture plates and culture protocol**

1. Coverslips were sterilised with hot air over 160<sup>0</sup>C for 4 hours. Sterile coverslips were placed into each well of a 24-well plate.
2. As each well is approximately 1.76 cm<sup>2</sup> a seeding density of 2.1x10<sup>4</sup> cells/cm<sup>2</sup> was required, therefore 3.7 x 10<sup>4</sup> cells in a 0.5 ml of  $\alpha$ -MEM with 10% FBS was added to each well. Cells were incubated overnight in a 37° C and 5% CO<sub>2</sub> incubator.
3. Each well is approximately 1.76 cm<sup>2</sup> and requiring 3.7 x 10<sup>4</sup> cells/well. Seed cells at a density of 2.1 x 10<sup>4</sup> cells/cm<sup>2</sup>.
4. At 50 - 70% confluency, replace the medium in each well with 0.5 ml of osteogenic differentiation medium to induce osteogenesis.
5. Replace with 0.5 ml of fresh osteogenic differentiation medium (0.5 ml/well) every 3 - 4 days.
6. After 14 - 21 days (or when cells start to detach), osteocytes can be fixed in 4% paraformaldehyde and saved for immunostaining.

### **2.17.3 Fixation and staining osteocytes for immunofluorescence**

1. Wash the cells twice with 1 ml of PBS.
2. Fix the cells with 0.5 ml of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells three times with 0.5 ml of 1% BSA in PBS for 5 minutes.
4. Permeabilise and block cells with 0.5 ml of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, dilute the reconstituted mouse anti-human osteocalcin antibody in PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 µg/ml.
6. After blocking, incubate cells with 300 µL/well of mouse anti-human osteocalcin antibody working solution overnight at 2 - 8° C.
7. Wash the cells three times with 0.5 ml of PBS containing 1% BSA for 5 minutes.
8. Dilute the secondary antibody according to the manufacturer's suggestion in PBS containing 1% BSA.
9. Incubate the cells with secondary antibody at 300 µl /well in the dark for 60 minutes at room temperature.
10. Wash the cells three times with 0.5 ml of 1% BSA in PBS for 5 minutes.
11. Carefully remove the cover slips with forceps and mount cell side down onto a drop of mounting medium on a glass slide.

#### **2.17.4 Fixation and staining for Alizarin Red S**

##### **A) Preparation of Alizarin Red S solution**

Weigh 2 gm of Alizarin Red S (Sigma: A5533) and dissolve in 100 ml of UHP water to make 2% alizarin red S. Mix well and adjust the pH to 4.1~4.3 with 10% ammonium hydroxide. The pH is critical, so make fresh or check pH if the solution is more than one month old.

##### **B) Alizarin staining**

1. After 21 days under differentiating condition, remove media from 24 well plate and rinse once with PBS for twice. Fix cells with 4% formaldehyde solution for 30 minutes.
2. After fixation, rinse wells twice with PBS and stain cells with 2% Alizarin Red S solution (pH 4.2) for 2 to3 minutes.
3. Rinse wells three times with PBS, and mount cover slip with 1% glycerin in PBS.

## **2.18 Molecular biology techniques**

### **2.18.1 RNA isolation from cells by TriReagent**

Approximately  $1 \times 10^7$  cells were trypsinised, washed once with PBS, pelleted and lysed using 1 ml of TriReagent (Sigma, T-9424). The samples were allowed to stand for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes and then stored at  $-80^{\circ}\text{C}$ . When required, the sample was allowed to stand at room temperature for 5-10 min. To this 0.2 ml of chloroform per ml of TriReagent was added. Samples were shaken vigorously for 15 sec. and allowed to stand for 15 min at room temperature. The resulting mixture was centrifuged at 9,260 G or 13,000 rpm in a microfuge for 15 min at  $4^{\circ}\text{C}$ . The colorless upper aqueous phase (containing RNA) was removed into a fresh RNase-free 1.5 ml eppendorf tube. To this 0.5 ml of ice-cold isopropanol (Sigma, I9516) was added. The samples were mixed and incubated at room temperature for 5-10 min. The eppendorf tubes were then centrifuged at 7,890 G or 12,000 rpm for 30 min at  $4^{\circ}\text{C}$  to pellet the precipitated RNA. Taking care not to disturb the RNA pellet, the supernatant was removed and the pellet was subsequently washed by the addition of 750  $\mu\text{l}$  of 75% of ethanol and vortexed. Centrifugation was followed at 7,500 rpm for 5 min at  $4^{\circ}\text{C}$ . The supernatant was removed and the wash step was repeated. The RNA pellet was then allowed to air-dry for 5-10 min and subsequently was re-suspended in 15  $\mu\text{l}$  to 25  $\mu\text{l}$  of RNase free water. To facilitate dissolution repeated pipetting was done.

## 2.18.2 RNA quantification using NanoDrop

The NanoDrop ND-1000 is a full-spectrum (220-750nm) spectrophotometer that measures 1  $\mu$ l samples with high accuracy and reproducibility. It uses a sample retention technology that relies on surface tension alone to hold the sample in place eliminating the need for cuvettes and other sample containment devices. In addition, the NanoDrop has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard cuvette spectrophotometer).

To quantify an RNA sample, 1 $\mu$ l of the sample is pipetted onto the end of a fibre optic cable (the receiving fibre, **figure 2.18A**). A second fibre optic cable (the source fibre, **figure 2.18 B**) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. The gap is controlled to a 1mm path **figure 2.18C**. A pulsed xenon flash lamp provides the light source and a spectrometer utilising a linear CCD array is used to analyse the light after passing through the sample. The instrument is controlled by special software run from a computer, and the data is logged in an archive file on the computer.

When measurement of the sample is complete, the sample can be simply wiped away using a soft laboratory wipe. This is sufficient to prevent sample carryover because each measurement pedestal is a highly polished end of a fibre optic cable, with no cracks or crevices for leftover sample to reside.

**Figure 2.18:** Samples are quantified by loading 1µl onto the receiving fibre (A), the source fibre, connected to the sampling arm (B) is brought down into contact with the sample allowing a 1mm gap between the upper and lower pedestal (C), through which the light is passed. (Pictures adapted from ND-1000 Spectrophotometer users manual V 3.1.0).



This was typically in the range of 1.8-2.0. A ratio of <1.6 indicated that the RNA may not be fully in solution. The RNA was diluted to 1µg/µl stocks for the subsequent reverse transcription (RT) protocol (Section 2.19).

## 2.19 REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

### 2.19.1 Reverse transcription of RNA from limbal-corneal epithelial cells (cDNA Synthesis)

mRNA was copied to cDNA by reverse transcriptase using an oligo dT primer. First-strand cDNA was synthesised by reverse transcription (RT) by mixing the following components in a 0.5 ml eppendorf tube (Eppendorf, 0030 121 023) and heated at 70°C for 10 min followed by cooling on ice. **Table 2.19** shows the protocol for preparation cDNA from limbal-corneal epithelial cells RNA.

**Table: 2.19 first strand cDNA from cell RNA**

Volume	Reagents
1 $\mu$ l	Oligo dT (0.5 $\mu$ g/ $\mu$ l) (MWG)
1 $\mu$ l	RNA (1 $\mu$ g/ $\mu$ l)
3 $\mu$ l	Nuclease free water

This step gets rid of RNA secondary structures and allows the oligo dT to bind the poly (A)<sup>+</sup> tail of the RNA. While this mixture was incubated, the following reaction mix was generated (all volumes listed in master mix assume 1  $\mu$ g total RNA)

**Table : 2.19.1 Reverse transcription reaction mixture**

Volume	Reagent
2 $\mu$ l	10X buffer (Sigma, B8559)
1 $\mu$ l	RNasin (40U/ $\mu$ l) (Sigma, R2520)
1 $\mu$ l	dNTP (deoxynucleotide triphosphate) (10mM of each dNTP) (Sigma, DNTP100)
10 $\mu$ l	Nuclease free water
1 $\mu$ l	MMLV-RT (200 U/ $\mu$ l) (Sigma, M1302)
15 $\mu$ l	Total

Once the RNA mixture had cooled (~2 min) 15  $\mu$ l of the master mix **table 2.19.1** was added and mixed by flicking. The mixture was centrifuged to collect the material in the bottom of the

tube and then incubated at 37°C for 1hr. The resultant cDNA was stable overnight at 4°C but for prolonged storage, it was maintained at -20°C.

### 2.19.2 Polymerase chain reaction (PCR)

The cDNA was analysed for the expression of genes of interest by PCR. The standardised PCR mix is listed below and did not change with any of the PCRs carried out in this thesis. 2.5 µl cDNA was added to the following reaction mixture **table 2.19.2**. The samples were mixed and centrifuged before being placed on the thermocycler (Biometra).

Volume	Reagents
12.25 µl	Nuclease free water
2.5 µl	10X PCR buffer (Sigma, P2317)
1.5 µl	25 mM MgCl <sub>2</sub> (Sigma, M8787)
4 µl	1.25 mM dNTP
0.5 µl	each of the forward and reverse primers (250 ng/µl) for the target gene (MWG)
0.25 µl	Taq Polymerase (5 U/µl) (Sigma, D4545).
20 µl	<b>Total</b>

**Table: 2.19.2 PCR reaction mixture**

A typical PCR protocol is outlined below. However, annealing temperatures can vary from primer set to primer set.

Step	Temperature Hold	Time	Cycle
Denature	95°C	3 Minutes	
Denature	95°C	15 Seconds	} * 35 Cycles
Annealing	52-62°C	30 Seconds	
Extension	72°C	30 Seconds	
Final Extension	72°C	7 Minutes	

PCR products were stored at 4°C until they were analysed by gel electrophoresis.

\* 35 cycles were sufficient when amplifying gene transcripts from cell RNA.

### 2.19.3 Primers for limbal-corneal epithelial cells

Primers designs were done using Primer Express from Applied BioSystems (<http://www.appliedbiosystems.com>) and the primers were ordered from MWG (<http://www.mwg-biotech.com/>). The primers were designed across the introns, making them specific for detection of RNA. Primers with Gene ID, annealing temperature, product size in **table 2.19.3**.

**Table 2.19.3** List of primer sequence with gene ID, annealing temperature, and PCR product size

Gene/ID	Primer sequence	Annealing T <sub>m</sub> in °C	DNA Fragment Size (bp)
<b>CK12/ NM_000223.3</b>	F: GAAGAAGAACCACGAGGATG R: TCTGCTCAGCGATGGTTTCA	54	146
<b>CK3/ NM_057808</b>	F: CGTACAGCTGCTGAGAATGA R: CTGAGCGATATCCTCATACT	53	261
<b>ΔNp63/ NM_001114980 .1</b>	F: CTGGAAAACAATGCCAGAC R: GGGTGATGGAGAGAGAGCAT	55	198
<b>ABCG2/ NM_004827.2</b>	F: GGTTTCCAAGCGTTCATTCAA R: TAGCCCAAAGTAAATGGCACCTA	54	112
<b>GAPDH/ NM_002046.3</b>	F: GCTCAGACACCATGGGGAAGGT R: GTGGTGCAGGAGGCATTGCTGA	61	472

#### 2.19.4 Gel electrophoresis of PCR products

Typically, 2% agarose (Sigma, A9539) gels were used for analysis of PCR products. The gels were prepared and run in 1X TBE (10.8 g Tris base, 5.5 g Boric Acid, 4 ml 0.5 M EDTA (pH 8.0) and made up to 1 L with UHP) and melted in a laboratory microwave. Upon cooling, the gel was supplemented with ethidium bromide (10 mg/ml) to allow visualisation of the DNA. Ethidium bromide is a dye that binds to double stranded DNA by interpolation (intercalation) between the base pairs. Here it fluoresces when irradiated in the UV part of the spectrum. The gel was then poured in to the electrophoresis unit (Biorad) and allowed to set. Sample wells were formed by placing a comb into the top of the gel prior to hardening.

To run the samples, 2 µl of 6X loading buffer (50% Glycerol, 1 mg/ml bromophenol blue, 1 mM EDTA) was added to 10 µl PCR product and the mixture was loaded to the gel with an appropriate size marker (Sigma, D0672). The gels were electrophoresed at 120-150 V for 1 - 2hrs. (Depending on size of the target gene, *i.e.* to get adequate separation). Once the internal control and target bands have migrated to the required extent, the gel was taken to the gel analyzer (an EpiChemi II Darkroom, UVP Laboratory Products).

## **2.20 Quantitative real-time polymerase chain reaction (qRT-PCR)**

Real-time PCR was mainly used to provide quantitative measurements of gene transcription. The technology was used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions.

TaqMan probes are oligonucleotides that have fluorescent reporter dyes attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent dye thus, increasing the fluorescence in each cycle, proportional to the amount of probe cleavage.

RNA was isolated (**Section 2.20.1**) and cDNA synthesised as described in **section 2.20.2**. In order to exclude any amplification product derived from genomic DNA or any other contaminant that could contaminate the RNA preparation, total RNA without reverse

transcription was used as a negative control. Water on its own was amplified as a negative control to rule out presence of any contaminating RNA or DNA in it.

### **2.20.1 Preparation of total RNA from cells using RNeasy Mini Prep Kit**

High quality RNA was isolated from cells using the RNeasy mini-kit (Qiagen, 74104). Cell pellets for RNA extraction (stored at  $-80^{\circ}\text{C}$ ) were re-suspended in 1.2 ml of buffer RLT (supplemented with  $10\mu\text{l/ml}$  of  $\beta$ -mercaptoethanol) and vortexed to loosen the pellets. The samples were completely homogenised by passing the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. One volume (1.2 ml) of 70% ethanol was added to the homogenised samples and mixed well by pipetting. This mixture was then loaded in  $700\mu\text{l}$  aliquots on to an RNeasy mini column, which was placed in a collection tube and centrifuged at  $8,000\times G$  for 15 sec (this was continued until the entire mixture had been passed through the column). Once all the homogenised cells had been passed through the column, the washes were carried out. Initially  $700\mu\text{l}$  RW1 was loaded on to the column and centrifuged at  $8,000\times G$  for 15 sec. This was closely followed by two washes in buffer RPE (also followed by centrifuging at  $3,507\times G$  or 8000 rpm for 15 sec). To completely dry the spin column, it was placed in a fresh collection tube and centrifuged at full speed for 1 min. The RNA was eluted by passing two lots of  $25\mu\text{l}$  RNase free water (supplied) through the column by centrifuging it at  $3,507\times G$  or 8,000 rpm for 1 min. The eluted RNA was then quantified as described in **section 2.18.2**.

## 2.20.2 High capacity RNA-to-cDNA

Total RNA was copied to cDNA by reverse transcriptase using high capacity RNA-to-cDNA Kit (Applied Biosystems, PN 4387406), preparation of RNA-to-cDNA protocol as follows:

1. Total 2 µg of RNA per 20µl reaction used.
2. Allow the kit components to thaw on ice.
3. RNA sample along with RT buffer, enzyme mix with required quantity of water is prepared per reaction as represented in below table.

Component	Component Volume/Reaction (µL)	
	+RT reaction	-RT reaction
2X RT Buffer	10.0	10.0
20X Enzyme Mix	1.0	-
RNA Sample	Up to 9 µl	Up to 9 µl
Nuclease-free H <sub>2</sub> O	To 20 µl (Quantity Sufficient)	To 20 µl (Quantity Sufficient)
<b>Total Per Reaction</b>	20.0	20.0

4. Aliquot RT reaction mix into 0.5ml tubes.
5. Briefly centrifuge the tubes to spin down the contents and to eliminate any air bubbles.
6. Incubate the reaction at 37<sup>0</sup>C for 60 minutes. Stop the reaction by heating to 95<sup>0</sup>C for 5 minutes and hold at 4<sup>0</sup>C in the thermal cycler.
7. The cDNA is ready for use in real-time PCR application. cDNA can be stored for long-term storage in a freezer (-15<sup>0</sup>C to-25<sup>0</sup>C).

### 2.20.3 Evaluation of cDNA

cDNA was quantified by using NanoDrop ND-1000 as described in **section 2.18.2**. The total of 100ng cDNA was used for real-time PCR for all samples per 20- $\mu$ l amplification reaction.

### 2.20.4 Preparation of PCR reaction mix

For each sample (in triplicate), pipet the following into a nuclease free 0.5-ml micro centrifuge tube:

PCR Reaction mix component	Volume 20 $\mu$ L -per Single reaction
20 $\times$ TaqMan <sup>®</sup> Gene Expression Assay	1.0
2 $\times$ TaqMan <sup>®</sup> Gene Expression Master Mix	10.0
cDNA template (100 ng)	4.0
RNase-free water	5.0

Transfer 20  $\mu$ l of PCR reaction mix into each well of 96 well reaction plates. Seal the plate with the appropriate adhesive cover, and centrifuge the plate briefly to eliminate any air bubbles then load the plate into the instrument. qRT-PCR primers and their assay ID used in this study for cultured limbal-corneal epithelial cells listed in **table 2.20**.

Primer Pair/ Gene Symbol	Gene Name	Assay ID	Supplier
KRT3	Keratin 3	Hs00365080_m1	Applied Biosystems
KRT12	Keratin 12	Hs00165015_m1	
TP63	Tumor protein p63	Hs00978340_m1	
ITGB4	Integrin, beta 4	Hs00236216_m1	
ITGA6	Integrin, alpha 6	Hs01041011_m1	
TJP1	Tight junction protein 1 (zona occludens 1)	Hs00268480_m1	
ACTB	Actin, beta	Hs99999903_m1	

**Table 2.20** qRT-PCR primers and their assay ID used for cultured limbal-corneal epithelial cells.

### 2.20.5 Experiment parameters for TaqMan<sup>®</sup> Gene Expression Assay

After the PCR reaction mix is ready in 96 well plates, a plate document/experiment was setup and PCR was run with below **table 2.20.1** values on Applied Biosystems 7500 Fast Real-Time PCR System (Fast).

Thermal Cycling Conditions		
Stage	Temp (°C)	Time (mm:ss)
<b>Hold</b>	95	0:20
<b>Cycle (40 Cycles)</b>	95	0:03
	60	0:30

**Table 2.20.1** Experiment parameter for TaqMan<sup>®</sup> Gene Expression Assay

## 2.20.6 qPCR-Data analysis

Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. The relative quantification method may be used to calculate relative changes in gene expression determined from real-time quantitative PCR experiment.

The choice of calibrator for the relative quantification method depends on the type of gene expression experiment (Livak and Schmittgen, 2001). In this experiment, the limbal-corneal epithelial cultures, condition A no-3T3 was used as the calibration sample to compare other conditions.  $\beta$ -actin was used as the endogenous control for this experiment. The purpose of the endogenous control gene is to normalize for the base line of gene expression in each sample. Using the relative quantification method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the condition A no-3T3 control.

The calculations of  $\Delta C_T$ ,  $\Delta\Delta C_T$  and RQ (relative quantification) were done using the following formulas.

$$\Delta C_T = C_T (\text{Gene}) - C_T (\text{Beta actin})$$

$$\Delta\Delta C_T = \Delta C_T (\text{Sample}) - \Delta C_T (\text{Calibrator sample, condition A no-3T3})$$

$$\text{RQ (Fold Change)} = 2^{-\Delta\Delta C_T}$$

Note- For RQ values  $<1$ , the reciprocal RQ value is taken to quantify negative fold change.

Relative quantification of gene expression was calculated in three biological replicate samples subject to six different treatment conditions (condition A, B, C, D, E and F). Technical triplicates were also performed for each biological replicate for each gene. Average  $C_T$  values from the three technical replicates were calculated for each biological replicate for each gene. The average  $C_T$  of each gene in each biological sample was normalized to endogenous control expression to generate the  $\Delta C_T$  value. The  $\Delta C_T$  was then normalized to the calibrator sample-condition A, to generate the  $\Delta\Delta C_T$  value.  $\Delta\Delta C_T$  values were then inputted into the equation  $2^{-\Delta\Delta C_T}$  to generate a value for relative quantification (RQ). RQ values  $>1.5$  indicate increased fold change relative to calibrator sample, while RQ values  $<1.5$ , indicate reduced expression relative to calibrator sample.

To determine fold of down-regulation, the reciprocal RQ value is calculated. Mean fold change is then graphed. Error bars in the graph show standard deviation of fold change between biological triplicate samples.

## **2.21 Statistical analysis**

The statistics applied to analyze the significance of our research findings in colony forming efficacy (CFE) were done by using a student t-test. The error bar in the graphs represents standard deviation (SD) or standard error mean (SEM) as mentioned in experiments where ‘n’ equal to greater than two, and ‘mean value’ was derived in experiments, where ‘n’ is equal to two. Number of biological samples used in experiments was notified in each experiment in this thesis.

## 3.0 Section

---

# Results

### 3.1 Standardisation of primary culture of human limbal-corneal epithelial cells

There are two main culture techniques available to culture limbal-corneal epithelial cells, the first is the plating the whole piece of limbal tissue or limbal explants, approximately 1 mm by 1mm size (Tsai *et al.*, 2000a, Tsai *et al.*, 2000b, Kolli *et al.*, 2008). The second is by enzymatically isolating the limbal epithelial cells from the limbal tissue (the suspension method). Enzymatic isolation can be performed either by using dispase to separate the limbal epithelium from the stroma or trypsinisation of the limbal epithelium to isolate single limbal epithelial cells (Pellegrini *et al.*, 1997, Koizumi *et al.*, 2002). The above mentioned methods have been used to culture limbal epithelial cells successfully, either on a 3T3 fibroblast feeder layers or on amniotic membrane. In some studies, explants growth is better than suspension growth and in other studies the opposite is found (Kim *et al.*, 2004, Zito-Abbad *et al.*, 2006).

A preliminary study was conducted for four primary culture techniques to assess which would be the most consistent in generating cell sheets with good morphology and marker expression.

The four methods investigated were:

- 1) **Enzymatic digestion of limbal tissue and isolated cells grown on irradiated 3T3 feeder layer.** In brief, limbal tissue was dissected from the corneo-scleral rim. The limbal tissue was then enzymatically digested with 0.25% trypsin for 1 hour at 37<sup>0</sup>C.

The separated single cells and small clumps were added to growth media to arrest trypsin activity, and cells were seeded onto 3T3 feeder cells.

- 2) **Tissue explants on tissue culture plates.** In this technique, the limbal tissue was dissected from the corneo-scleral rim, and limbal tissue was chopped into 2 to 3mm pieces. These 2-3 mm limbal tissue fragments were placed on moist 6 well inserts and allow settling for 15 minutes before adding growth media.
- 3) **Tissue Explants on tissue culture with a gelatine coating:** This culture method was also a modification of culture method 2; with the limbal explants added to inserts pre-coated with gelatine (0.025%).
- 4) **Tissue explants on tissue culture plastic with an irradiated 3T3 feeder layer.** This is a modification of culture method 2; limbal explants were plated on pre-coated irradiated 3T3 feeder ( $2.4 \times 10^4$  cells per  $\text{cm}^2$ ) layers.

### 3.1.1 Assessment of cell culture morphology and marker expression

The cultures were grown in culture media for 15-16 days, till a confluent cell sheet was observed. During the culture period cell growth and morphology was monitored with phase contrast microscopy.

The ideal limbal-corneal culture has been reported as one that is 3-5 cells thick, with a cobblestone-like cell morphology, positive for markers such as cytokeratin 3 (CK3), and cytokeratin 19 (CK19) (Koizumi *et al.*, 2001b, Koizumi *et al.*, 2002).

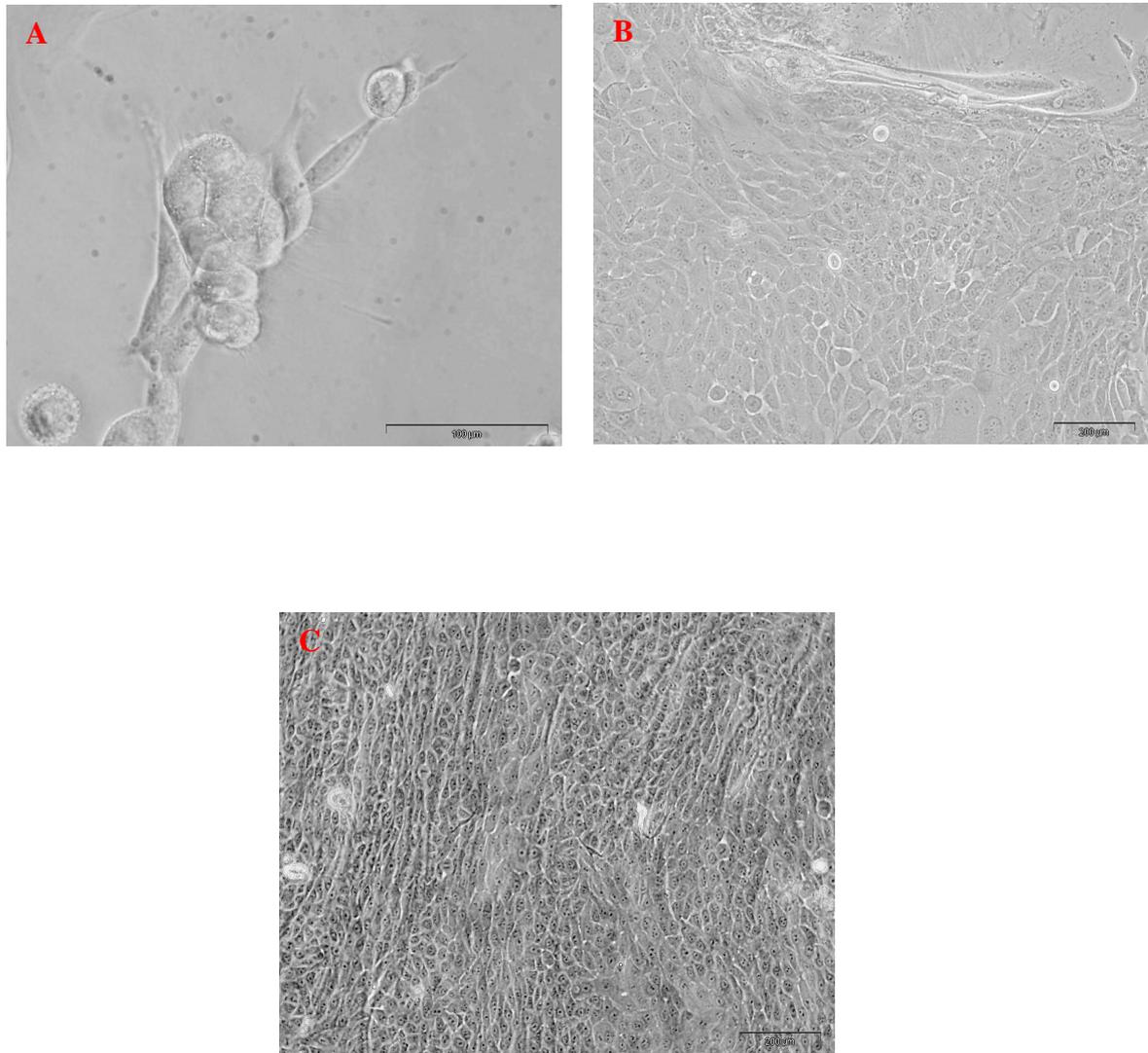
**Table 3.1** shows the success rates in generating primary cultures for each of the four techniques. Enzymatic digestion of limbal tissue generated only two cultures from six attempts. In contrast method 2; tissue culture plates, resulted in four primary cultures out of five attempts. Similarly method 3, tissue explants on tissue culture plastic with a gelatine coating, also resulted in four primary cultures and of five attempts. Method 4, tissue explants on tissue culture plastic with a 3T3 feeder layer, was marginally most successful in generating primary cultures, with all five attempts.

Culture method	Number of patient samples used	Number of patient samples grown
(1) Enzymatic digestion with 3T3 feeder layer	6	2
(2) Explants on tissue culture plates	5	4
(3) Explants on tissue culture plate with gelatine coating	5	4
(4) Explants on tissue culture plastic with 3T3 feeder layer	5	5

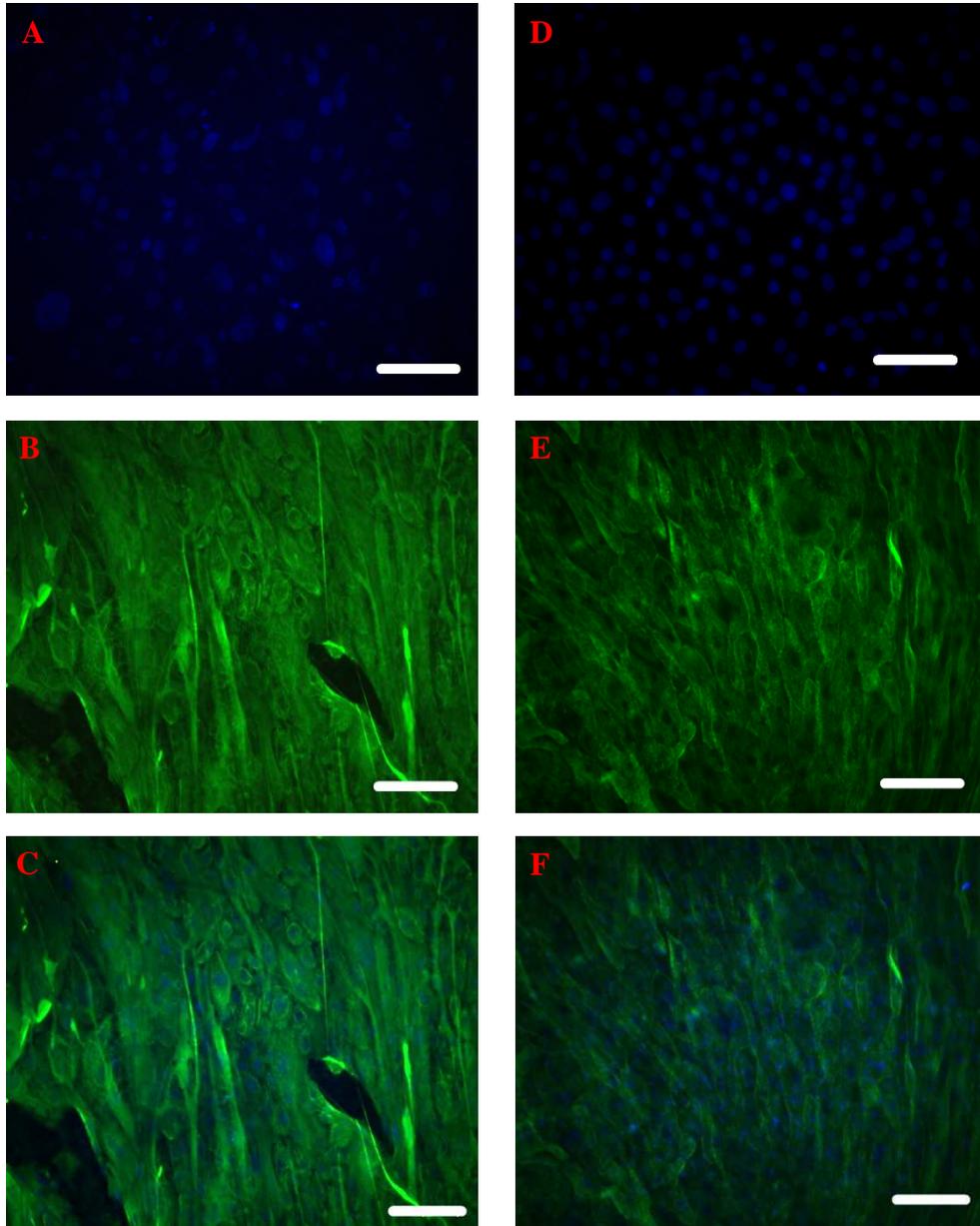
**Table- 3.1** Different culture methods used for culturing limbal-corneal stem cells

The typical morphology of primary cultures of limbal-corneal epithelial cells generated by method 1, enzymatic digestion of limbal tissue, with isolated cells grown on an irradiated 3T3 feeder layer is shown in **figure 3.1**. Initially at day 2, a small cluster of attached epithelial cells are observed. By day 8 this cluster had proliferated to form a sheet of cells with a cobble-stone morphology. The cell sheet appeared to be advancing into the 3T3 feeder cells and was growing on the feeder cells **figure 3.1B**. At day 16 **figure 3.1C** the primary culture was confluent with a large number of cells with small cobble-stone morphology. The primary culture also appeared to be multilayered with cells showing squamous morphology on the apical layer. Immunofluorescence analysis of these primary cultures for the corneal epithelial markers CK3 and CK19 is shown in **figure 3.2**. The primary culture showed positive staining for both markers.

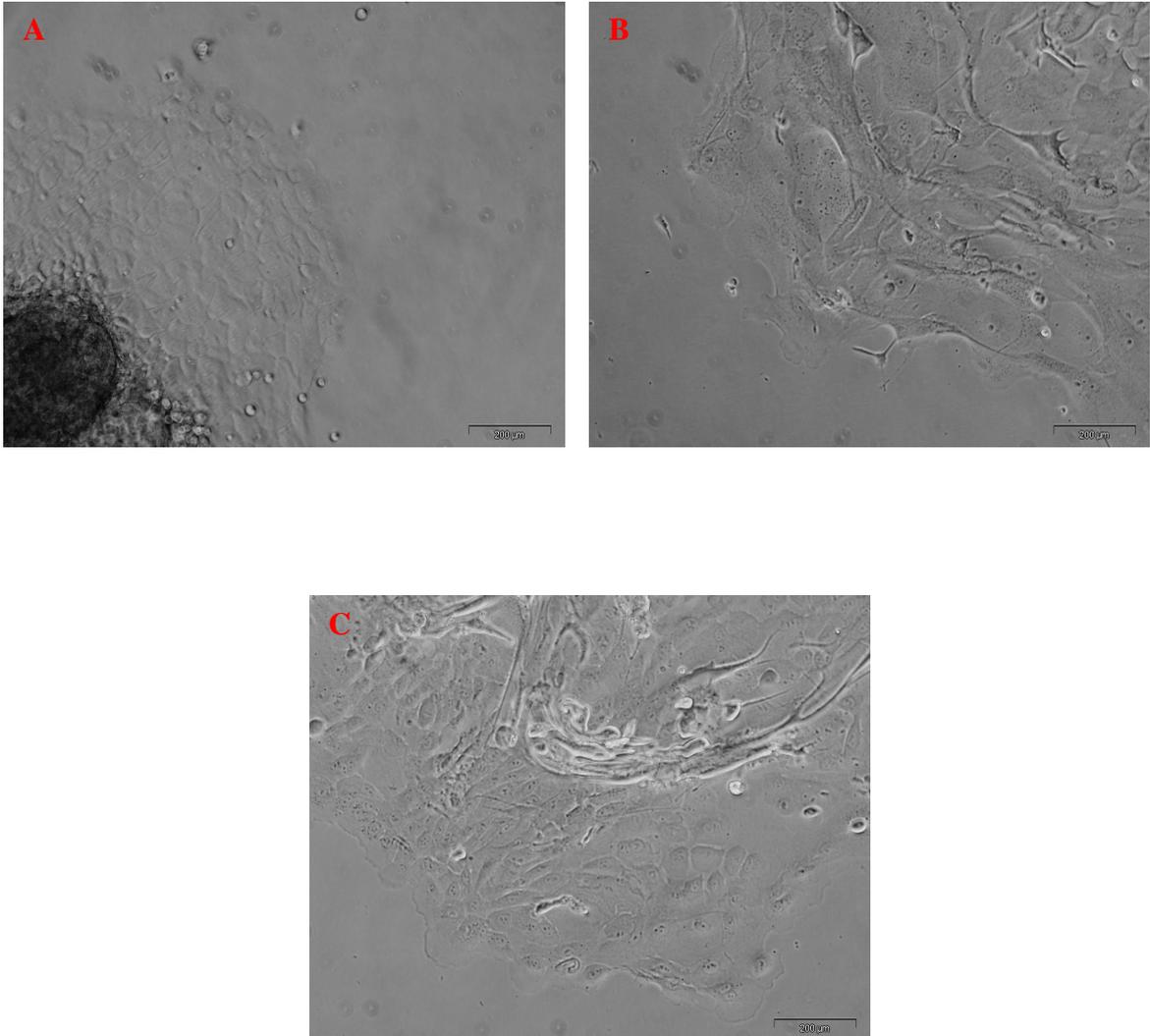
**Figure 3.3** shows the typical morphology of primary cultures of limbal-epithelial cells generated by method 2; tissue explants on cell culture plastic. Initially at day 2 small groups of epithelial cells can be observed growing out from the explants. At day 8 a cell sheet had formed with squamous type morphology. By day 16 the cell sheet covered 80-100% of the well. The culture was comprised predominantly of cells with large squamous type morphology and appeared to be monolayer. Un-measured observations suggested that these cultures had a slow growth rate. Immunofluorescence analysis of these primary cultures for the corneal epithelial markers CK3 and CK19 is shown in **figure 3.4**. The primary culture showed strong positive staining for both markers.



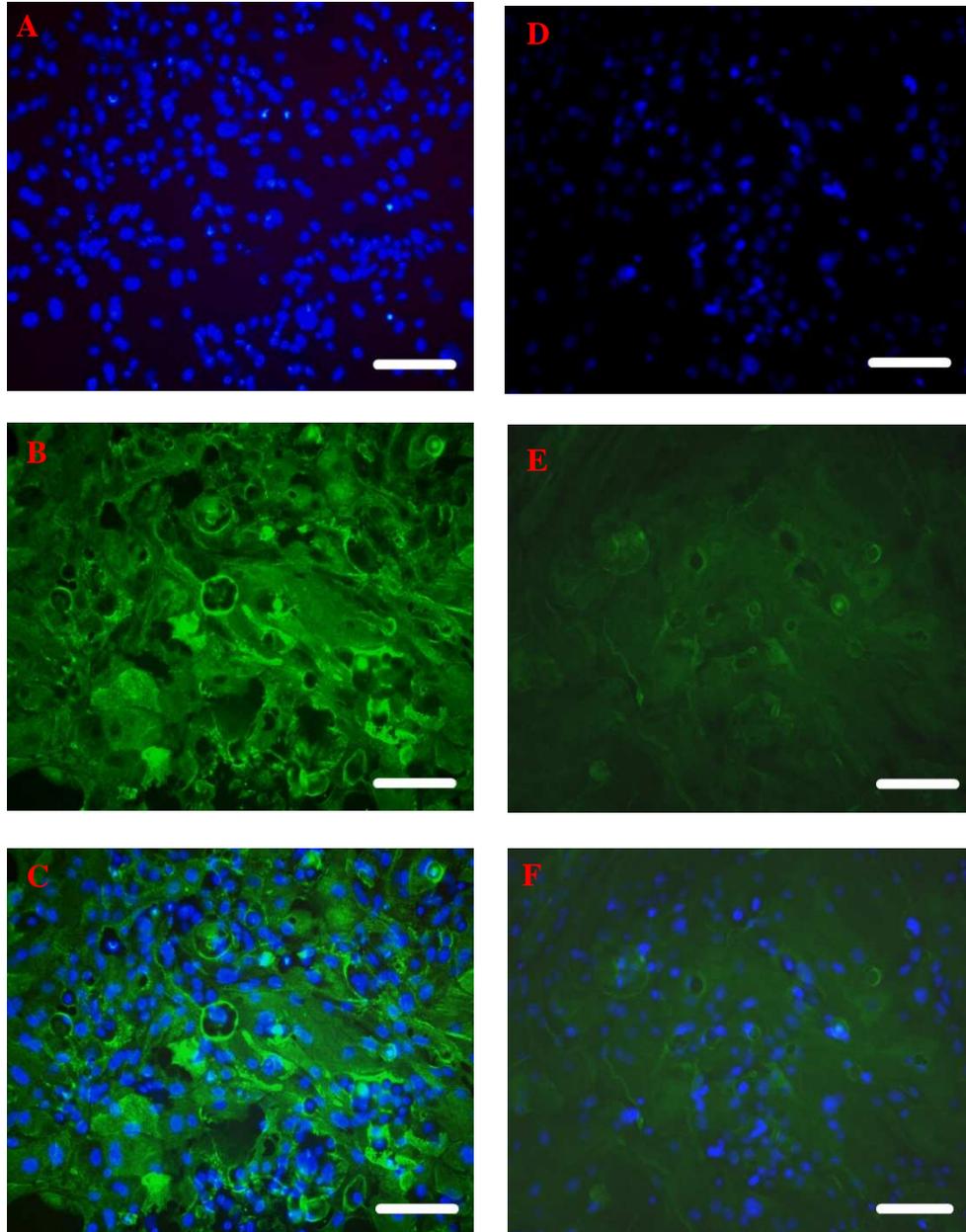
**Figure: 3.1** Phase contrast microscopic assessment of cell suspension culture on 3T3 feeder layer, limbal-corneal epithelial cells as early as day 2 (20x) (A), and cells further divided to form a monolayer by day 8 (10x) (B), a complete compact monolayer of cells at day16 (10x)



**Figure:3.2** Immunofluorescence staining images of CK3 and CK19, cell suspension culture on plastic with 3T3 feeder layer at day 16, DAPI stained nuclei (blue) (A and D) , CK3(green) (B) and CK19 (green) ( E), and merge images CK3 (C), CK 19 (F), Scale bars 100µm.



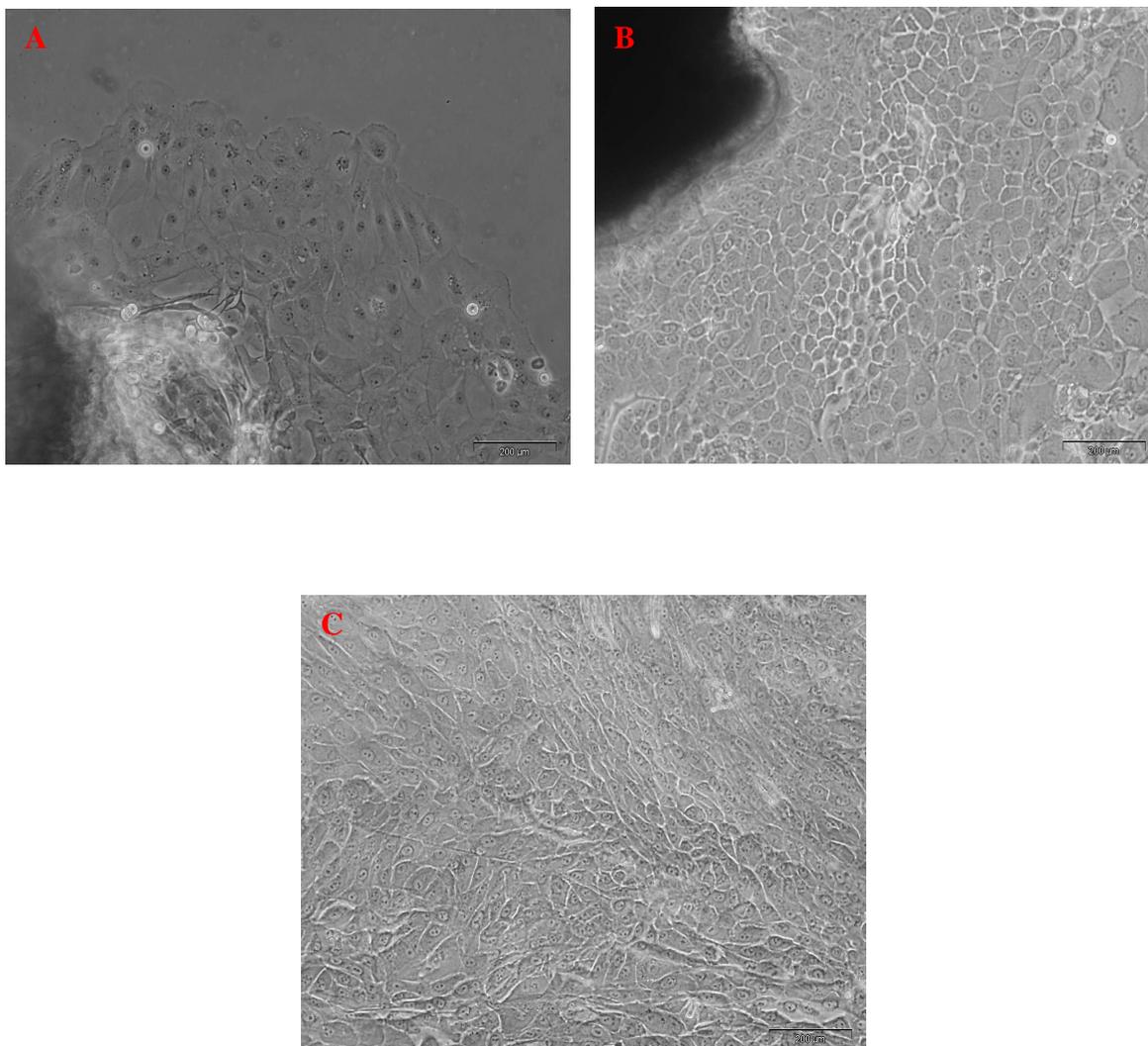
**Figure:3.3** Phase contrast microscopic assessment of explants cultured on tissue culture plastic, explant out growth at day 2 (A), the monolayer is spreading at day 8 (B). At day 16 the monolayer is spread-out with flattened cells and stretched cells on the top layer (C), Scale bar



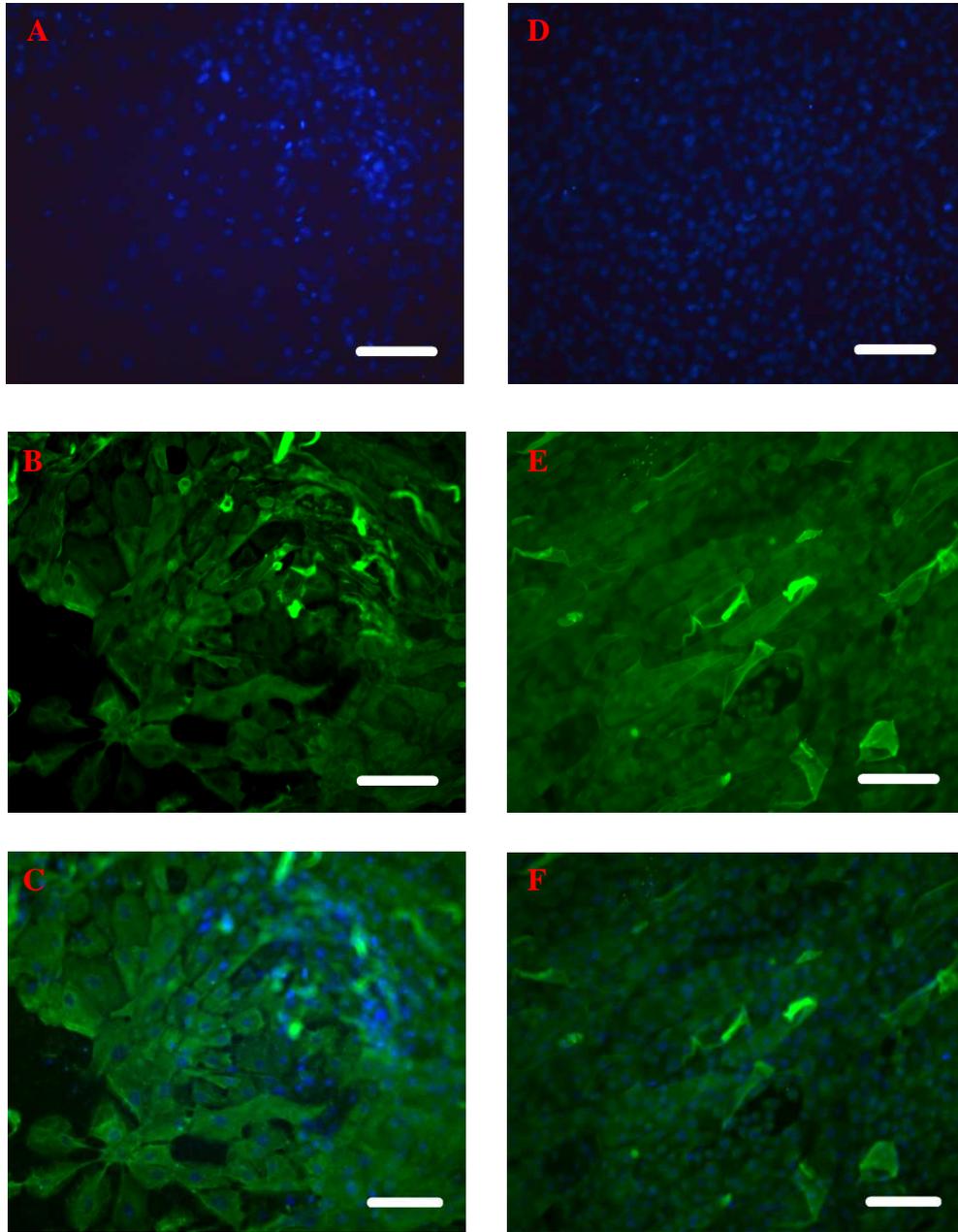
**Figure:3.4** Immunofluorescence staining images of CK3 and CK19 on explant cultures on tissue culture plates at day 16, DAPI stained nuclei (blue) (A and D) , CK3 (green) (B) and CK19 (green) (E), and merge images CK3 (C), CK 19 (F), Scale bars 100µm.

**Figure 3.5** represents limbal-corneal epithelial explants grown on gelatine coated plates, show cell out growth at day 2, with compact cell-cell boundaries and monolayer by day 8, the proliferative cells attained 2-3 cells thick by day 16, and cells were positive for corneal epithelial markers CK3 and CK19, **figure 3.6**.

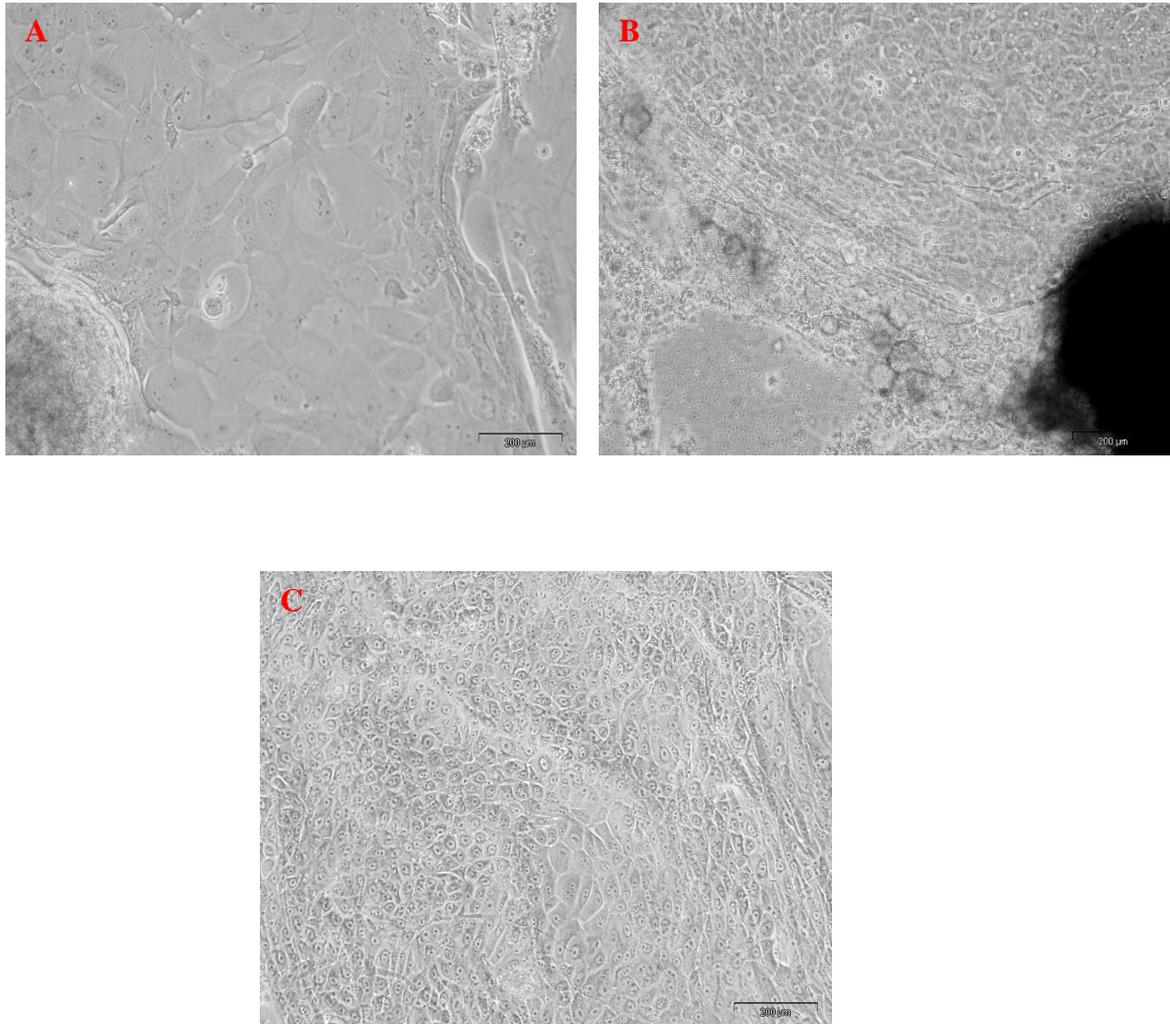
**Figure 3.7** represents the typical morphology of limbal-corneal epithelial cells generated using primary culture method. Tissue explants on tissue culture plastic with 3T3 feeder layer. At day 2 cells were observed growing from explants. These cells continued to grow forming cell sheets, with compact cuboidal cells. By day 16, 100% of the well was covered with a cell sheet. These cultures appeared to be multilayered, with cells having a cuboidal morphology on the bottom layer, and large squamous type cells on the apical layer. Immunofluorescence analysis was positive for both CK3 and CK19 **figure 3.8**.



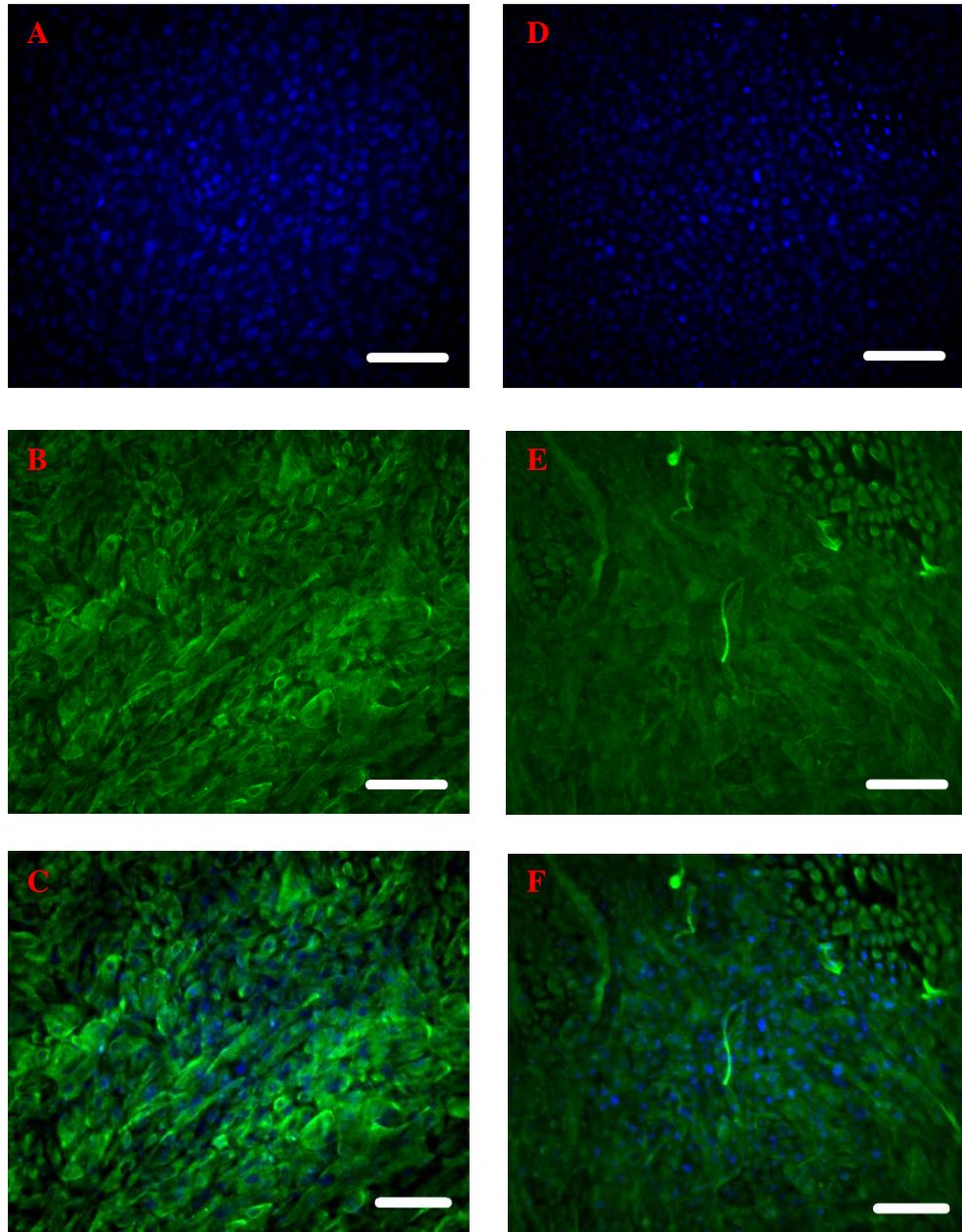
**Figure: 3.5** Phase contrast microscopic assessment of explants on gelatine coated plates, monolayer is started at day 2(A), and growing monolayer at day 8 (B), by day 16 a complete monolayer with compact cells is seen (C). Scale bar 200μm



**Figure: 3.6** Immunofluorescence staining images of CK3 and CK19 on explant cultures on gelatine coated plates at day 16, DAPI stained nuclei (blue) (A and D) , CK3(green) (B) and CK19 (green) (E), and merge images CK3 (C), CK 19 (F), Scale bars 100 $\mu$ m



**Figure: 3.7** Phase contrast microscopic assessment of explants on tissue culture plastic with 3T3 feeder layer (arrow), formation of monolayer at day 2 (A), and growing monolayer at day 8 (B), by day 16 a complete monolayer with compact cells (C). Scale bar 200μm



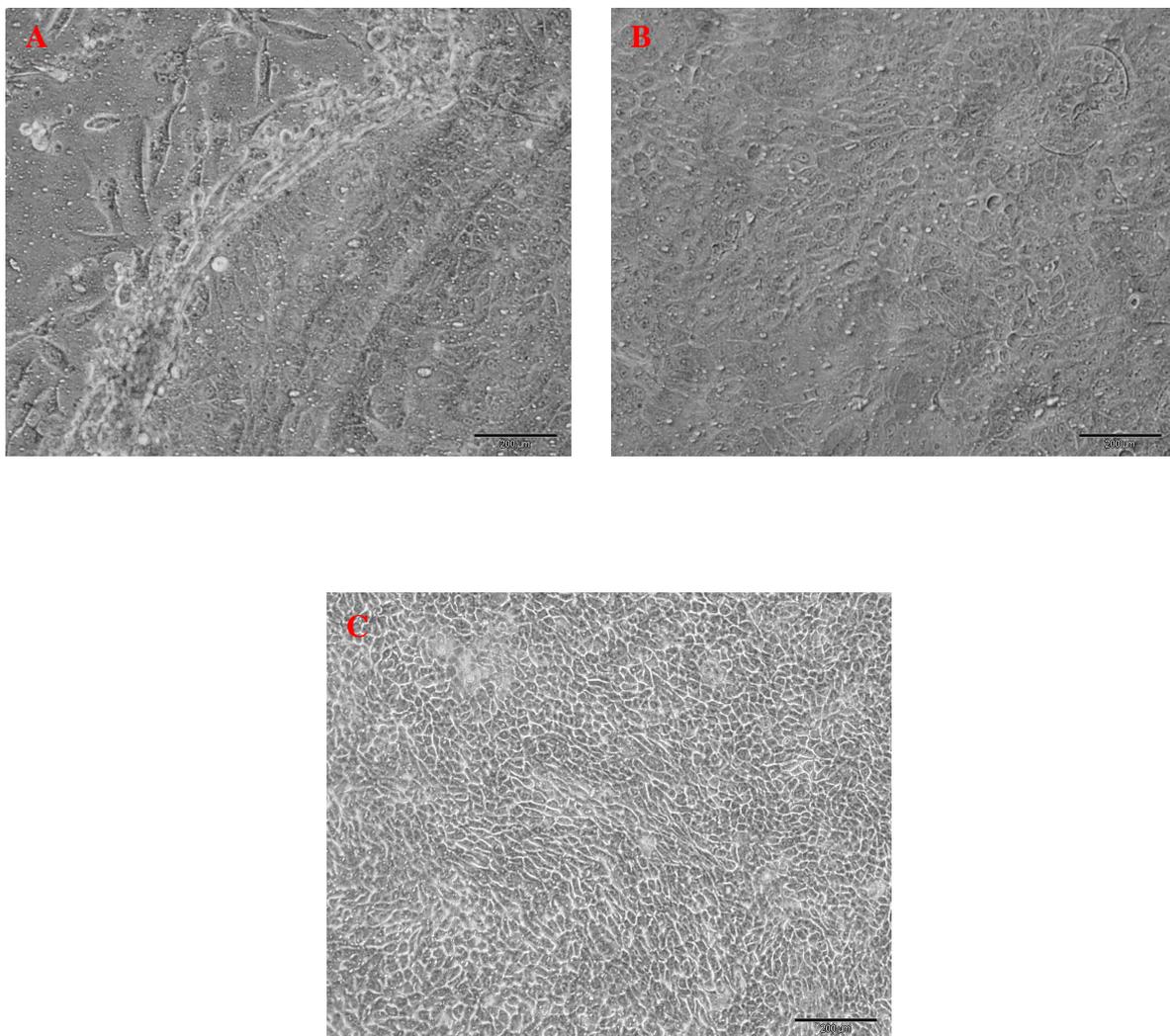
**Figure: 3.8** Immunofluorescence staining images of CK3 and CK19 on cell explants cultured with 3T3 feeder layer at day 16, DAPI stained nuclei (blue) (A and D) , CK3 (green) (B) and CK19 (green) (E), and merge images CK3 (C), CK 19 (F), Scale bars 100µm.

### **3.1.2 Explants culture on trans-well cell culture inserts with 3T3 feeder cells**

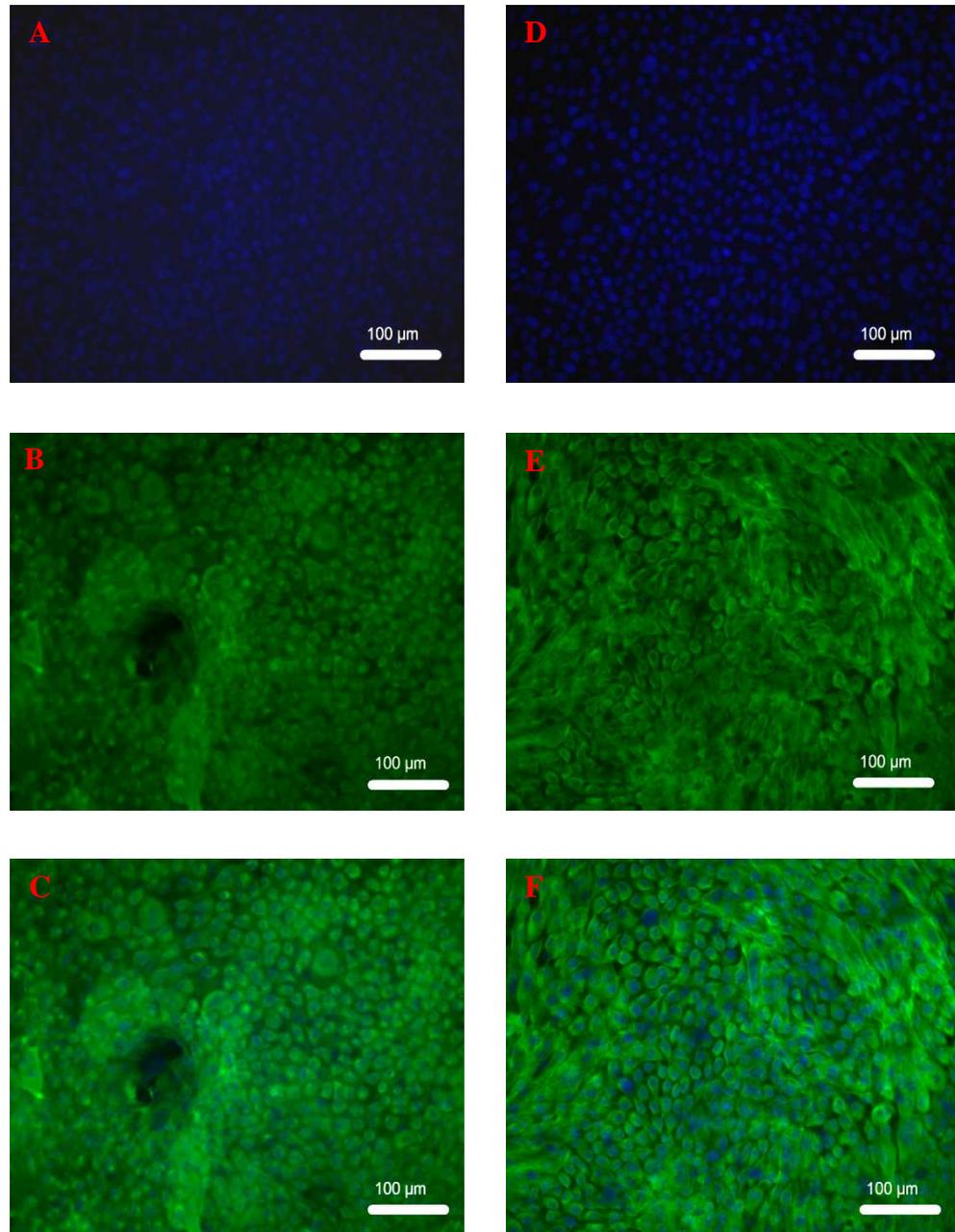
The generation of limbal-corneal epithelial cultures by method 4; tissue explants with 3T3 feeder layer was judged to be the most successful method. This was based on all five-culture attempts being successful, the small cuboidal cell morphology present in the cell sheet and staining for the corneal markers CK3 and CK19.

The exact role of feeder cells in the cultures of limbal-corneal epithelial cells is unknown. As a preliminary experiment, method 4- tissue explants with 3T3 feeder cells was adapted to a 0.4-micron trans-well insert. The cultures were characterised on morphology and expression of CK3 and CK19.

**Figure 3.9** shows the limbal-corneal epithelial cells grown on trans-well inserts with 3T3 feeder cells, limbal explants show growth of monolayer by day 5, proliferative cells formed compact epithelial sheet by day 8, and multilayered cell sheet with compact epithelial sheet appeared by day 20. The cell sheets were positive for corneal epithelial markers CK3 and CK19, as shown by immunofluorescence **figure 3.10**.



**Figure: 3.9** Phase contrast microscopic assessment of explants on trans-well inserts with 3T3 feeder cells, initial formation of monolayer is seen at day 5 (A), and growing monolayer at day 8 (B), by day 20 a complete monolayer with compact cells seen (C). Scale bar 200 $\mu$ m



**Figure: 3.10** Immunofluorescence staining images of CK3 and CK19 on explant cultures in trans-well inserts with 3T3 feeder cells at day 16. DAPI stained nuclei (blue) (A and D), CK3 (green) (B) and CK19 (green) (E), and merge images CK3 (C), CK 19 (F), Scale bars 100μm

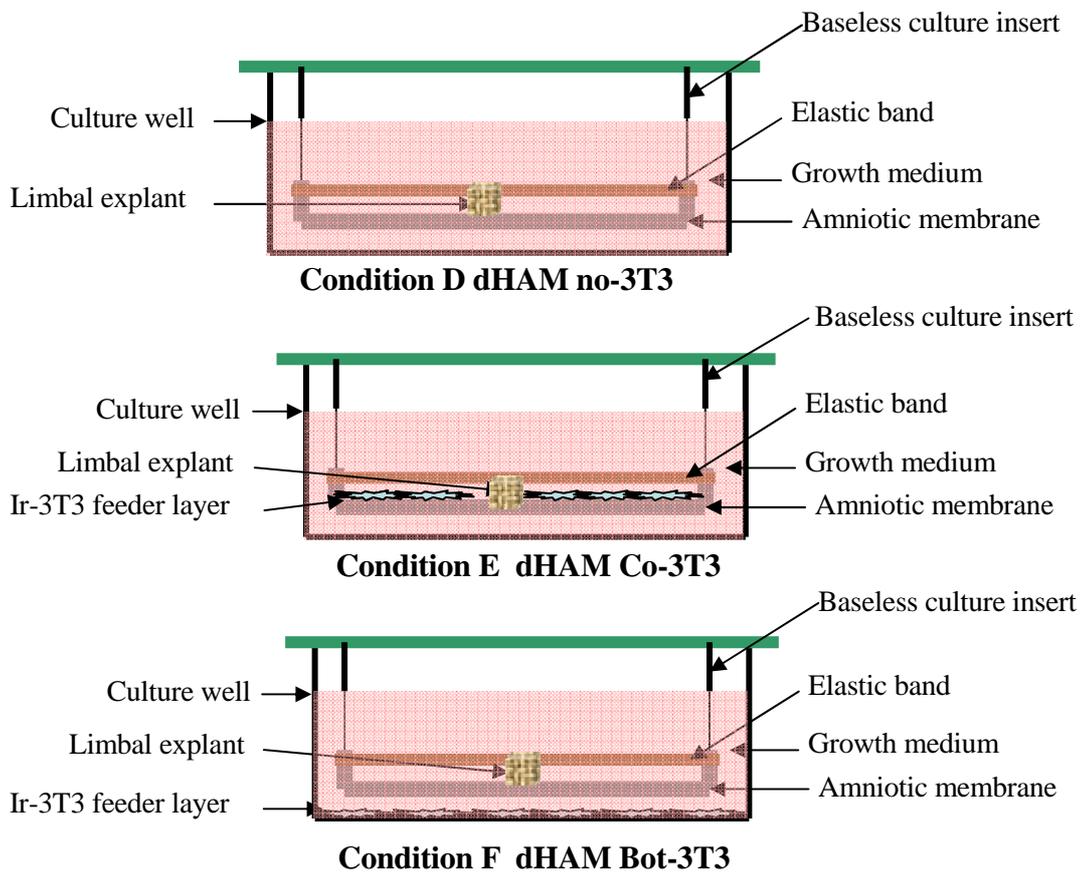
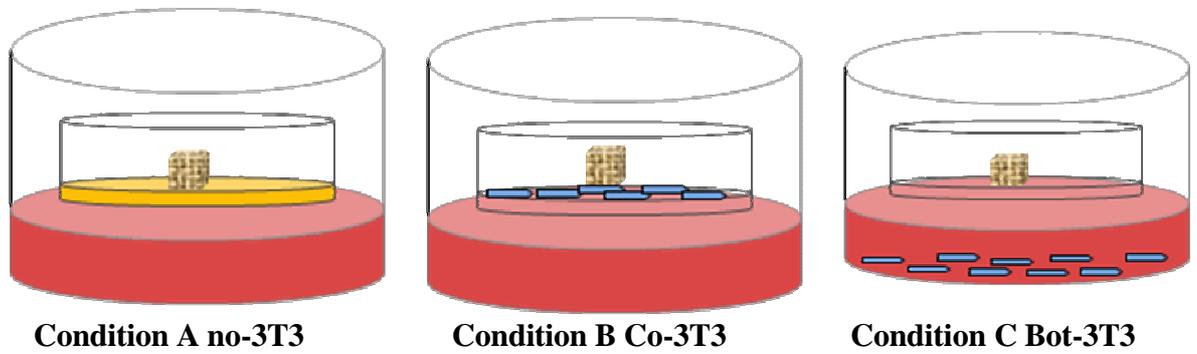
### **3.2 The influence of culture environment on primary limbal-corneal epithelial cultures**

The preliminary experiments in **section 3.1** indicated that explants with 3T3 feeder layers were the most successful at generating limbal-corneal epithelial cultures. Sun and Green 1977 were the first to demonstrate that corneal epithelial cells could also be successfully cultured and sub-cultured using 3T3 feeder cells (Sun and Green, 1977). Since then numerous studies have shown that 3T3 feeder cells are beneficial in the culture of limbal-corneal epithelial cells (Nakamura *et al.*, 2006b, Koizumi *et al.*, 2001a, Koizumi *et al.*, 2001b, Nakamura *et al.*, 2004b, Balasubramanian *et al.*, 2008, Zito-Abbad *et al.*, 2006, Cristovam *et al.*, 2008). The exact mechanism by which 3T3 feeder cells provide this beneficial effect is unknown. It is either possible that an extracellular matrix or the release factors such as FGF and transforming growth factor- $\alpha$  into the medium or through direct cell-to-cell contact or through a combination of these mechanisms.

Similarly, human amniotic membrane (HAM) has been also used for the culture of limbal-corneal epithelial cells. It encourages proliferation and retention of stem cell markers (Grueterich *et al.*, 2002a, Grueterich *et al.*, 2002b, Grueterich *et al.*, 2003a, Koizumi *et al.*, 2001b, Koizumi *et al.*, 2001a, Koizumi *et al.*, 2007, Lindberg *et al.*, 1993, Sangwan *et al.*, 2003a, Sangwan *et al.*, 2003b, Sangwan *et al.*, 2005). Both 3T3 feeder cells and HAM have been used individually (Sangwan *et al.*, 2003a, Sangwan *et al.*, 2003b, Sangwan *et al.*, 2005, Sangwan *et al.*, 2006) and in combination (3T3+HAM) for the cultivation of limbal-corneal epithelia for clinical use (Grueterich *et al.*, 2003a, Grueterich *et al.*, 2003b, Koizumi *et al.*, 2007, Sudha *et al.*, 2008)

However, the clinical application of these cultures requires not alone cell growth but also maintenance of stem cell properties. There are only limited studies on which culture environment is optimum for generating limbal-corneal epithelial cultures with high levels of stem cell markers.

To investigate the role of culture environment in determining the number of cells with stem cell characteristics six culture conditions were investigated **figure 3.2.1** diagrammatic representations of culture systems used in this study.



**Figure 3.2.1** Diagrammatic representation of six culture conditions.

- A) Condition A no-3T3:** Limbal-corneal epithelial cells were cultured on a plastic trans-well membrane without 3T3 feeder cells. This was taken as reference point for a poor culture environment.
- B) Condition B Co-3T3:** In this condition, the limbal-corneal epithelial cells are in contact with 3T3 feeder cells, with supply of soluble factors secreted by feeder cells.
- C) Condition C Bot-3T3:** In this condition, there is no physical contact between limbal-epithelial cells and 3T3 feeder cells, this provides only soluble factors secreted by feeder cells through membrane pores (trans-well insert pore size is 0.4 $\mu$ m).
- D) Condition D dHAM no-3T3:** In this condition, limbal-corneal epithelial cells were grown without any 3T3 feeder cells, but only with HAM substrate.
- E) Condition E dHAM Co-3T3:** In this condition, the limbal-epithelial cells are grown on HAM substrate and in contact with 3T3 feeder cells, with supply of soluble factors secreted by feeder cells.
- F) Condition F dHAM Bot-3T3:** in this condition, cells are grown on HAM, but there is no physical contact between limbal-corneal epithelial cells and 3T3 feeder cells, this provides only a soluble factors secreted by feeder cells.

However, application of limbal-corneal epithelial cells to clinical application requires a suitable substrate or carrier for transplantation. This study aims to investigate the role of 3T3 feeder layers in maintenance of limbal-corneal epithelial cells on denuded human

amniotic membrane (dHAM) as an extracellular matrix, which can be directly used for clinical application. Limbal epithelial cells are always cultured on the epithelial side of the amniotic membrane rather than the stromal side.

**Table 3.2.1a** illustrates the number of samples used for the culture of limbal-corneal epithelial cells in cell culture inserts for conditions A, B and C. Details of donor age, time between death and enucleation (D-Enuc-), preservation to culture time (Pre-cult-), growth start in three culture conditions, and termination day.

The average age of donor tissue 55.1 years, with maximum age of 69 years, and minimum age of 19 years, we have not observed any obvious difference in growth/success rate in cultured limbal-epithelial cells in relation to age, and no significant difference between time of preservation and culture time on growth of limbal-corneal epithelial cells. In cell, culture inserts for all conditions the average day for growth to start was 2.6 with maximum of 6 days delay in growth and average termination day is 15.5 days with maximum of 20 days in culture to attain epithelial sheet.

S.No	Donor Age	D-Enuc-In Hours	Pre-Cult-In Days	Growth Start			Termination Day
				Condition A no-3T3	Condition B Co-3T3	Condition C Bot-3T3	
1	62	09:04	7	2†	2†	2†	15
2	19*	08:32	7	3	3	3	15
3	69**	10:07	16	2	2	2	16
4	55	05:19	4	2	2	2	15
5	61	16:42	4	2	2	2	16
6	54	03:37	5	3	3	3	16
7	47	12:44	3	5	5	5	15
8	NA	08:25	6	2	2	2	16
9	51	08:05	7	4	4	4	16
10	65	04:35	3	2	2	2	15
11	59	04:43	7♦	3	3	3	14
12	67	07:05	7	2	2	2	15
13	66	16:08	5	2	2	2	15
14	41	07:41	5	2	2	2	15
15	69	04:59	5	2	2	2	15
16	62	04:05	20♦♦	6††	6††	6††	20♠♠
17	NA	12:10	7	3	3	3	15
18	33	07:12	7	3	3	3	17
19	NA	08:35	9	2	2	2	15
20	NA	08:35	7	2	2	2	14
21	NA	09:49	14	2	2	2	14
22	NA	16:30	7	3	3	3	14
23	NA	09:27	7	2	2	2	15
24	66	13:29	7	2	2	2	16
25	66	13:29	7	2	2	2	16
26	39	07:17	5	2	2	2	12♠
27	59	05:23	18	4	NA	NA	NA
28	67	11:37	7	3	3	3	17
29	NA	06:11	10	3	3	3	16
30	62	04:06	4	2	2	2	17
31	NA	18:05	7	2	2	2	14
32	30	13:06	7	NA	NA	NA	Contamination
33	NA	04:07	7	2	2	2	14
34	66	02:58♠	8	3	3	3	15
35	42	07:00	7	3	3	3	15
36	NA	26:00♠♠	10	2	2	2	17
37	61	03:55	8	2	2	2	17
38	NA	08:43	7	3	3	3	20♠♠
39	51	07:05	6	3	3	3	18
<b>Average</b>	<b>55.1</b>	<b>09:08</b>	<b>12.55</b>	<b>2.6</b>	<b>2.6</b>	<b>2.6</b>	<b>15.5</b>

\*Minimum\*\*Maximum age; NA- Not applicable.

♠Minimum♠♠Maximum time in Death and Enucleation (D-Enuc-)

♦Minimum♦♦Maximum time difference in Preservation and culture time (Pre-Cult-)

†Minimum time ††Maximum time for growth start in culture condition.

♠Minimum day ♠♠ Maximum day of termination

**Table 3.2.1a** Time-lapse details for samples used in first three culture conditions. (Cell culture inserts).

**Table 3.2.1b** illustrates the number of samples used for the culture of limbal-corneal epithelial cells on dHAM conditions D, E and F. Details of donor age, time between death and enucleation (D-Enuc-), preservation to culture time (Pre-Cult-), growth start in three culture conditions, and termination day.

The average age of donor tissue 46.8 years with maximum age of 66 years, and minimum age of 30 years, we have not observed any obvious difference in growth/success rate in cultured limbal-corneal epithelial cells in relation to age, and no significance difference between time of preservation and utilization time on growth of limbal-corneal epithelial cells. In dHAM cultures for all conditions the average day for growth start is 5.1 with maximum of 8 days delay in growth and average termination day is 20 days with maximum of 23 days in culture to attain epithelial sheet.

The end point to characterise limbal-corneal epithelial cells in all culture conditions is 1-2 cells thick cell sheet covering all the insert/dHAM. In insert cultures the approximate day for reaching cell sheet is the 17<sup>th</sup> day ( $16^{\text{th}} \pm 2$ ), whereas in HAM cultures the approximate day for reaching cell sheet to the edge of insert is 20<sup>th</sup> day ( $20^{\text{th}} \pm 2$ ).

**Table 3.2.1b:** Time-lapse details of samples used for second three culture conditions (denuded human amniotic membrane with and without 3T3 cells).

S.No	Donor Age	D-Enuc- In Hours	Pre- Cult- In Days	Growth Start			Termination Day
				Condition D dHAM no-3T3	Condition E dHAM Co-3T3	Condition F dHAM Bot-3T3	
1	NA	16:20	7	5	5	5	14♠
2	NA	20:25	7	5	5	5	14
3	NA	05:10	8	5	5	5	20
4	NA	07:22	5♦	4	4	4	18
5	63	10:20	5	5	5	5	23
6	NA	12:17	5	5	5	5	23♠♠
7	NA	12:17	5	5	5	5	23
8	39	07:17	5	5	5	5	20
9	59	05:23	10♦♦	5	5	5	22
10	NA	17:58	6	5	5	5	20
11	NA	12:57	5	5	5	5	20
12	NA	25:44	5	8††	8††	8††	16
13	46	04:56	6	5	5	5	20
14	28	07:17	6	5	8	5	22
15	NA	04:07	7	3	3†	3	20
16	NA	14:11	7	3	3	3	19
17	NA	05:58	5	5	5	5	22
18	66**	04:00	7	5	5	5	22
19	30*	13:06	7		NA		Contamination
20	38	16:00	5	6	6	6	20
21	53	12:00	8	5	5	5	22
<b>Average</b>	<b>46.88</b>	<b>10:03</b>	<b>5.44</b>	<b>5.1</b>	<b>5.1</b>	<b>5.1</b>	<b>20</b>

\*Minimum\*\*Maximum age; NA- Not applicable.

♠Minimum and ♠♠Maximum time in Death and Eucleation (D-Enuc-)

♦Minimum and ♦♦Maximum time difference in preservation and culture (Pre-Cult-)

†Minimum time and ††Maximum time for growth start in culture condition.

♠Minimum day and ♠♠ Maximum day of termination

### 3.2.1 Outgrowth of limbal-corneal epithelial cells

The impact of six different culture conditions on rate of epithelial growth was assessed by measuring the distance of the cell sheet formed over time. Cultures were terminated when the cell sheet had reached the edge of inserts or dHAM. The graph in **figure 3.2.2** shows quantitative analysis of the area of epithelial outgrowths.

Condition A no-3T3 had no observable outgrowth at day 2, by day 5 the average area of cell outgrowth was  $2.5 \pm 0.5\text{mm}^2$ , raising to average of  $10 \pm 1.4\text{mm}^2$  by day 8, thereafter the proliferating cells attained average growth rate of  $35 \pm 5.8\text{mm}^2$  by day 14, and covered trans-well insert by day 17 with average of  $40.7 \pm 0.5\text{mm}^2$ .

Condition B Co-3T3 had no observable outgrowth at day 2, whereas by day 5 the cell outgrowth was  $4.1 \pm 1.03\text{mm}^2$ , with increased outgrowth to  $12.2 \pm 0.5\text{mm}^2$  by day 8, and  $38.5 \pm 5.7\text{mm}^2$  by day 14, with full coverage of the trans-well insert by day 17 with  $41.5 \pm 0.5\text{mm}^2$ .

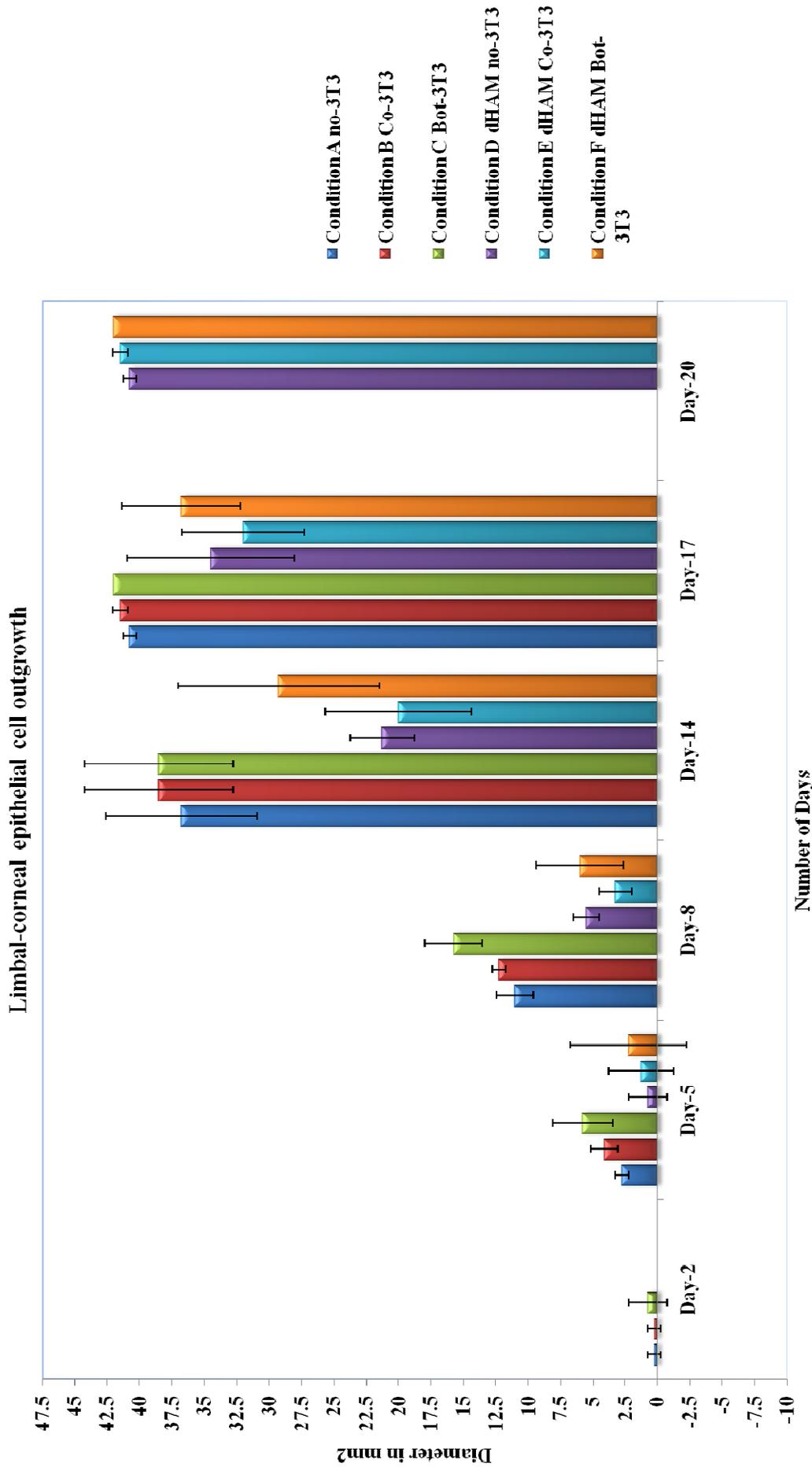
Condition C Bot-3T3 had visible outgrowth by day 2 with  $0.75 \pm 1.5\text{mm}^2$ , outgrowth increased by day 5 to  $5.8 \pm 2.3\text{mm}^2$ , which is higher than condition A no-3T3 and B Co-3T3, similarly, the outgrowth was greater than condition A and B by day 8 with  $15.7 \pm 2.2\text{mm}^2$ , covering entire trans-well insert by day 17 with  $42 \pm 0.1\text{mm}^2$ .

In condition, D dHAM no-3T3 growth was observed on day 5 with average of  $0.75 \pm 1.5\text{mm}^2$ , with exponential increase in outgrowth rate to average of  $5.5 \pm 1\text{mm}^2$  by day 8,  $21.2 \pm 2.5 \text{mm}^2$  by day 14,  $34.5 \pm 6.4\text{mm}^2$  by day 17 and  $40.7 \pm 0.5\text{mm}^2$  by day 20.

In condition E dHAM Co-3T3, explants outgrowth was observed on day 5 with average of  $1.25 \pm 2.5\text{mm}^2$ , whereas the outgrowth was just  $3.2 \pm 1.2\text{mm}^2$  by day 8, which is less than condition D dHAM no-3T3, and increased outgrowth to  $20 \pm 5.6\text{mm}^2$  by day 14,  $32 \pm 4.6\text{mm}^2$  by day 17 and  $41.5 \pm 0.5\text{mm}^2$  by day 20 observed.

In condition F dHAM Bot-3T3, explants outgrowth was observed on day 5 with average of  $2.2 \pm 4.5\text{mm}^2$ , which is higher than conditions D and E, with outgrowth of  $6 \pm 3.3\text{mm}^2$  by day 8, and increased outgrowth to  $29.2 \pm 7.8\text{mm}^2$  by day 14,  $36.7 \pm 4.5\text{mm}^2$  by day 17 and  $42\text{mm}^2$  by day 20.

No obvious growth was observed in limbal-corneal epithelial cultures grown on dHAM with all three-culture conditions at day  $5 \pm 2$ ; this could be due to technical difficulty in observing cell outgrowth due to thickness of membrane. Furthermore, it took until day  $20 \pm 2$  for these conditions to reach termination point.

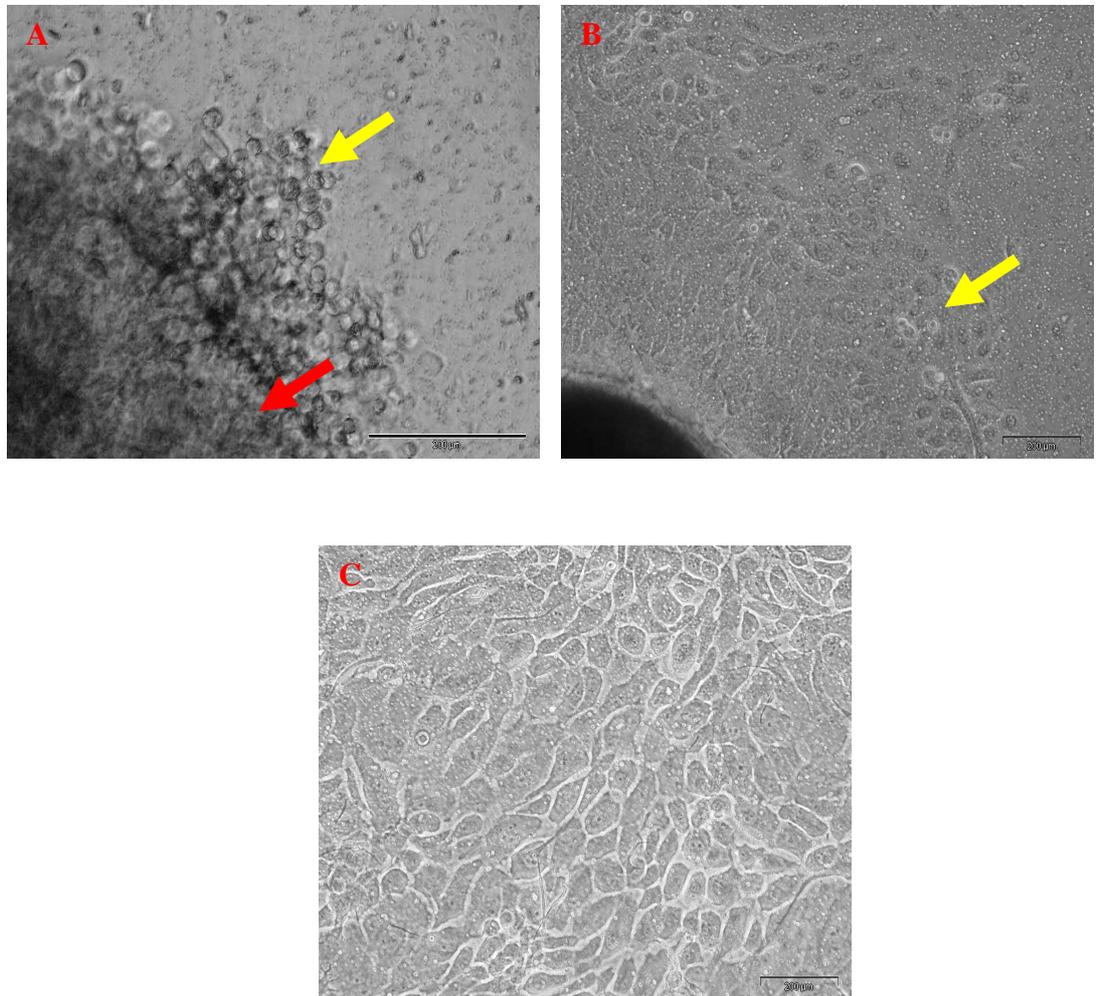


**Figure: 3.2.2** Graphical representation of the distance migrated by cells from limbal explants in six culture conditions. Mean  $\pm$ S.D and n=6 (Six biological samples (sample number- 15 to 20) for condition A,B,C and six biological samples (Sample number – 46, 47, 48, 53, 69, 84) for conditions D,E and F).

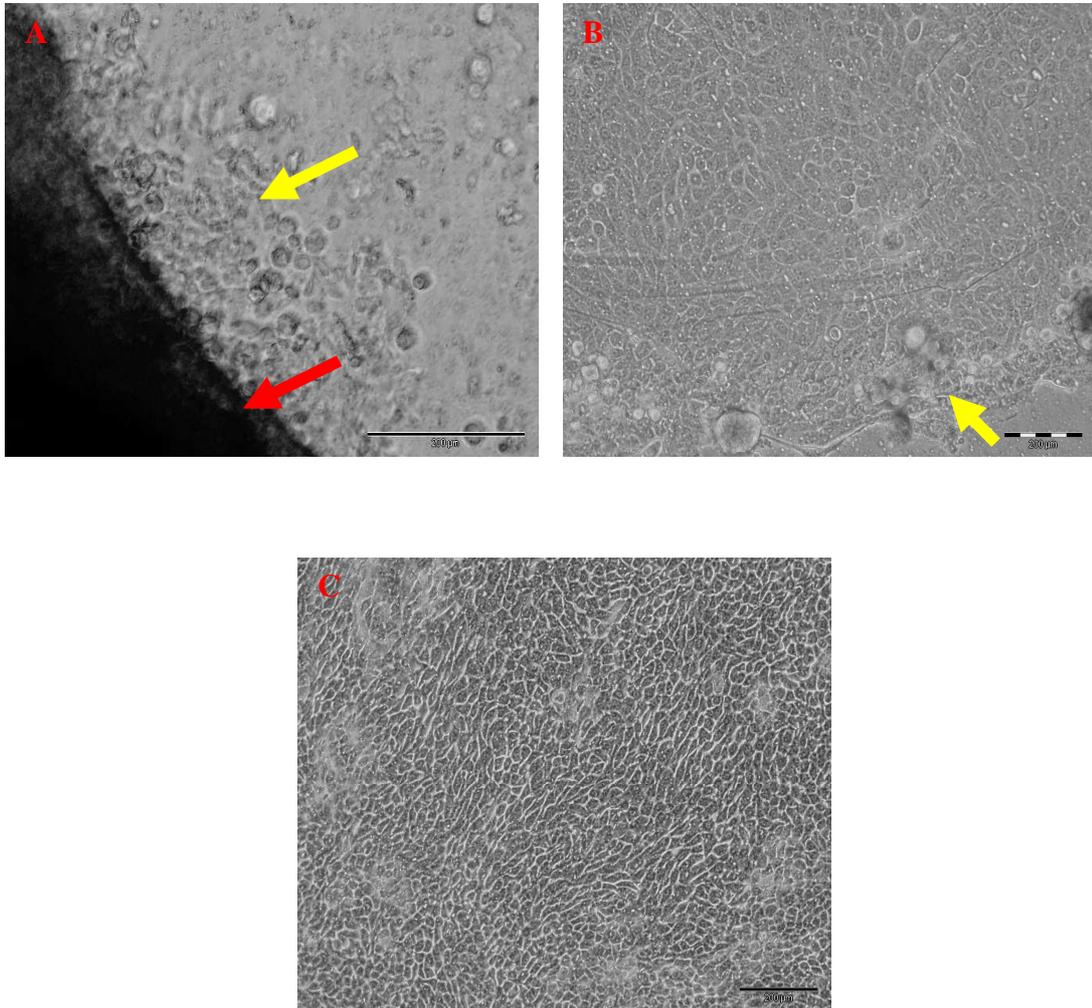
### **3.2.2 Morphology of limbal-corneal epithelial cells**

The morphology of expanded epithelial cells in trans-well inserts and dHAM were observed under phase contrast microscope at different time intervals. For expanded cells in condition A no-3T3 (**figure 3.2.3**), the cells appear large with polygonal borders and showed discontinuous cell expansion with irregular leading edges (**figure 3.2.3 b**).

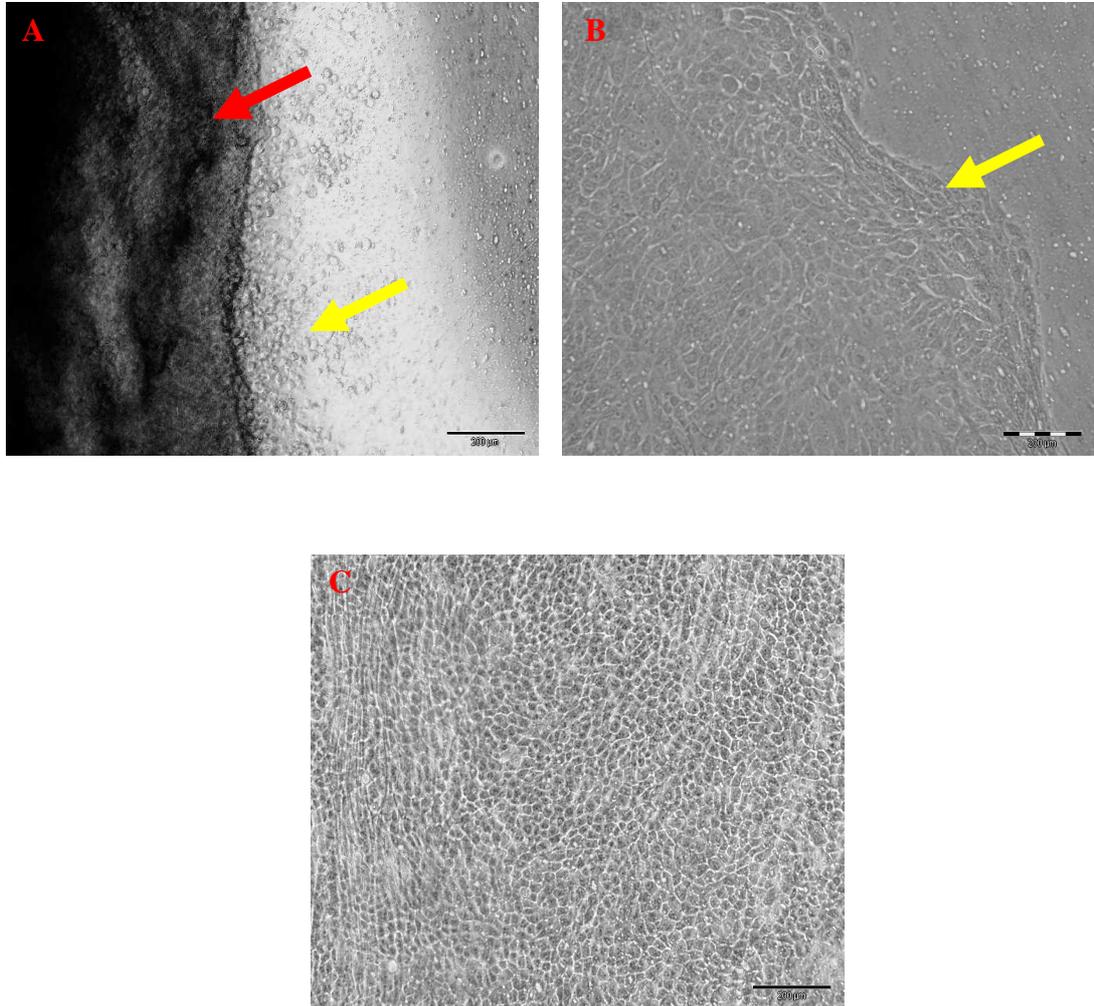
Condition B Co-3T3 and condition C Bot-3T3 both showed small, compact and uniform cells with round cell borders and continuous expansion area with semi-circular and regular margins (**figure 3.2.4** and **3.2.5**). No obvious difference was observed in condition D dHAM no-3T3, condition E dHAM Co-3T3 and Condition F dHAM Bot-3T3, where the cell morphology was small and compact with regular size (**figure. 3.2.6; 3.2.7** and **3.2.8**).



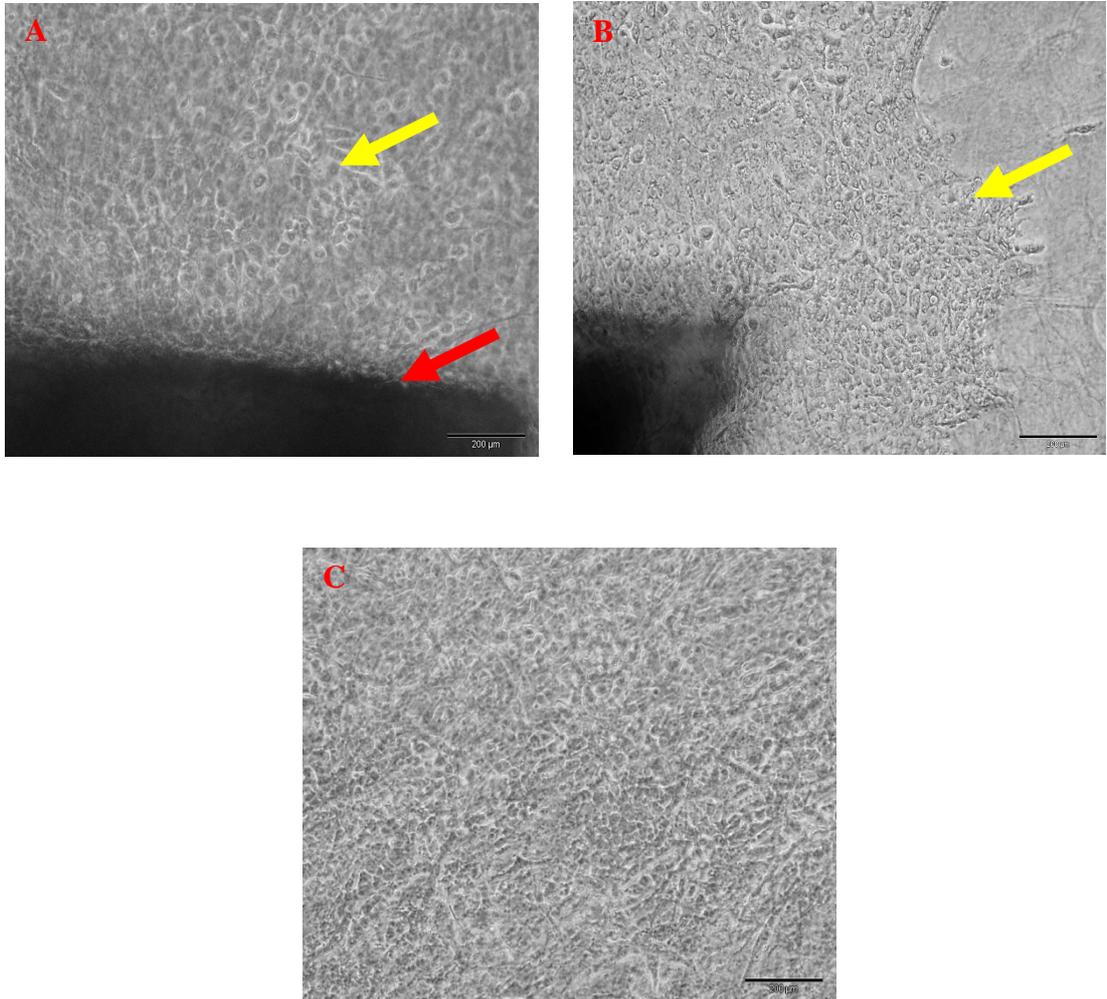
**Figure: 3.2.3:** Phase contrast microscopic assessment of explants on trans-well membrane with no 3T3 cells (Condition A No-3T3): explant (red arrow) on trans-well membrane with cell outgrowth (yellow arrow) at day 2 (20x) (A), monolayer outgrowth from explant at day 8, edge of the monolayer (yellow arrow) (10x) (B), and complete monolayer in trans-well insert at day 16 (10x) (C).



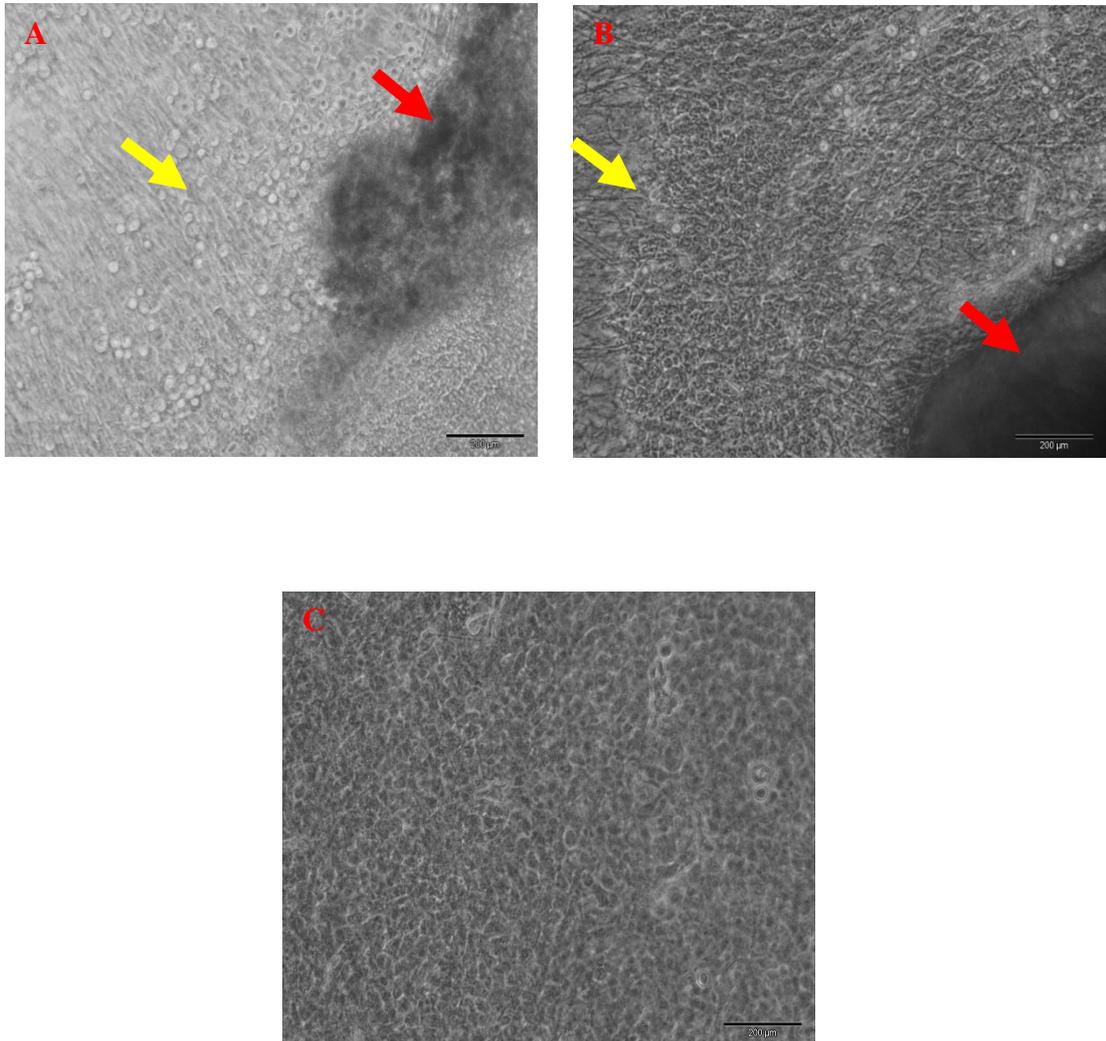
**Figure: 3.2.4** : Phase contrast microscopic assessment of explants on trans-well membrane co-culture with 3T3 cells (Condition B co-3T3): explant (red arrow) on trans-well membrane with cell outgrowth (yellow arrow) at day 2 (20x) (A), monolayer out growth from explant at day 8, edge of the monolayer (yellow arrow) (10x) (B), and complete monolayer in trans-well insert at day 16 (10x) (C).



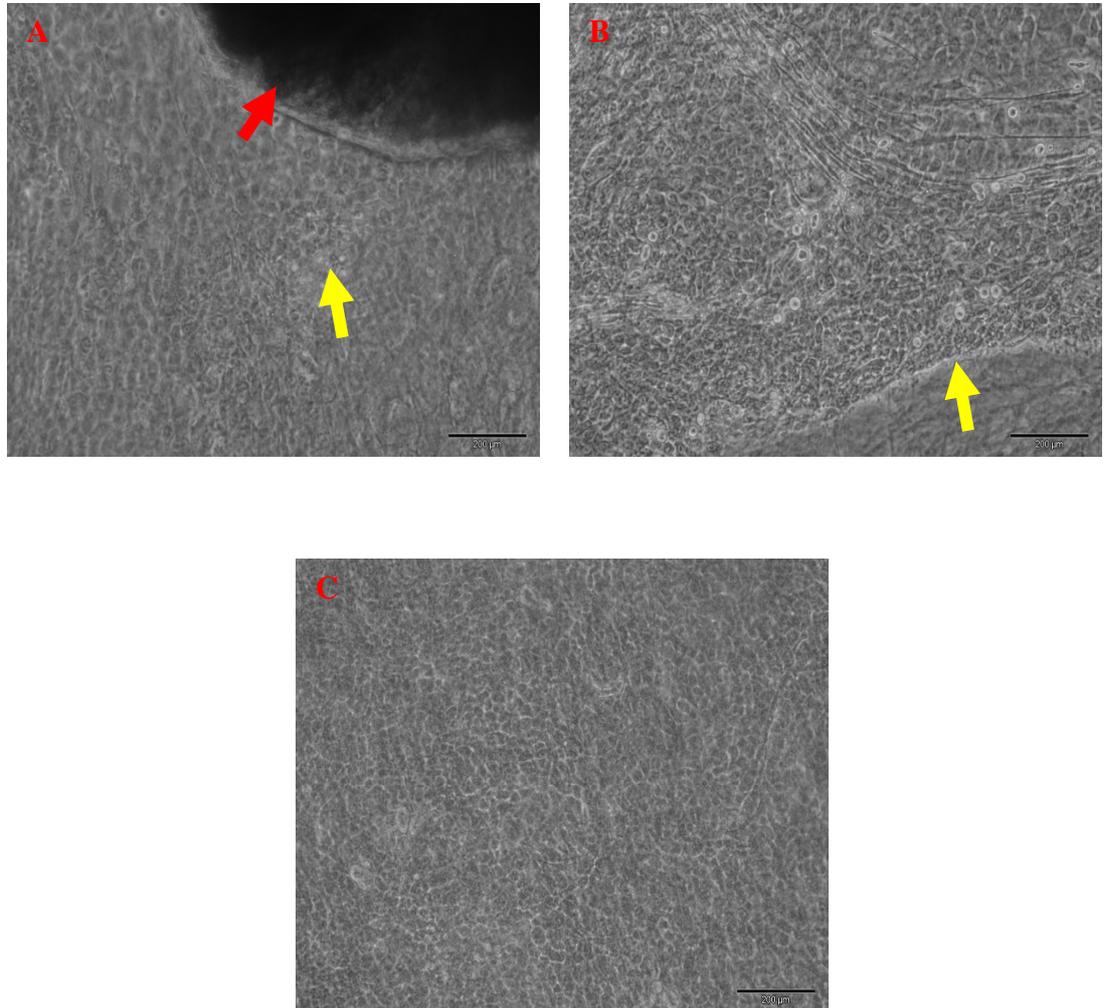
**Figure: 3.2.5** : Phase contrast microscopic assessment of explants on trans-well membrane with 3T3 separated (Condition C Bot-3T3): explant (red arrow) on trans-well membrane with cells (yellow arrow) outgrowth at day 2 (10x) (A), a monolayer out growth from explant at day 8, edge of the monolayer (yellow arrow) (10x) (B), and complete monolayer in trans-well insert at day 16 (10x) (C).



**Figure: 3.2.6:** Phase contrast microscopic assessment of explants on denuded human amniotic membrane (dHAM) with no 3T3 cells (Condition D dHAM no-3T3): explant (red arrow) on dHAM with cell outgrowth (yellow arrow) at day 5 (A), monolayer outgrowth from explant at day 14, edge of the monolayer (yellow arrow) (B), and complete monolayer on HAM insert at day 20 (C). Scale bars 200  $\mu\text{m}$ .



**Figure: 3.2.7:** Phase contrast microscopic assessment of explants on denuded human amniotic membrane (dHAM) with Co- 3T3 cells (Condition E dHAM Co-3T3): explant (red arrow) on dHAM with cell outgrowth (yellow arrow) at day 5 (A), monolayer outgrowth from explant at day 14, edge of the monolayer (yellow arrow) (B), and complete monolayer on HAM insert at day 20 (C). Scale bars 200 µm.

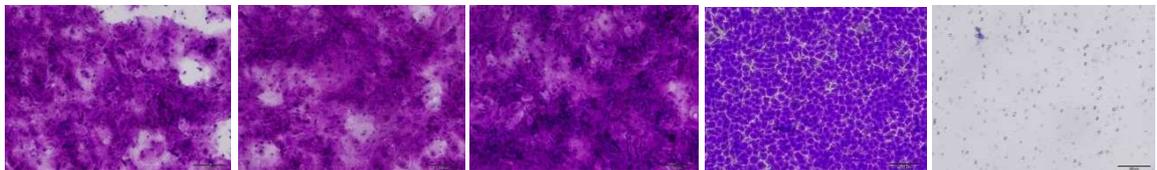
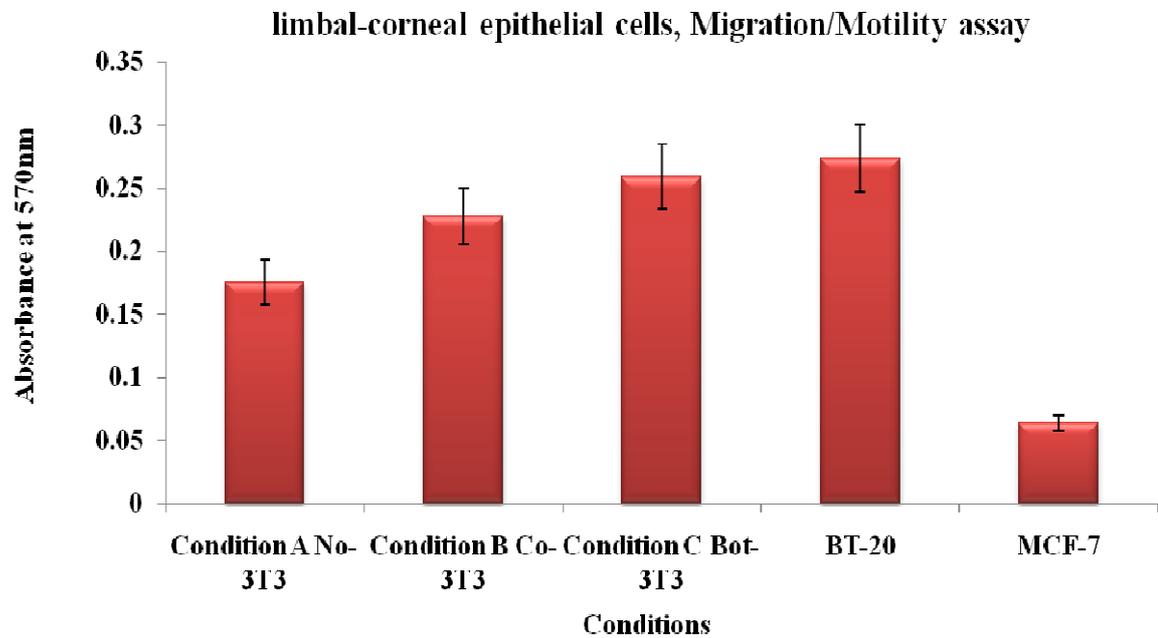


**Figure: 3.2.8:** Phase contrast microscopic assessment of explants on denuded human amniotic membrane (dHAM) with Bot-3T3 cells (Condition F dHAM Bot-3T3): explant (red arrow) on dHAM with cell outgrowth (yellow arrow) at day 5(A), monolayer outgrowth from explant at day 14, edge of the monolayer (yellow arrow) (B), and complete monolayer on HAM insert at day 20 (C). Scale bars 200 μm.

### 3.3 Migration/motility assay of limbal-corneal epithelial cells

The processes involved in the healing of corneal epithelial wounds can be divided into three distinct components: cell migration, cell proliferation, and cell adhesion. All three components are part of a continuous process but the contribution of each can vary depending on the size and depth of the wound and nature of injury (Dua *et al.*, 1994). A migration/motility assay was performed on limbal-corneal epithelial cells for first three conditions (Conditions A, B and C) grown on cell culture trans-well inserts.

**Figure 3.3** illustrates the motility/migration of limbal-corneal epithelial cells grown in condition A no-3T3, condition B Co-3T3, and condition C Bot-3T3, where condition C Bot-3T3 shows higher migration rate compared to condition A no-3T3, and condition B Co-3T3. Overall limbal-corneal epithelial cells are highly motile compared with negative control MCF-7 cell line.



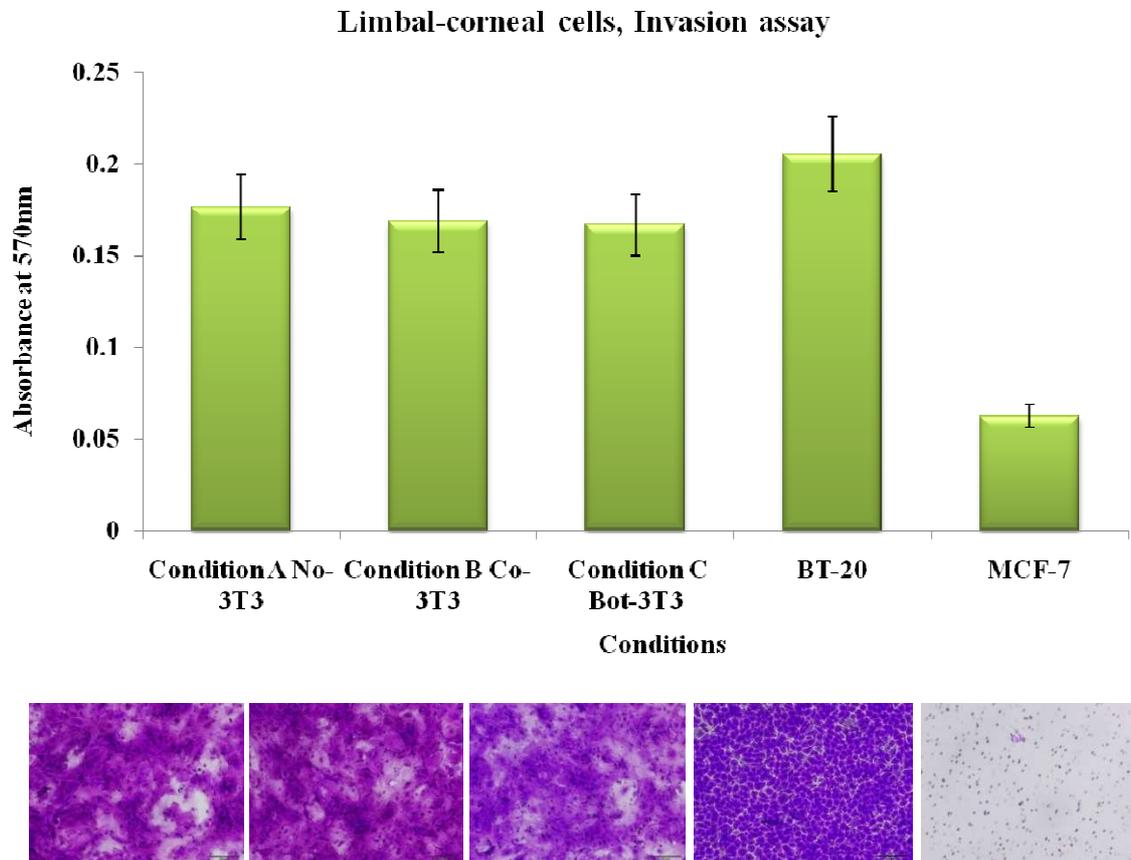
**Figure: 3.3** Graphical and crystal violet stained representation of motility/migration of cultured limbal-corneal epithelial cells grown on cell culture inserts with no3T3, Co-3T3 and Bot-3T3 conditions. n=2 biological replicates (sample number 15 and 18) error bars with mean values.

### **3.4 Invasion assay of limbal-corneal epithelial cells**

Invasion assays were performed on primary cultures of limbal-corneal epithelial cells in conditions A, B and C to understand invasion property of cells, which is known to have role in wound healing mechanism. In this experiment the BT-20 cell line, a known highly invasive cell line, is used as a positive control and non-invasive cell line MCF7 as a negative control.

The three conditions, condition A no-3T3, condition B Co-3T3, condition C Bot-3T3, showed similar high level of invasion compared to the negative control MCF-7. Invasion level for the three limbal-corneal epithelial culture conditions was close to that of the positive control BT-20.

**Figure 3.4** illustrates the invasion of limbal-corneal epithelial cells grown in different culture conditions compared to positive and negative controls. Bright field pictures of invasive limbal-corneal epithelial cells stained with crystal violet.



**Figure: 3.4** Graphical and crystal violet stained representation of invasion of cultured limbal-corneal epithelial cells grown on cell culture inserts with no3T3, Co-3T3 and Bot-3T3 conditions. n=2 biological replicates, (Sample number 15 and 18) error bars with mean values.

### 3.5 Characterisation of limbal-corneal epithelial cells grown on cell culture inserts and denuded human amniotic membrane (dHAM)

The expression of limbal-corneal stem cell and differentiation markers were examined by immunofluorescence, western blot, flow cytometry, RT-PCR, and qRT-PCR analysis. A panel of markers were used to assess differentiation (**table 3.5a**) and stem cell markers (**table 3.5b**). Number of biological samples and sample number used for western blot and immunofluorescence were listed in table 3.5c and 3.5d.

**Table 3.5a** Limbal-corneal epithelial cell differentiation markers

Marker	Specification
<b>Cytokeratin 3</b>	Major cytokeratin in corneal epithelium
<b>Cytokeratin 12</b>	Major cytokeratin in corneal epithelium
<b>E-cadherin</b>	Cell-cell contact
<b>Connexin43</b>	Cell-cell contact (tight junction protein)
<b>ZO1</b>	Cell-cell contact
<b>Occludin</b>	Cell-cell contact

**T**

Marker	Specification
<b>Cytokeratin 19</b>	Limbal corneal epithelial cells
<b>Cytokeratin 14</b>	Limbal basal cells
<b><math>\Delta</math>Np63<math>\alpha</math></b>	Nuclear
<b>p63</b>	Nuclear
<b>ABCG2</b>	ATP-binding cassette transporter
<b><math>\alpha</math>-2 Integrin</b>	Membrane
<b><math>\beta</math>-1 Intergrin</b>	Membrane

**Table 3.5b** Limbal-corneal epithelial cell stem cell markers

The ideal characteristics required in a limbal-corneal epithelial cell cultures is low expression levels or absence of markers associated with differentiation and elevated expression levels of markers associated with limbal stem cells.

**Table- 3.5c** Sample number (biological sample) used for western blot and immunofluorescence in first three culture conditions (cell culture inserts with/without 3T3 cells).

Serial number	Sample number used for western blot analysis	Sample number used for immunofluorescence
1	15	10
2	16	11
3	18	15
4	19	16
5	20	18
6	26	19
7	29	20
8	30	29
9	34	30
10		31

**Table- 3.5d** Sample number (biological sample) used for western blot and immunofluorescence in second three culture conditions (Human amniotic membrane with/without 3T3 cells)

Serial number	Sample number used for western blot analysis	Sample number used for immunofluorescence
1	46	46
2	47	47
3	48	48
4	53	53
5	69	69
6	84	84

### **3.5.1 Expression of limbal-corneal epithelial differentiation cytokeratin 3 (CK3)**

The expression of the differentiation marker cytokeratin 3 (CK3) was investigated in all six conditions by immunofluorescence, western blot, and RT-PCR analysis.

#### **3.5.1.1 Immunofluorescence analysis of CK3 expression**

Immunofluorescence analysis was performed for CK3 with four biological replicates. Representative results were shown in **figure 3.5.1** and **3.5.2**.

The staining of cytokeratin 3, in condition B Co-3T3 showed slightly lower staining in the basal cells (**figure 3.5.1c**) than in the apical cell layer (**figure 3.5.1d**). This staining level in the basal cells of condition B Co-3T3 appears to be slightly lower than that observed in condition A no-3T3. In contrast the staining for CK3 in the apical cells of condition B appears to be similar to the apical cell layer of condition A no-3T3.

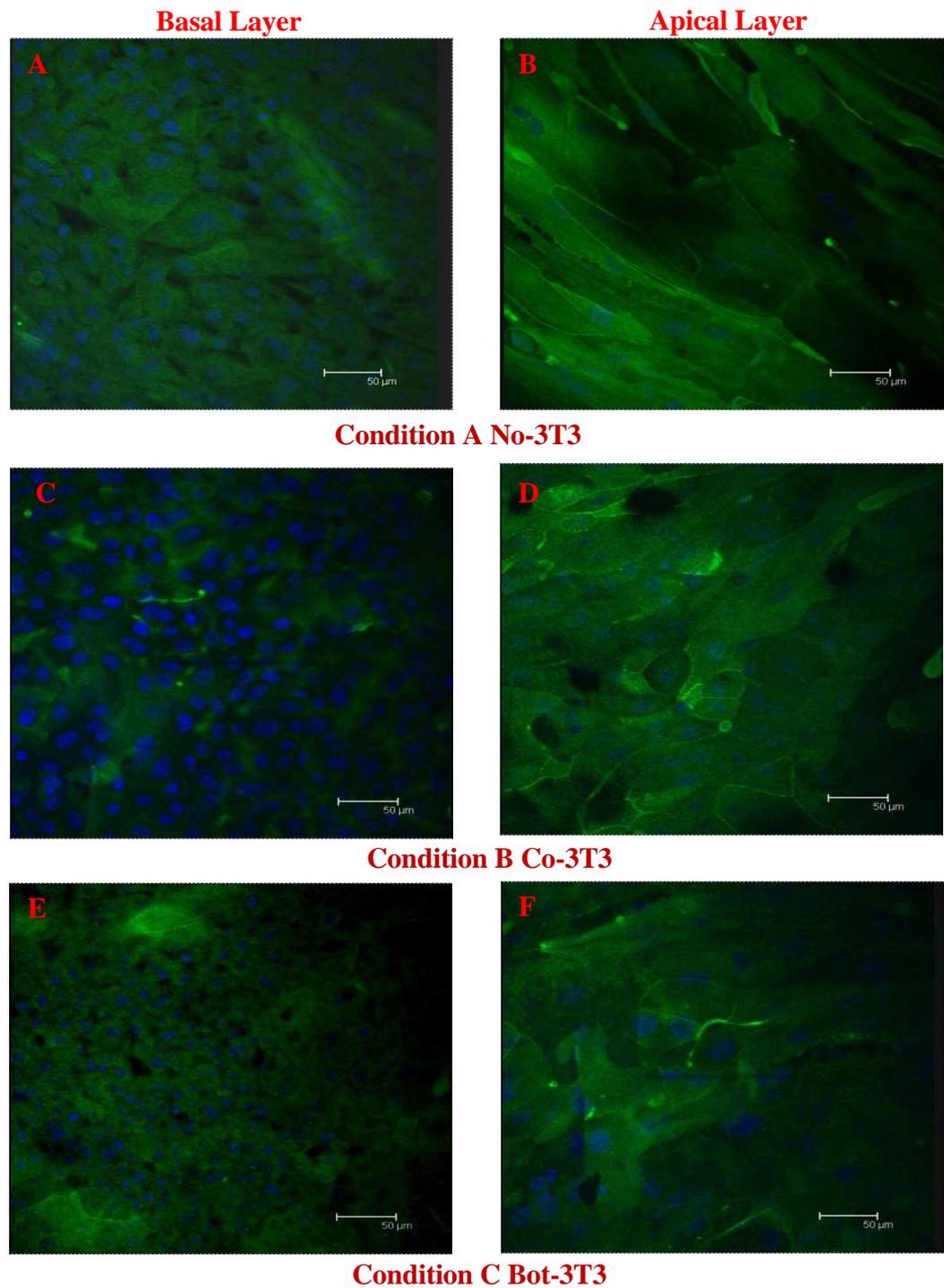
The staining of CK3 in condition C Bot-3T3 also showed slightly lower staining in the basal cell layer (**figure 3.5.1e**) than in the apical cell layer (**figure 3.5.1 f**). The level of staining for CK3 in the basal cell layer of condition C Bot-3T3 appeared similar to condition B Co-3T3 with slightly lower levels than that observed in condition A no-3T3.

The staining for CK3 in condition D dHAM no-3T3 showed lower staining in the basal cell layer (**figure 3.5.2a**), than apical cell layer (**figure 3.5.2b**). The level of staining for CK3 in the basal layer of condition D dHAM no-3T3 appears to show lower levels of staining than in the

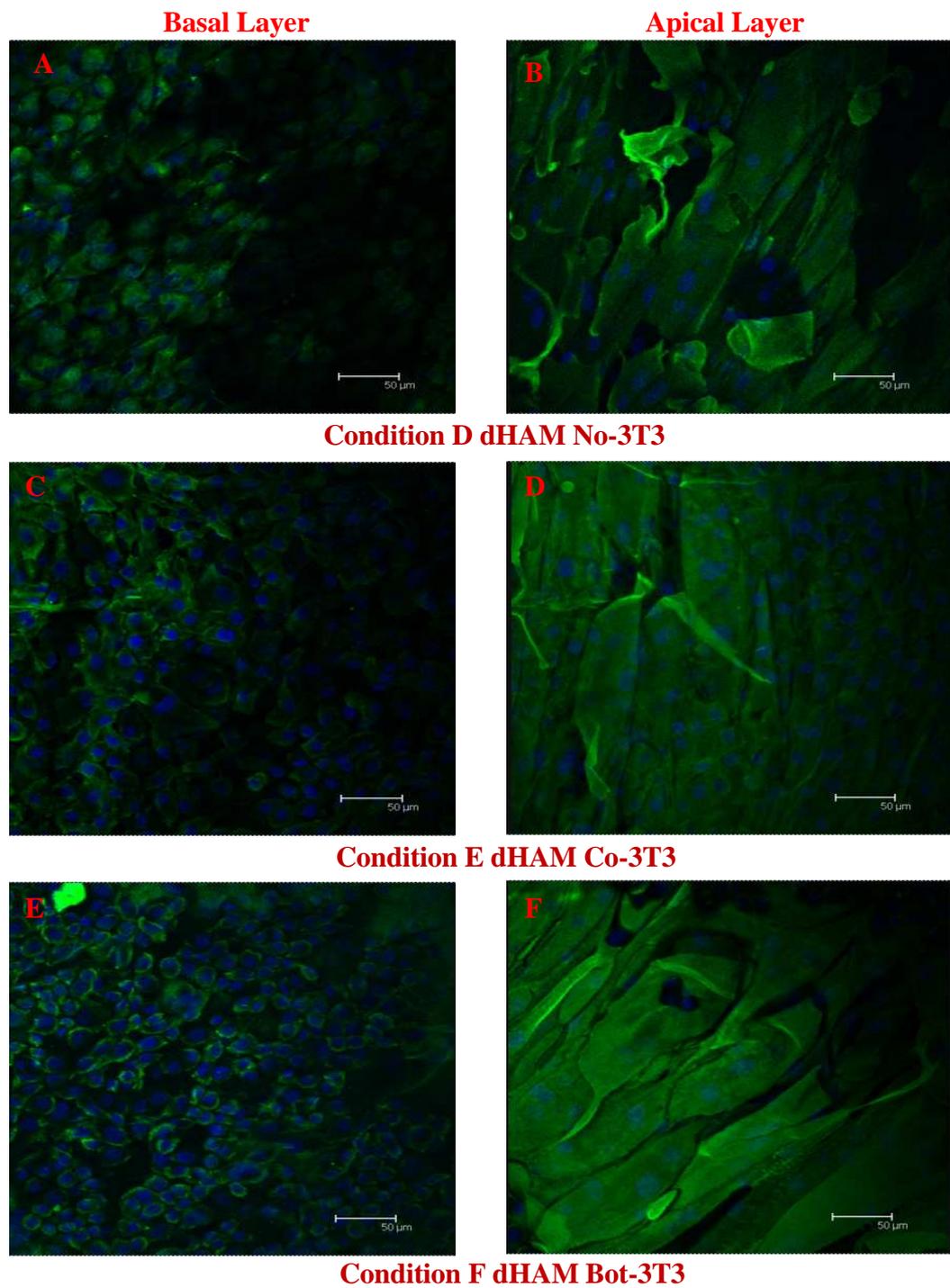
basal cell layers of condition A no-3T3 as well as condition B Co-3T3 and condition C Bot-3T3. The level of CK3 staining on the apical cell layer of condition D dHAM no-3T3 appears similar to that observed in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3.

In condition E dHAM Co-3T3, CK3 staining was lower in basal cell layer (**Figure 3.5.2c**) than the apical cell layer (**figure 3.5.2d**). The observed level of staining for CK3 in the basal cell layer appears to be similar to that of condition D dHAM no-3T3 and lower than that observed in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3. The level of staining in the apical cells of condition E dHAM Co-3T3 appeared to be similar to that observed in other conditions.

In condition F dHAM Bot-3T3, the staining for CK3 appeared to be lower in the basal cell layer (**figure 3.5.2e**) than in the apical cell layer (**figure 3.5.2f**). The level of expression of CK3 in the basal layer appeared to similar to that observed in condition D dHAM no-3T3 and condition E Co-3T3 and slightly lower than that observed in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3. The observed level of staining in the apical layer of condition F dHAM Bot-3T3 appeared similar to that observed in the other conditions.



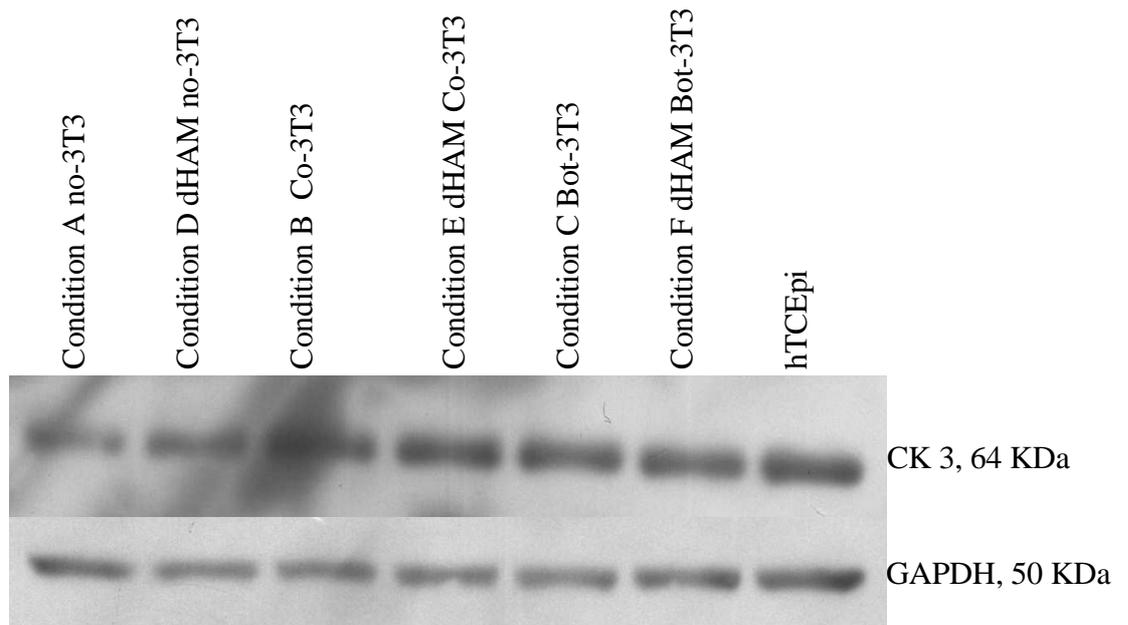
**Figure 3.5.1** Confocal images of immunofluorescence staining for CK3 in limbal-corneal epithelial cells grown on cell culture inserts with conditions A, B and C in basal and apical layers, nuclei (Blue) & CK3 (Green); Scale bars 50μm.



**Figure: 3.5.2** Confocal images of immunofluorescence staining for CK3 in limbal-corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E and F in basal and apical layers, nuclei (blue) & CK3(green); Scale bars 50μm.

### **3.5.1.2 Western blot analysis of CK3**

Western blot analysis was performed on six culture conditions with 5 biological replicates; a representative blot is shown in **figure 3.5.3**. In contrast to the slight variations observed in CK3 as analysed by immunofluorescence, no obvious differences were observed in the six culture conditions for CK3.

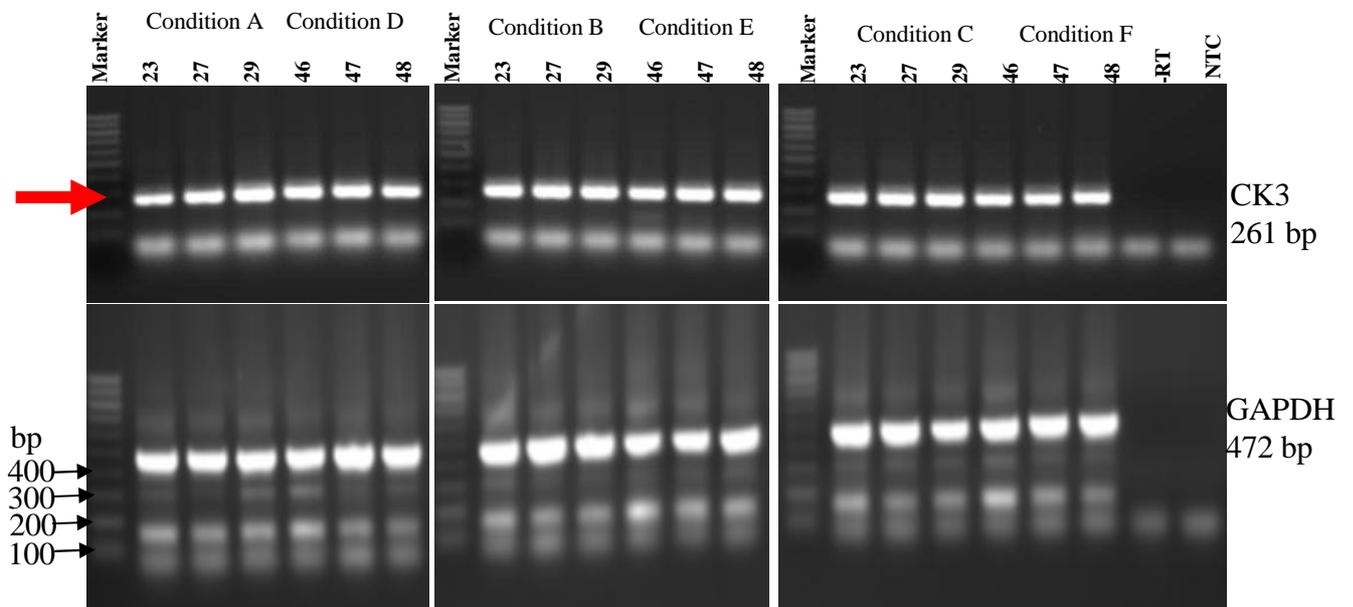


**Figure: 3.5.3:** Representative western blotting profile of CK3 in cultured limbal-corneal cells, where CK3 is positive in all six conditions. Condition A, B and C with sample number 18, and condition D, E & F with sample 47. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates).

### 3.5.1.3 RT-PCR analysis for CK3

The end point reverse transcription-polymerase chain reaction (RT-PCR) of CK3 (261 bp) for all six conditions has shown no differential expression across three biological replicates **figure**

### 3.5.4.



**Figure 3.5.4:** RT-PCR of CK3 (261 bp arrow) on limbal-corneal epithelial cells grown on cell culture inserts and dHAM, with three biological samples for condition. Sample 23, 27, and 29 cultured on cell culture inserts with condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 conditions. Samples 46, 47 and 48 cultured on dHAM with condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Internal control gene GAPDH (472 bp).

### **3.5.2 Expression of limbal-corneal epithelial differentiation marker cytokeratin 12 (CK12)**

Another major differentiation marker for corneal epithelial cells is CK12 (Kurpakus *et al.*, 1990), the level of expression of CK12 in limbal-corneal epithelial indicates the presence/absence of differentiated cells in the culture.

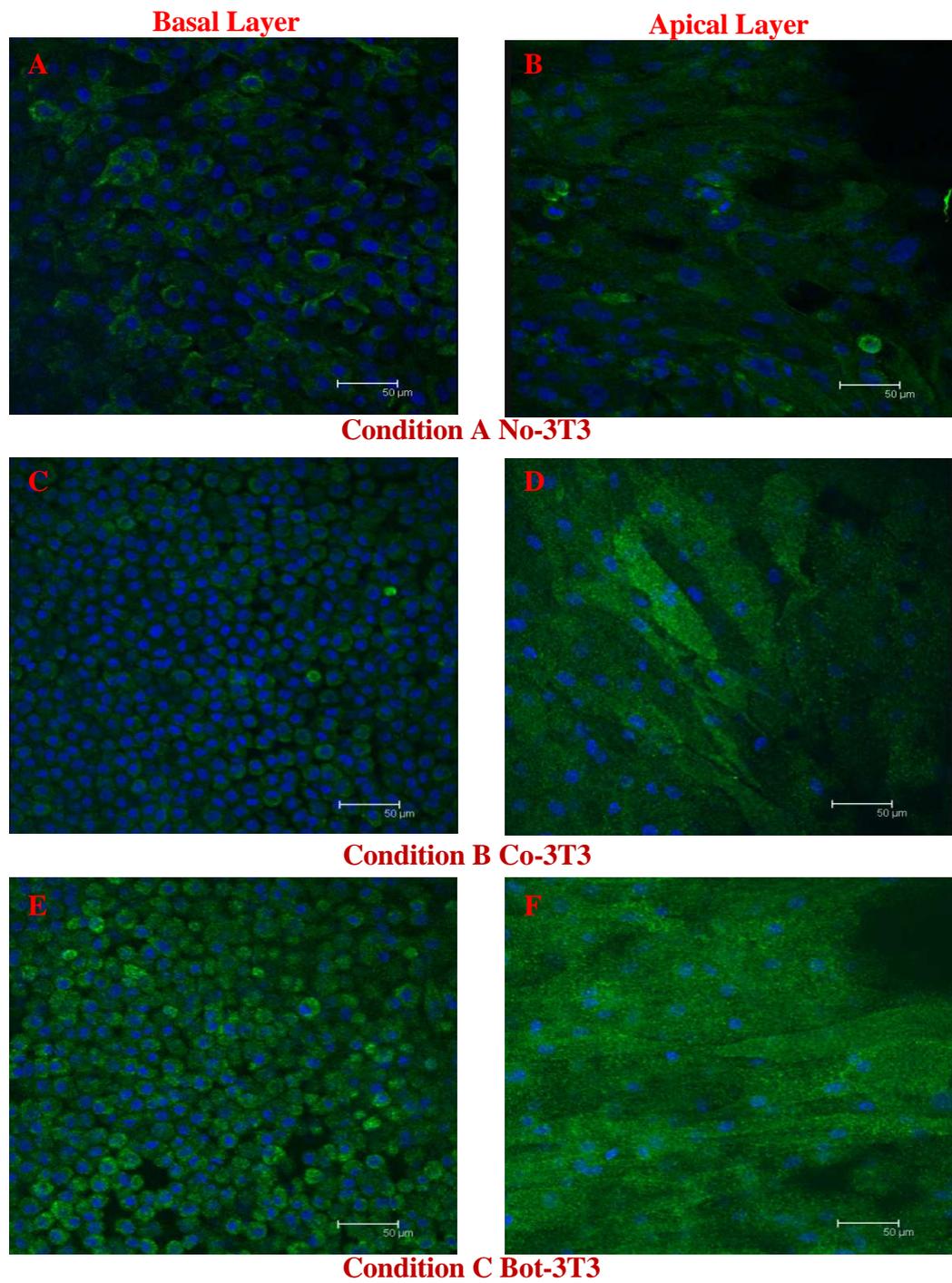
#### **3.5.2.1 Immunofluorescence analysis of CK12 expression**

The staining of CK12, in condition A no-3T3 showed slightly lower expression in basal cells compared to apical cells (**figure 3.5.5b**), with increased CK12 expression in apical cells in condition B Co-3T3 and condition C Bot-3T3. In contrast the CK12 expression was higher in basal cells of condition C Bot-3T3 compared to basal cells of condition B Co-3T3 and condition A no-3T3.

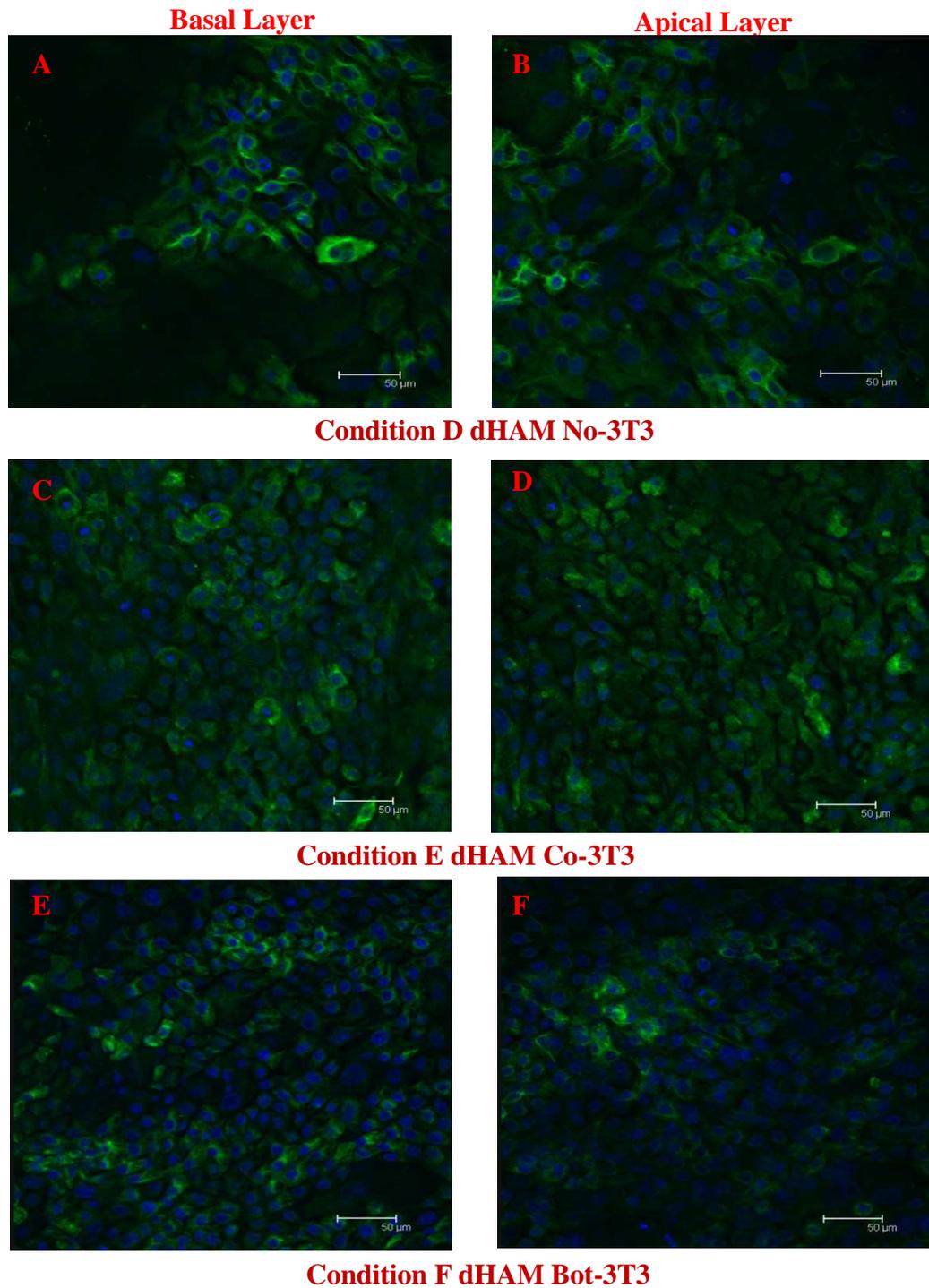
The staining of CK12 in condition D dHAM no-3T3 showed sporadic expression in basal and apical cells, whereas the expression of CK12 was slightly higher in condition E dHAM Co-3T3 in basal and apical cells compared to condition D dHAM no-3T3. Condition B Co-3T3 and condition E dHAM Co-3T3 show similar pattern of expression at basal and apical layers (**figure 3.5.5c, d and 3.3.6 c, d**).

In condition F dHAM Bot-3T3, the staining for CK12 appeared to be slightly higher in basal cells (**figure 3.5.6 e**) than in the apical cells (**figure 3.5.6 f**). Overall the level of expression

of CK12 in condition F dHAM Bot-3T3 appeared to be low in apical and basal cells compared to other culture conditions.



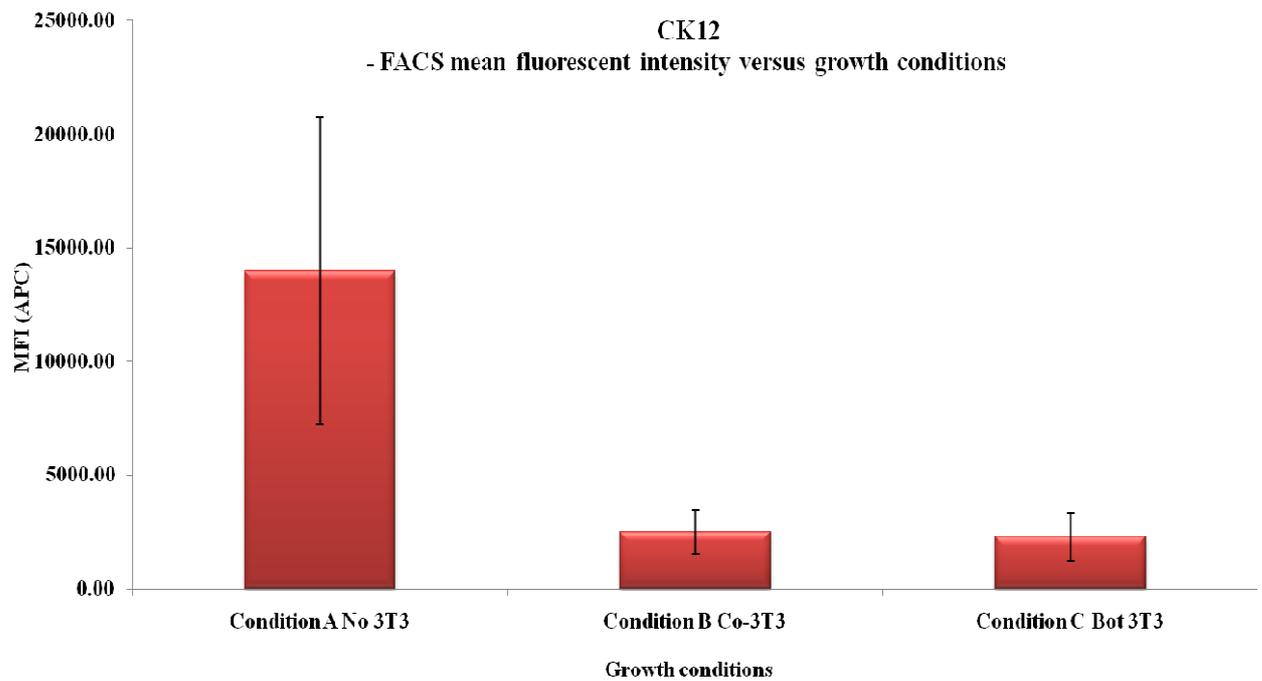
**Figure: 3.5.5** Confocal images of immunofluorescence staining for CK12 in limbal-corneal epithelial cells grown on cell culture inserts with conditions A, B and C in basal and apical layers, nuclei (blue) & CK12 (green); scale bars 50μm.



**Figure: 3.5.6** Confocal images of immunofluorescence staining for CK12 in corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E and F in basal and apical layers, nuclei (blue) & CK12 (green); scale bars 50 $\mu$ m.

### **3.5.2.2 Flow cytometry analysis of CK12**

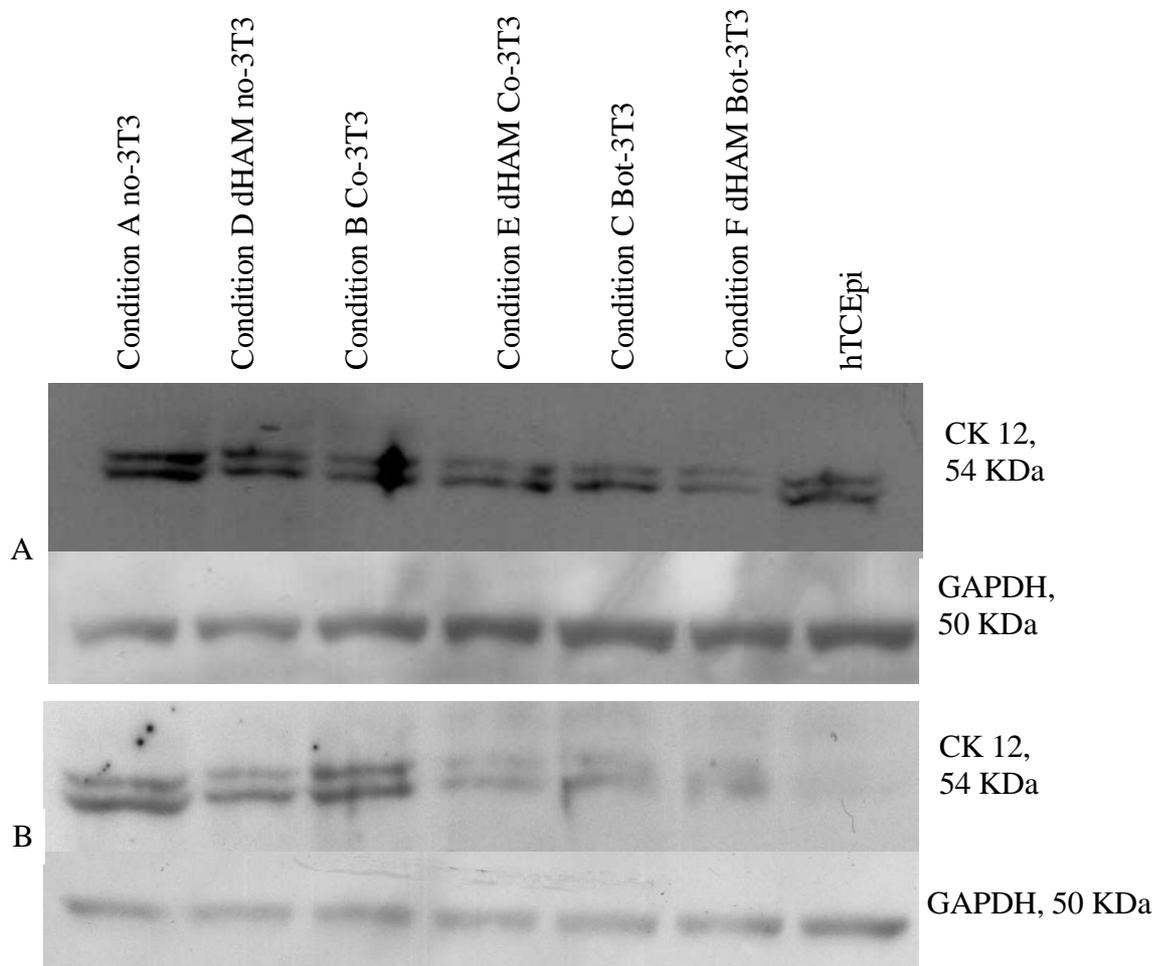
A flow cytometry analysis of CK12 in first three culture conditions (condition A, B and C) has shown positive staining, whereas, the mean fluorescent intensity (MFI) of CK12 is higher in condition A no-3T3, with decreased MFI in condition B Co-3T3 and condition C Bot-3T3 (**figure 3.5.7**).



**Figure: 3.5.7** Flow cytometry analysis of CK12 in limbal-corneal epithelial cells grown on trans-well inserts with conditions A, B and C. Graphical representation of mean fluorescent intensity versus growth conditions. Mean =  $\pm$  S.D and (n=2, sample 63 and 65)

### **3.5.2.3 Western blot analysis of CK12**

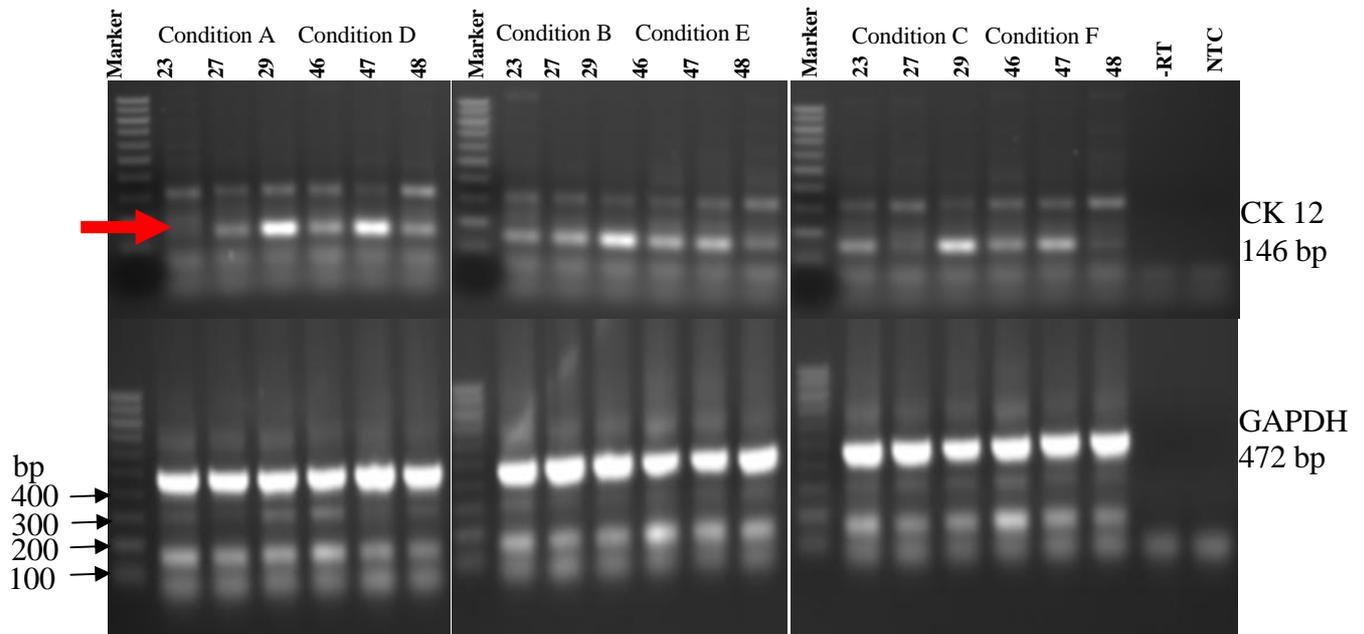
Total protein analysis of CK12 (54 kDa) was performed 6 times with 5 biological replicates. The differential expression in all six culture conditions was observed. Where condition A no-3T3 and condition B Co-3T3 expressed higher level of CK12 protein compared to condition D dHAM no-3T3, condition E dHAM Co-3T3 and Condition F dHAM Bot-3T3. In contrast to immunofluorescence condition C Bot-3T3 shown lower expression CK12 protein. The expression of CK12 is lower in condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 compared to other culture conditions (**figure 3.5.8**).



**Figure: 3.5.8:** Representative western blot profiles of CK12 in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 15, and condition D, E & F with sample 46. (B) Condition A, B and C with sample number 16, and condition D, E & F with sample 48. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=5 biological replicates)

#### **3.5.2.4 RT-PCR analysis of CK12**

The end point RT-PCR of CK12 (146 bp) for all six conditions has shown differential expression in three biological replicate samples. Expression of CK12 in sample 29 in trans-well insets shown higher expression in condition A no-3T3 compare to condition B Co-3T3 and condition C Bot-3T3. Similarly sample 47 with dHAM culture, shown decreased level of CK12 expression in condition F dHAM Bot-3T3 and condition E dHAM Co-3T3 compared to condition D dHAM no-3T3 (**figure 3.5.9**).



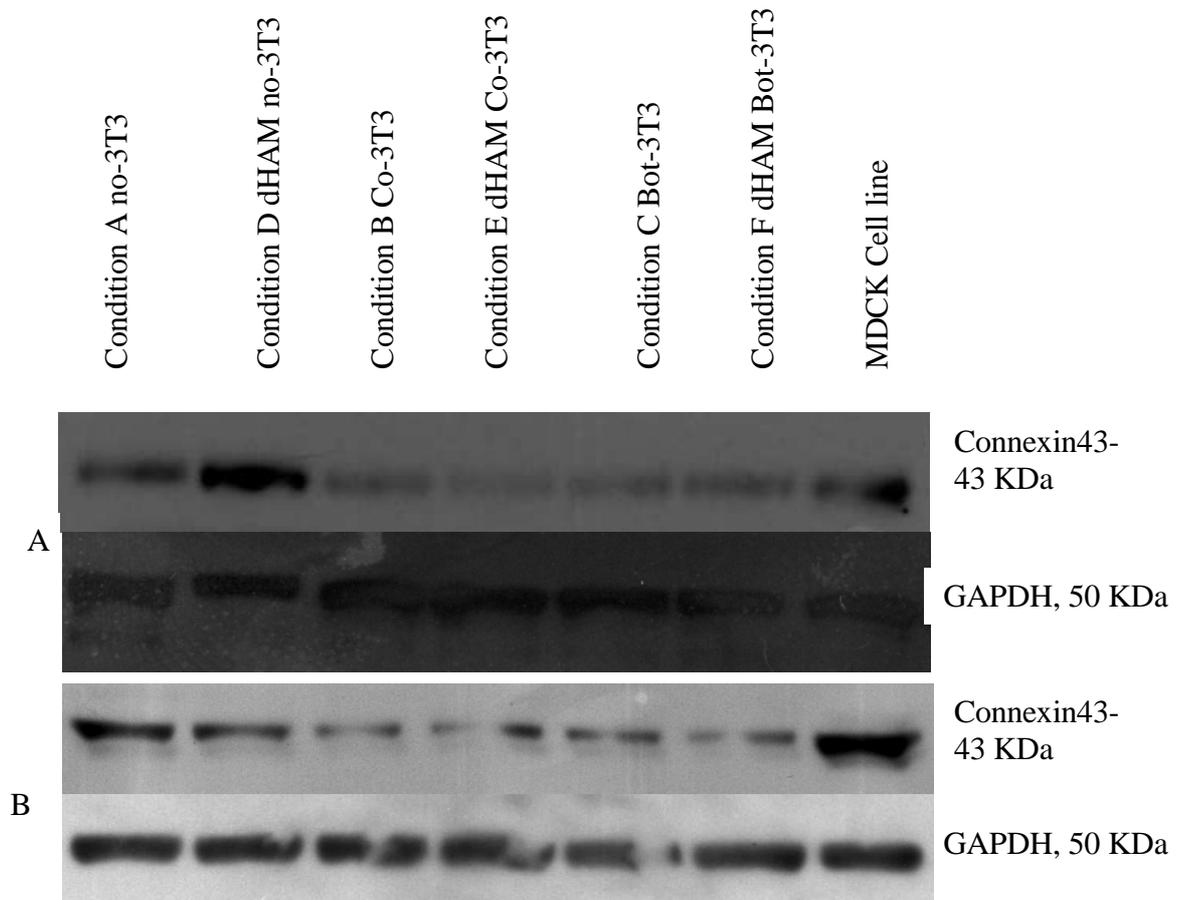
**Figure 3.5.9:** RT-PCR of CK12 (146 bp arrow) on limbal-corneal epithelial cells grown on cell culture inserts and dHAM, with three biological samples in each condition. Sample 23, 27, and 29 cultured on cell culture inserts with condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 conditions. Samples 46, 47 and 48 cultured on dHAM with condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Internal control gene GAPDH (472 bp).

### **3.5.3 Expression of connexin 43 (Cx43)**

In human *in vivo* corneal epithelium, only two gap junction proteins have been identified so far: connexin 43 and connexin 50 (Dong *et al.*, 1994). Connexin 43 is expressed by the corneal basal epithelium but not by limbal basal epithelium (Dong *et al.*, 1994). This implies that connexin 43 expression is acquired during the process of trans amplifying cell (TAC) differentiation.

#### **3.5.3.1 Western blot analysis of connexin 43 (Cx43)**

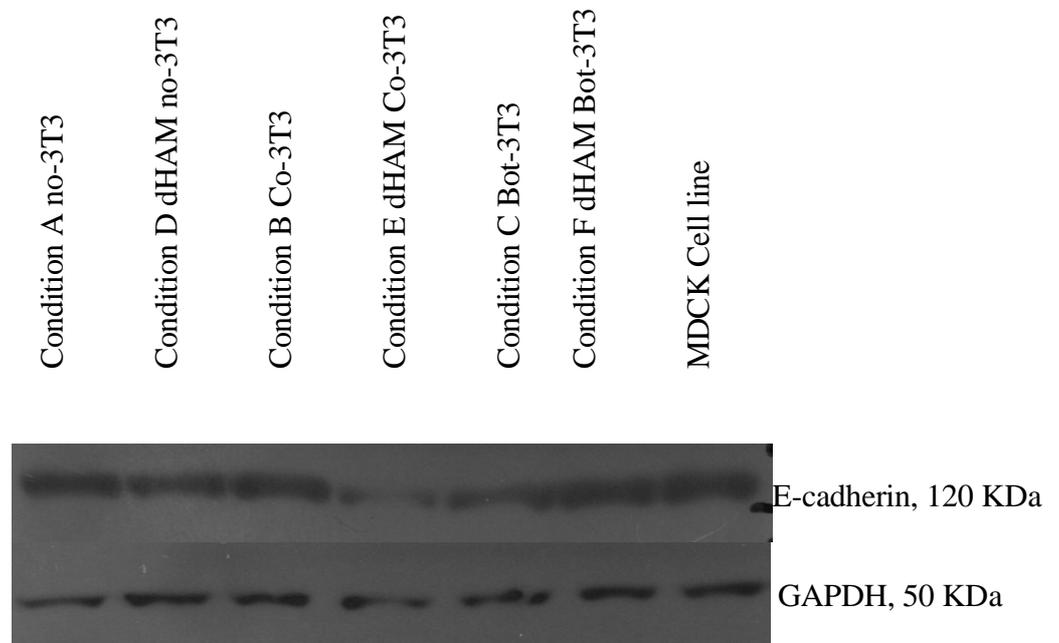
**Figure 3.5.10** illustrates the expression of connexin 43 protein (43 KDa) in cultured limbal-corneal epithelial cells grown in six different culture conditions with 4 biological replicates and 4 technical replicates. The expression of connexin 43 is higher in condition A no-3T3, compared to all other conditions. Limbal-corneal epithelial cells grown with 3T3 feeder cells in condition B Co-3T3 and condition C Bot-3T3 show less protein expression. Similarly expression of Cx43 is lower in culture with dHAM with 3T3 feeder cells. Decreased protein expression was observed condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 compared to condition D dHAM no-3T3.



**Figure: 3.5.10:** Representative western blot profile of Connexin43 in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 18, and condition D, E & F with sample 47. (B) Condition A, B and C with sample number 19, and condition D, E & F with sample 48. Madin-Darby canine kidney (MDCK) cell line as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates).

### 3.5.4 Expression of E-cadherin

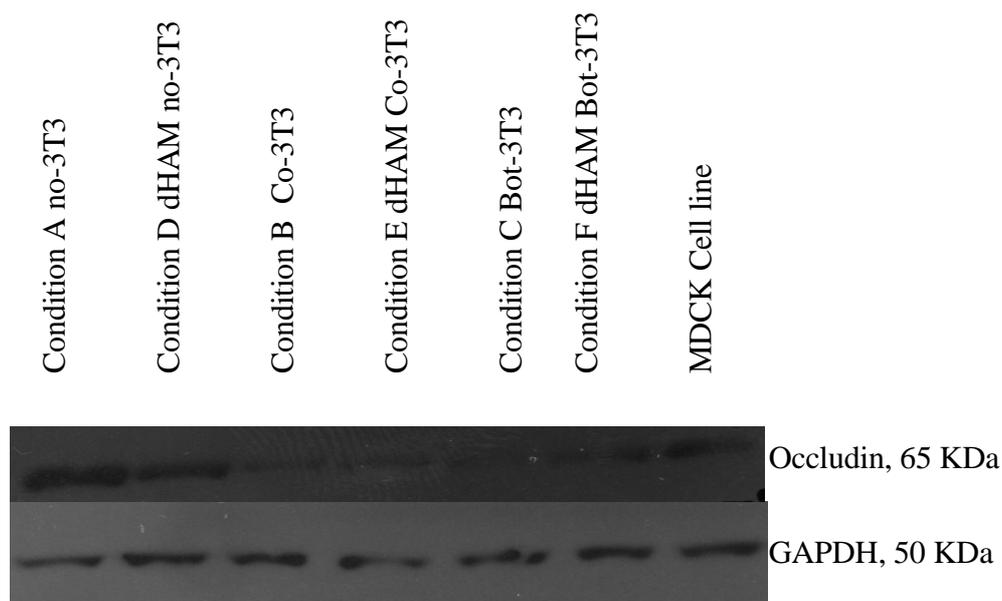
Expression of limbal-corneal epithelial cell differentiation marker- E-cadherin (120 KDa) was analysed with western blot analysis for all six conditions. **Figure 3.5.11** is representative of 4 technical and 4 biological replicates. Expression of tight junction protein E-cadherin was shown to have low level expression in condition E dHAM Co-3T3 and condition C Bot-3T3, compared to other four culture conditions.



**Figure: 3.5.11** Representative western blot of E-cadherin on cultured limbal-corneal cells on cell culture inserts and dHAM. Condition A, B and C with sample number 19, and condition D, E & F with sample 47. Madin-Darby canine kidney (MDCK) cell line used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates)

### 3.5.5 Expression of occludin

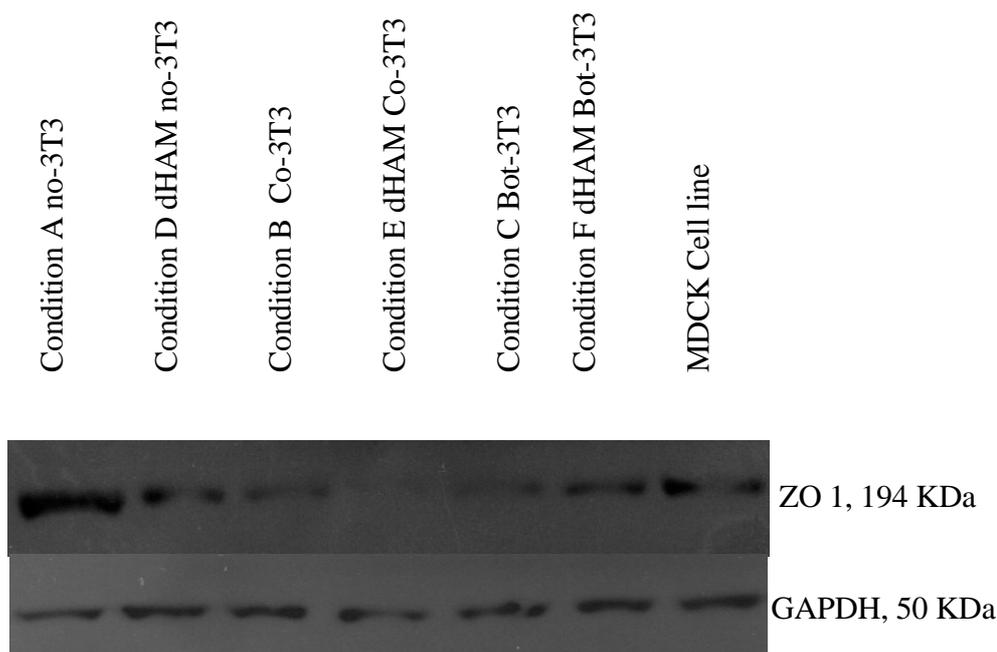
Expression of limbal-corneal epithelial cell differentiation marker occludin (65 KDa), was analysed with western blot analysis for all six conditions. **Figure 3.5.12** is representative of 4 technical and 4 biological replicates. Expression of tight junction protein occludin showed higher level in condition A no-3T3 and condition D dHAM no-3T3 compared to other four culture conditions, where significant decrease in condition E dHAM Co-3T3 and condition C Bot-3T3 was observed.



**Figure: 3.5.12** Representative western blot analysis of occludin, in cultured limbal-corneal cells on cell culture inserts and dHAM. Condition A, B and C with sample number 20, and condition D, E & F with sample 53. Madin-Darby canine kidney (MDCK) cell line used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates).

### 3.5.6 Expression of ZO1

Expression of limbal-corneal epithelial cell differentiation marker ZO1 (194 KDa), was analysed with western blot analysis for all six conditions. **Figure 3.5.13** is representative of 4 technical and 4 biological replicates. Expression of tight junction protein ZO1 showed higher level in condition A no-3T3 and decreased levels in condition D dHAM no-3T3. ZO1 protein showed very low expression in condition E dHAM Co-3T3 compared to all other culture conditions. Whereas, condition F dHAM Bot-3T3 shown similar expression level of condition D dHAM no-3T3.



**Figure: 3.5.13** Representative western blot analysis of ZO1, in cultured limbal-corneal cells on cell culture inserts and dHAM. Condition A, B and C with sample number 19, and condition D, E & F with sample 47. Madin-Darby canine kidney (MDCK) cell line used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates)

### **3.6 Expression of limbal-corneal epithelial stem cell markers in cultured limbal-corneal epithelial cells**

A number of stem cell associated markers were analysed in this study, such as- cytokeratin 19 (Cooper and Sun, 1986), CK14 (Lavker *et al.*, 2004), ATP binding cassette transporter protein ABCG2, and an isoform of p63 is  $\Delta\text{Np63}\alpha$  which has been shown to be more specific for limbal epithelial stem cells than other isoforms (Di Iorio *et al.*, 2005). Integrin  $\beta 1$  has been proposed as keratinocyte stem cell marker (Jones *et al.*, 1995). Integrin  $\beta 1$  and  $\alpha$ -2 are expressed most intensely in the basal cells with expression progressively lost in the more apical layers. The most intense staining for integrins is found where the basal aspect of the basal cells comes into contact with the basement membrane (Kurpakus *et al.*, 1991, Stepp *et al.*, 1993, Tervo *et al.*, 1991).

Cultured limbal-corneal epithelial cells were analysed for stem cell markers CK19, CK14 transporter molecule ABCG2, nuclear protein  $\Delta\text{Np63}\alpha$ ,  $\alpha$ -2 and  $\beta 1$  integrins by immunofluorescence, western blot and RT-PCR analysis.

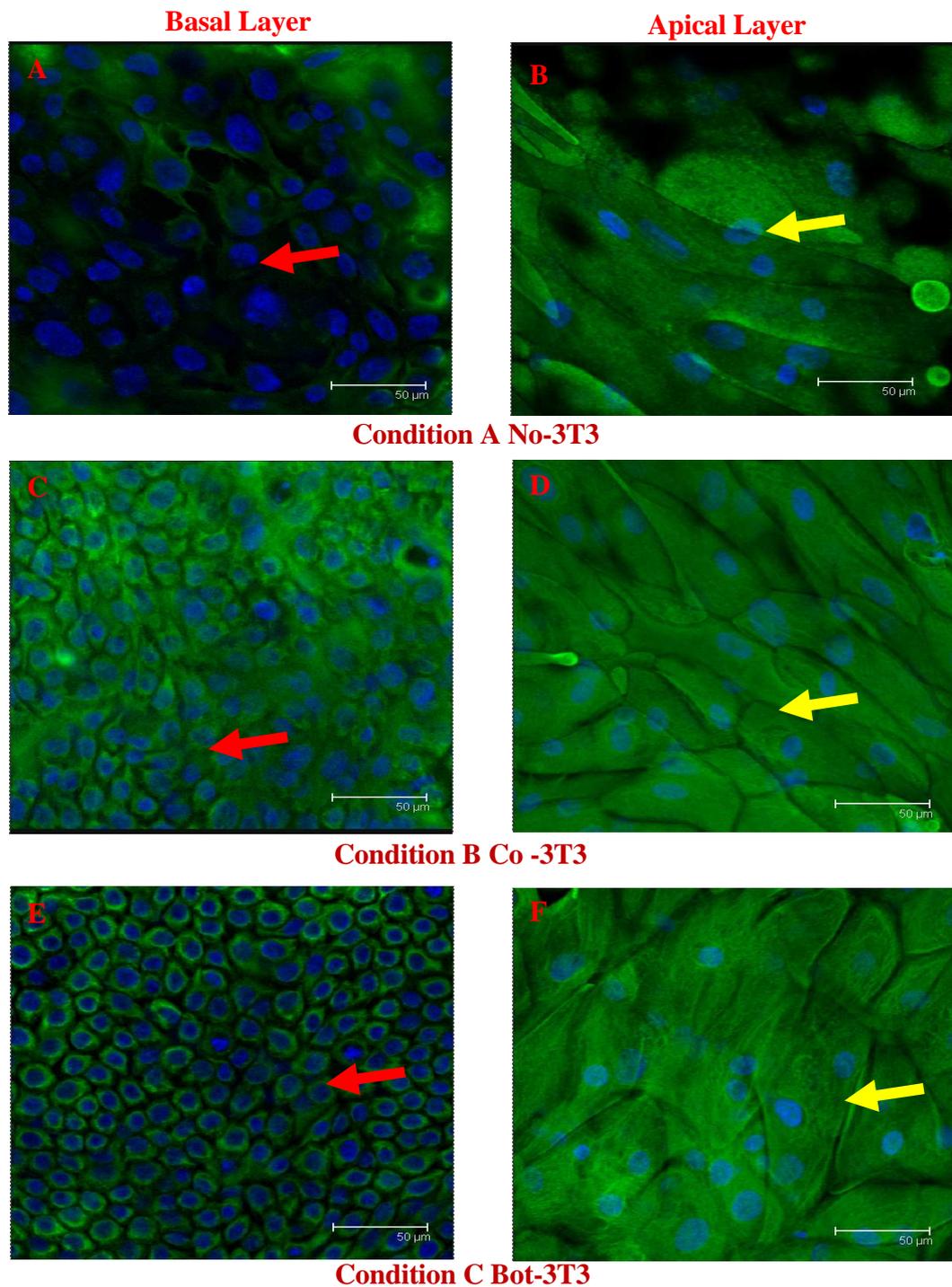
### **3.6.1 Expression of CK19**

Cytokeratins are the principle components of a complex network of intermediate filaments of epithelial cells (Watt, 1989), CK19 is considered as putative marker for limbal-corneal cells as it is expressed in the cytoplasm of the most differentiated cells in the central part of the cornea, in the suprabasal and epibasal layers *in vivo* (Cooper and Sun, 1986).

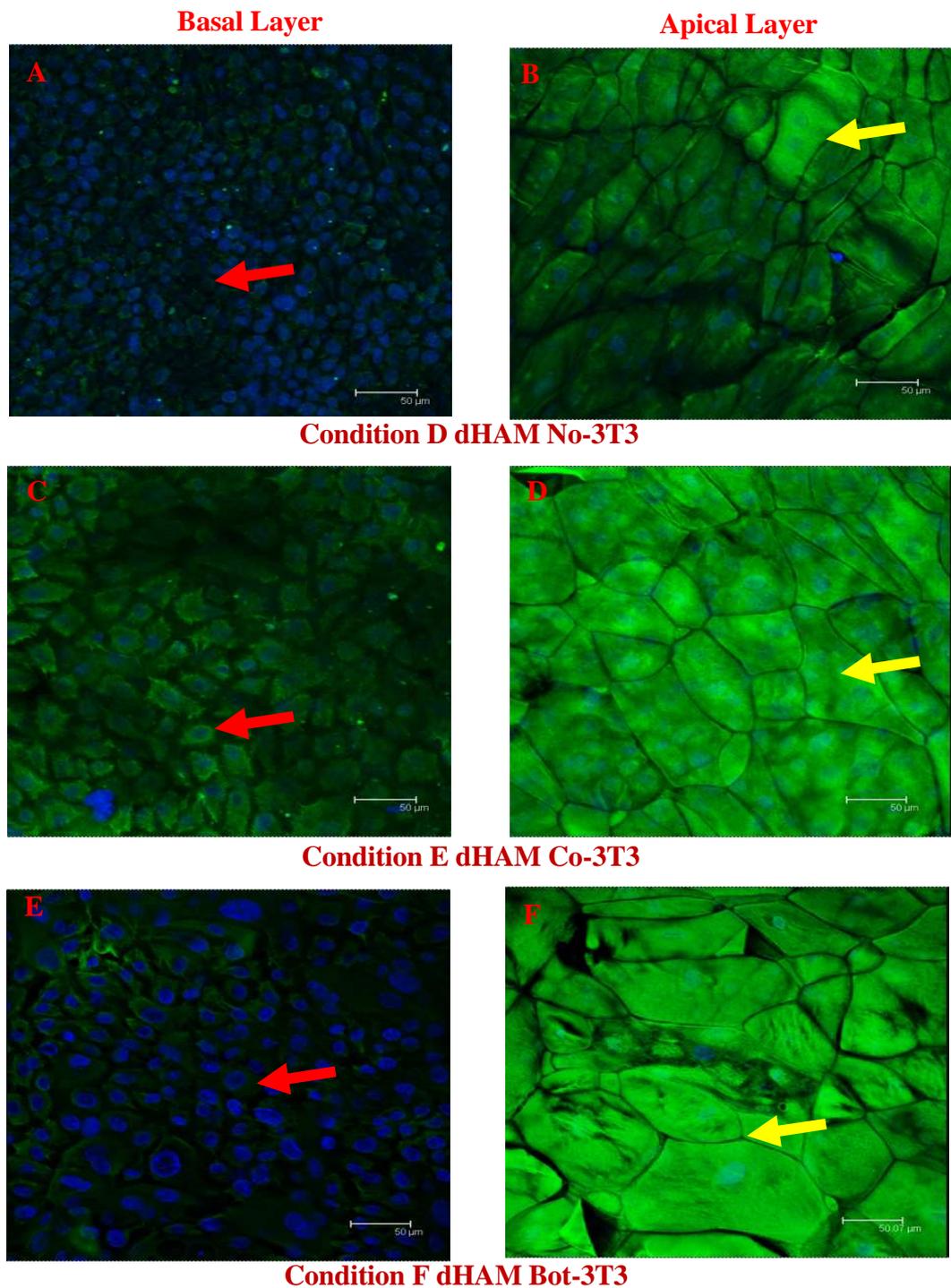
#### **3.6.1.1 Immunofluorescence analysis of CK19 expression**

All six growth conditions showed CK19 expression in the apical layers with large squamous cells showing stronger staining than the basal layer cuboidal compact cells. Condition A no-3T3 shown slightly lower staining in basal cells and higher staining in apical cells (**figure 3.6.1 a and b**). The staining pattern of CK19 was lower in basal cells of condition C Bot-3T3 compared to condition B Co-3T3 basal cells. Similarly the expression of CK19 was reduced in basal cells of condition D dHAM no-3T3 and condition F dHAM Bot-3T3 (**figure 3.6.2a and e**).

Interestingly expression of CK19 at basal cells of condition B Co-3T3 and condition E dHAM Co-3T3 is similar and higher than other culture conditions (**figure 3.6.1c and 3.6.2c**).



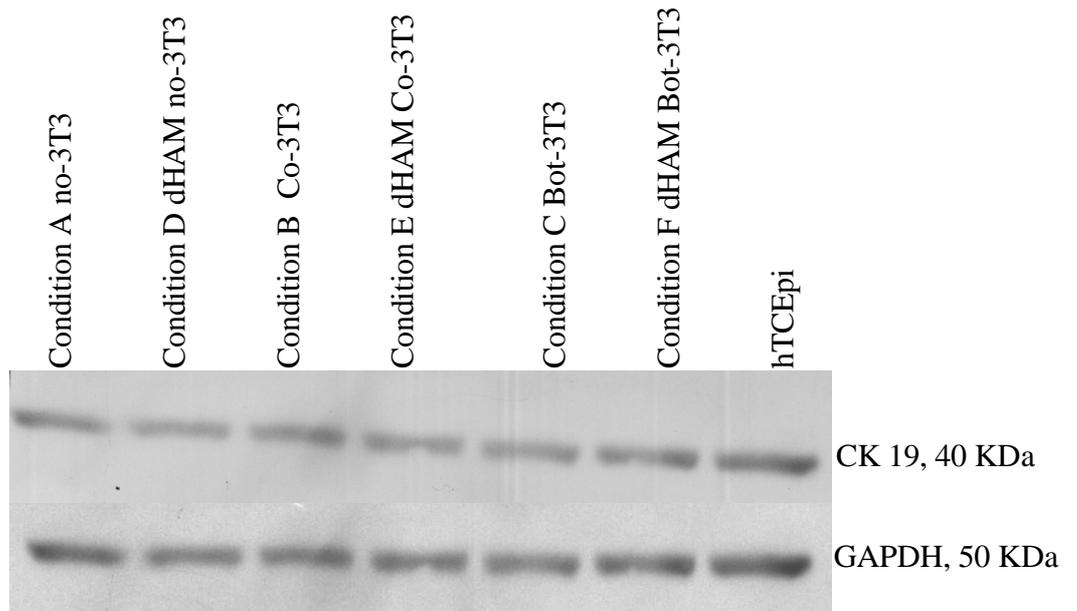
**Figure: 3.6.1** Confocal images of immunofluorescence staining for CK19 in corneal epithelial cells grown on cell culture inserts with conditions A, B and C in basal and apical layers; nuclei (blue) & CK19 (green); Scale bars 50 $\mu$ m. Red arrows example for spindle shaped and yellow arrows for polygonal shaped morphology of cells.



**Figure: 3.6.2** Confocal images of immunofluorescence staining for CK19 in corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E and F in basal and apical layers; nuclei (blue) & CK19 (green); scale bars 50 $\mu$ m. Red arrows example for spindle shaped and yellow arrows for polygonal shaped morphology of cells.

### 3.6.1.2 Western blot analysis of CK19

**Figure 3.6.3** illustrates representative of the expression of CK19 (40 KDa) total protein level in six culture conditions, with 5 technical replicates with 5 biological samples. In contrast to the immunofluorescence staining pattern of CK19 we observed no differential expression of total protein across six culture conditions, telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control.



**Figure: 3.6.3:** Representative western blot profile of CK19 in cultured limbal-corneal cells. Condition A, B and C with sample number 19, and condition D, E & F with sample 47. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=5 biological replicates).

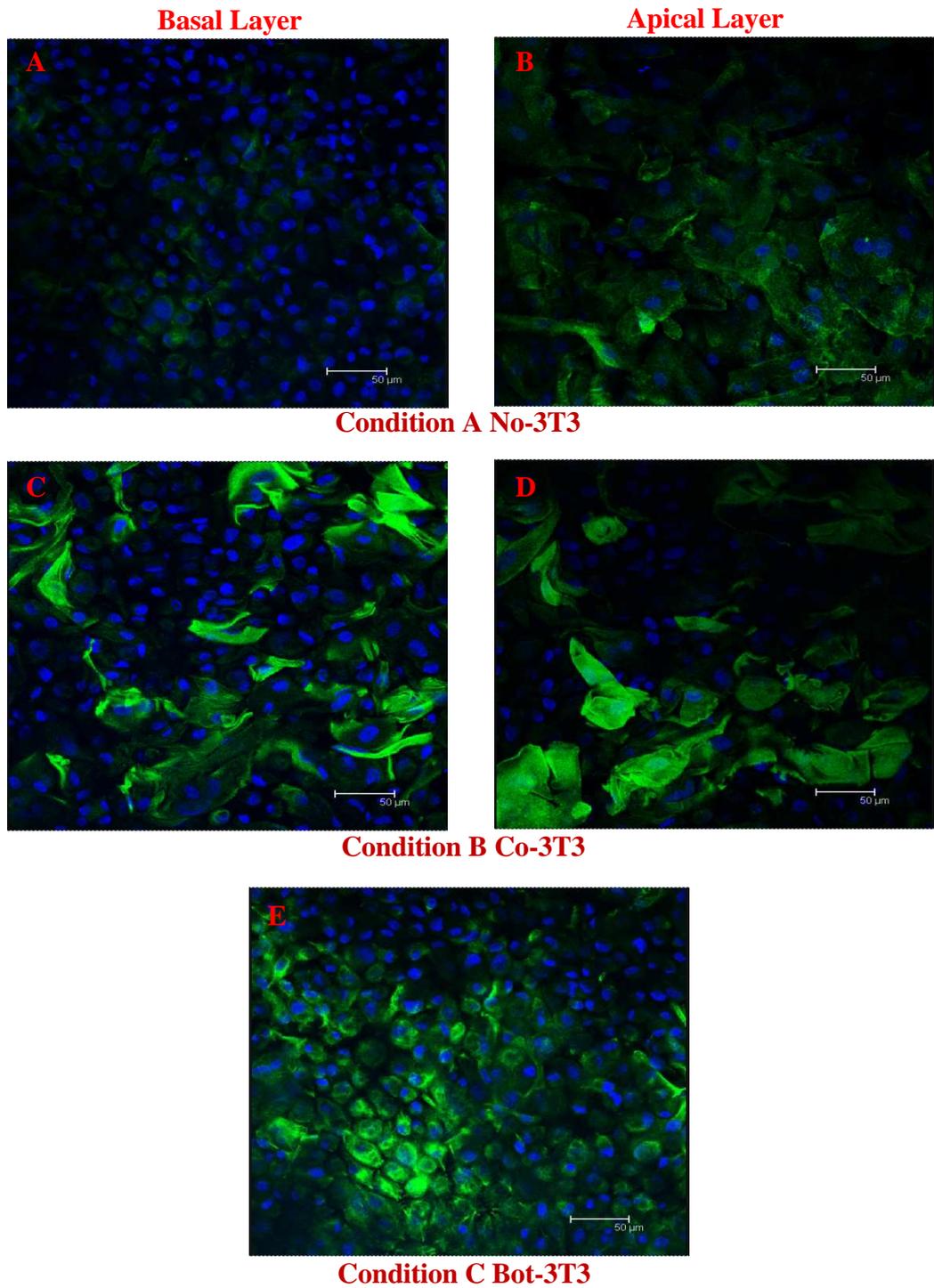
### 3.6.2 Expression of CK14

Cytokeratin 14 is expressed in all layers of the cornea during the foetal stage, but remains restricted to only a few basal cells in the adult *in vivo* (Lavker *et al.*, 2004, Tanifuji-Terai *et al.*, 2006).

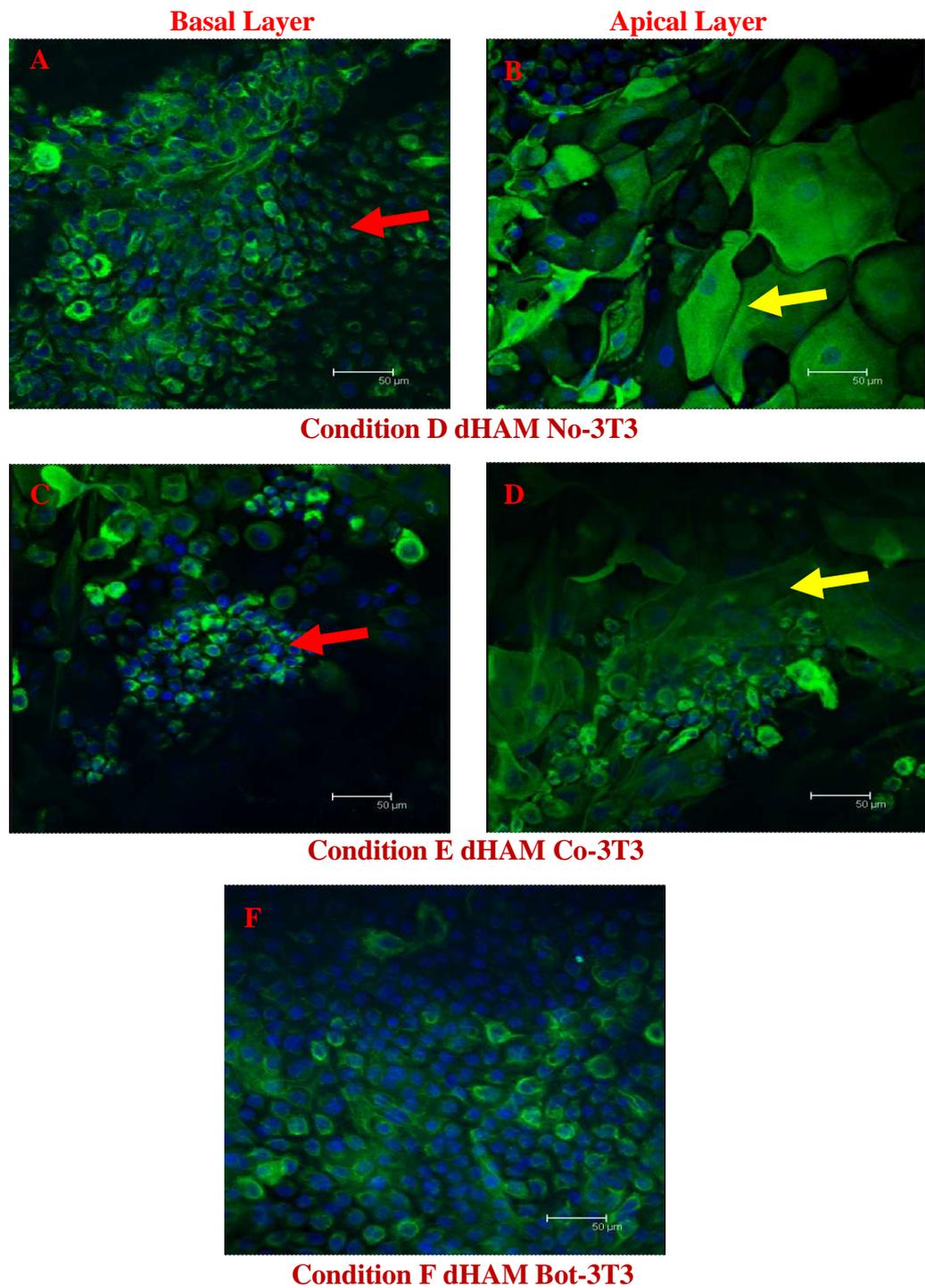
#### 3.6.2.1 Immunofluorescence analysis of CK14 expression

Immunofluorescence analysis of CK14 was performed three times. **Figure 3.6.4** and **3.6.5** show representative images of CK14 in culture limbal-corneal epithelial cells. Staining pattern of CK14 in all cultured conditions is sporadic, mix of high intense stained cells and negative cells.

Condition A no-3T3 has shown very low intensity stained cells at basal layer with increased expression at apical cells where large squamous cells were highly positive (**figure 3.6.4a** and **b**). In contrast condition B Co-3T3 and condition C Bot-3T3 have shown highly positive stained cells at basal layer (**figure 3.6.4c** and **e**). The expression of CK14 was increased in condition D dHAM no-3T3 at basal and apical cells with highly intense stain compared to other culture condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. CK14 expression was lower in condition F dHAM Bot-3T3 at basal and apical layer with sporadic stained cells (**figure 3.6.5e** and **f**).



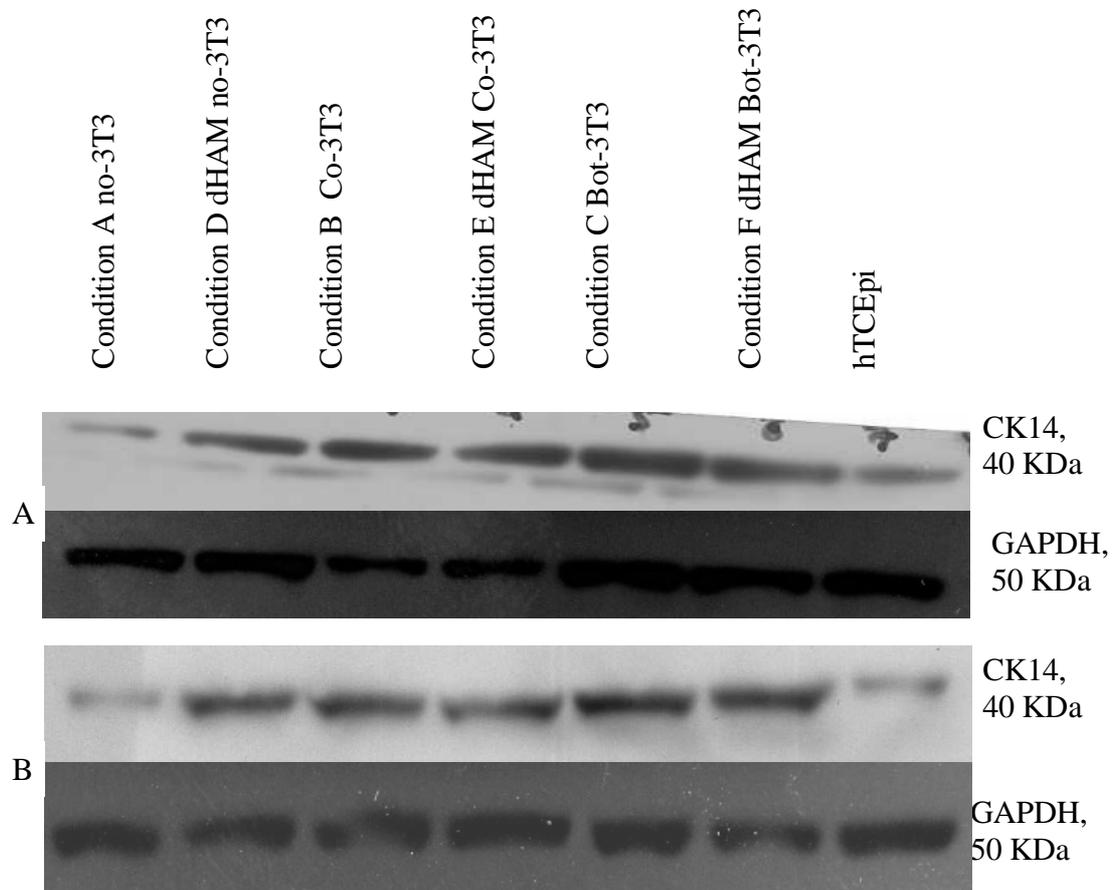
**Figure: 3.6.4** Confocal images of immunofluorescence staining for CK14 in corneal epithelial cells grown on cell culture inserts with conditions D, E basal and apical layers and F basal cells; nuclei in (blue) & CK14 (green); scale bars 50 $\mu$ m.



**Figure:3.6.5** Confocal images of immunofluorescence staining for CK14 in corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E basal and apical layers and F basal cells; nuclei in (blue) & CK14 (green); scale bars 50μm. Red arrows example for spindle shaped and yellow arrows for polygonal shaped morphology of cells.

### **3.6.2.2 Western blot analysis of CK14**

Western blot analysis was performed for CK14. **Figure 3.6.6** is representative of 5 biological and 4 technical replicates of growth conditions. Total protein analysis of CK14 (40 KDa) showed similar amounts of expression in condition B Co-3T3, condition C Bot-3T3, condition D dHAM no-3T3, condition E dHAM Co-3T3, and condition F dHAM Bot-3T3. However these five growth conditions showed an elevated expression compared to condition A no-3T3.



**Figure: 3.6.6** Representative western blot profile of CK14 in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 18, and condition D, E & F with sample 48. (B) Condition A, B and C with sample number 19, and condition D, E & F with sample 53. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=5 biological replicates)

### 3.6.3 Expression of stem cell marker $\Delta$ Np63 $\alpha$

The gene with the most striking effects on the development of stratified epithelia is p63 (Yang *et al.*, 1998). The p63 gene generates six isoforms (Yang and McKeon, 2000).  $\Delta$ Np63 sustains the keratinocyte proliferative potential (Parsa *et al.*, 1999) that is characteristic of stem cells.  $\Delta$ Np63 $\alpha$  is known to present in the limbus but absent from the corneal epithelium (Di Iorio *et al.*, 2005).

#### 3.6.3.1 Immunofluorescence expression of $\Delta$ Np63 $\alpha$

$\Delta$ Np63 $\alpha$  is positive and differentially expressed across six culture conditions. In condition A no-3T3 localisation of  $\Delta$ Np63 $\alpha$  was absent at apical cells, with appearance of positive cells at basal layer, whereas  $\Delta$ Np63 $\alpha$  showed lower staining of apical cells in condition D dHAM no-3T3 (**figure 3.6.7c** and **3.6.11c**). In condition B Co-3T3 and condition C Bot-3T3 a sporadic stain of  $\Delta$ Np63 $\alpha$  was observed in apical cells, with strong staining of basal cells with compact morphology in both conditions, E-cadheren was used as a membrane stain, which illustrates the cell morphology, (**figure 3.6.7; 3.6.8; 3.6.9**); cross sections **figure 3.6.10**.

Similarly, localisation of  $\Delta$ Np63 $\alpha$  in limbal-corneal epithelial cells grown on dHAM with/without 3T3 feeder cells has shown differential expression in basal and apical cells. **Figure 3.6.11 (cross section 3.6.14)**. The expression of  $\Delta$ Np63 $\alpha$  in condition D dHAM no-3T3 showed strong staining in basal cells and decreased staining of apical cells. In condition E dHAM Co-3T3 culture  $\Delta$ Np63 $\alpha$  expression was strong in apical and basal cells with

compact cuboidal cells, **figure 3.6.13**, (**cross section 3.6.14**). Condition F dHAM Bot-3T3 shows strong bright staining of  $\Delta\text{Np63}\alpha$  in basal cells compared to apical cells, where expression was sporadic, similar to conditions B Co-3T3 and condition C Bot-3T3, **figure 3.6.13; 3.6.8; 3.6.9** respectively and cross section **figure 3.6.15**.

### **3.6.3.2 Flow cytometry analysis of $\Delta\text{Np63}\alpha$**

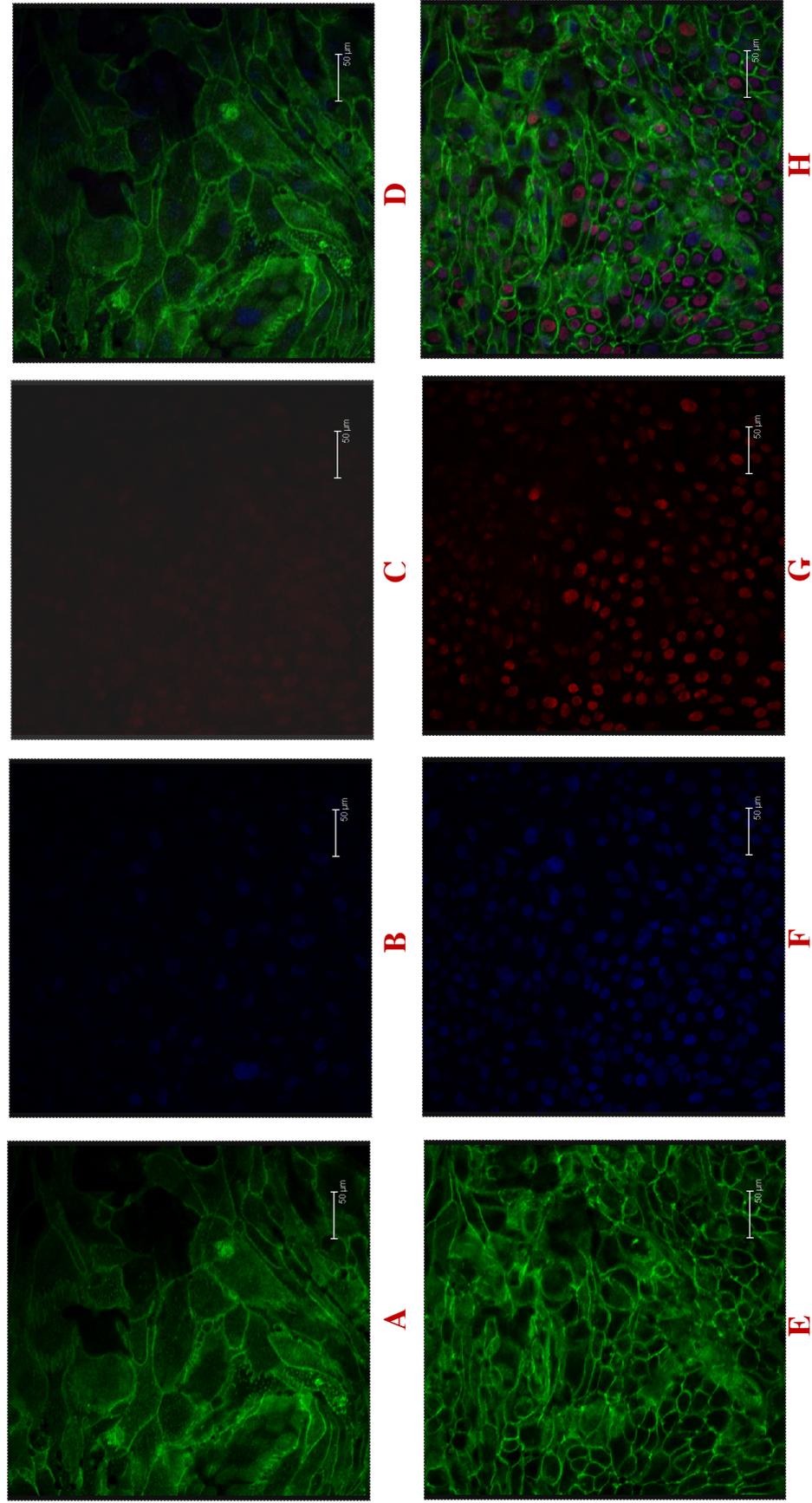
Flow cytometry analysis of  $\Delta\text{Np63}\alpha$  has showed positive staining in the three conditions condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 of limbal-corneal epithelial cells grown on trans-well inserts. The expression of  $\Delta\text{Np63}\alpha$  was differentially expressed in the three growth conditions (**figure 3.6.15**). Condition B Co-3T3 showed a higher mean fluorescent intensity (MFI) compared to condition A no-3T3. Condition C Bot-3T3 showed the high MFI of the three culture conditions assayed.

### **3.6.3.3 Western blot analysis of $\Delta\text{Np63}\alpha$ and p63**

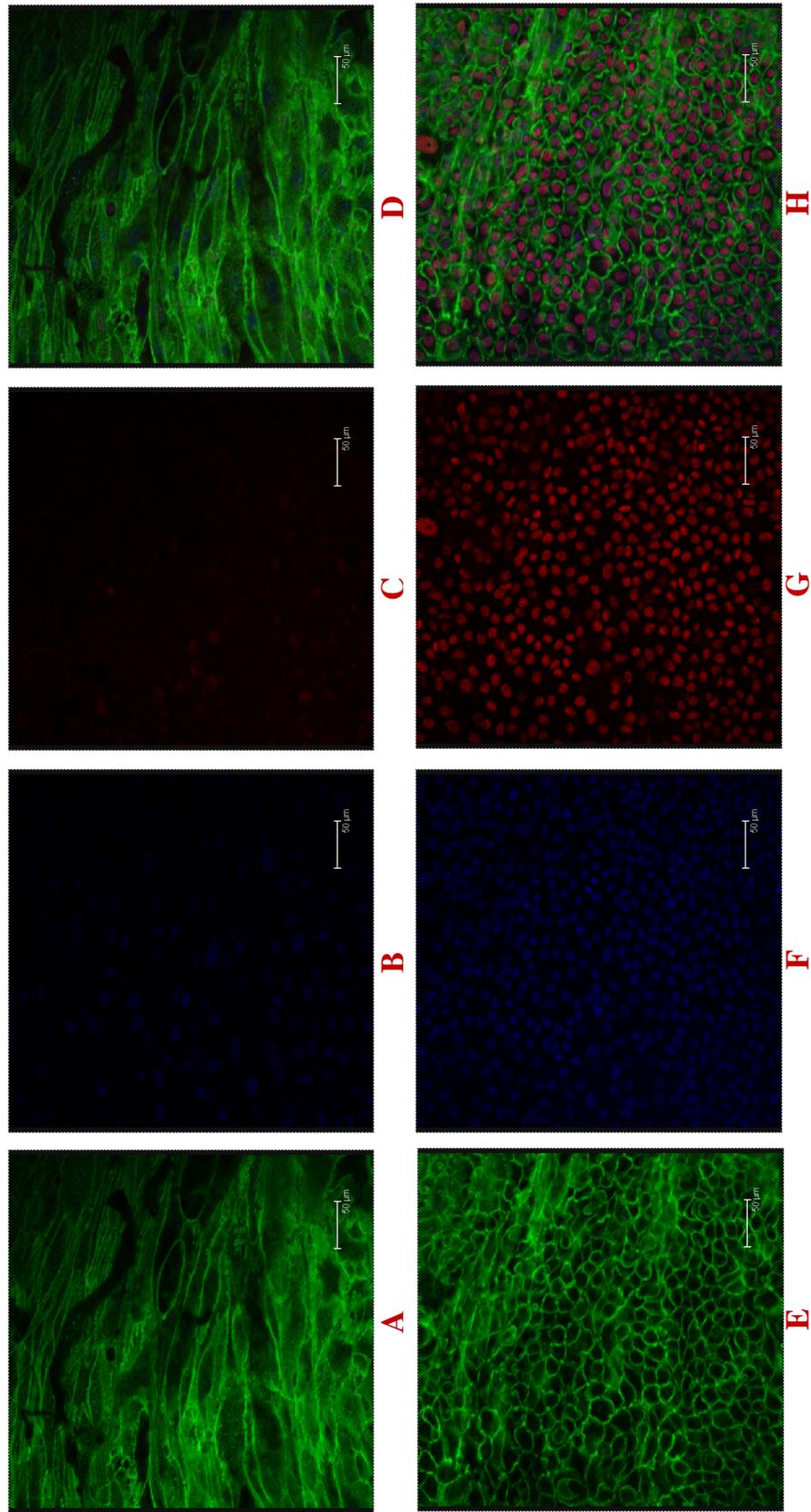
$\Delta\text{Np63}\alpha$  was analysed with five technical and six biological replicates. Differential expression of  $\Delta\text{Np63}\alpha$  (51 KDa) was observed in all six conditions. High levels of  $\Delta\text{Np63}\alpha$  protein was observed in condition C Bot-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3, compared to condition A no-3T3, condition B Co-3T3 and condition D dHAM no-3T3. A notable increase in expression was observed in condition E dHAM Co-3T3 compared to condition D dHAM no-3T3 (**figure 3.6.16**). No differential protein expression was observed in six culture conditions for marker p63 (**figure 3.6.17**)

#### **3.6.3.4 RT-PCR analysis of $\Delta$ Np63 $\alpha$**

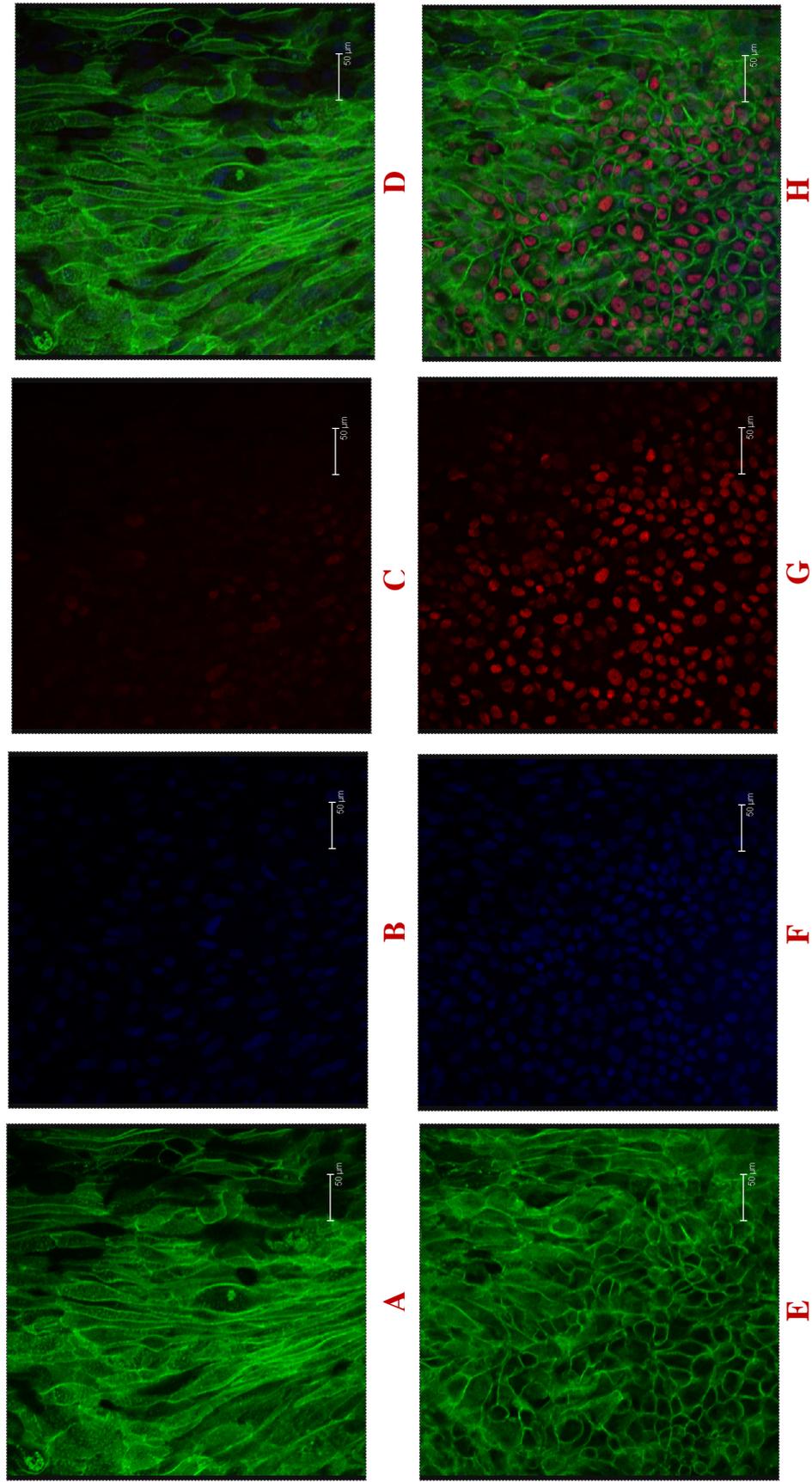
Total mRNA expression of  $\Delta$ Np63 $\alpha$  (198 bp) has shown no differential expression in three biological samples for condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3. Similarly, no differential expression noted in three biological samples for condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 **figure 3.6.18.**



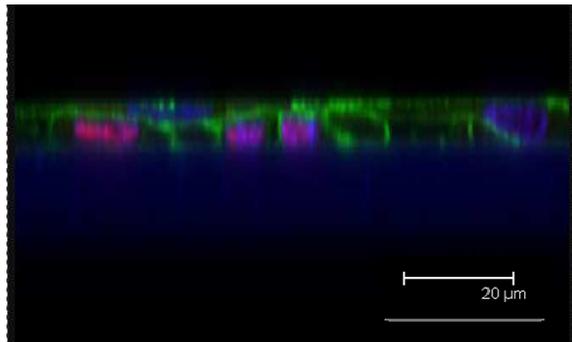
**Figure: 3.6.7** Confocal images of immunofluorescence staining for  $\Delta Np63\alpha$  in condition A no-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal) ,  $\Delta Np63\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu$ m.



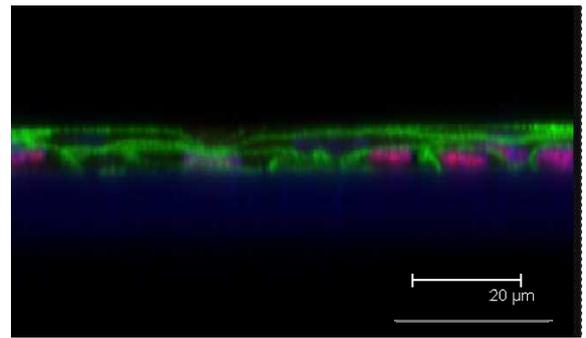
**Figure: 3.6.8** Confocal images of immunofluorescence staining for  $\Delta Np63\alpha$  in condition B Co-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B-basal),  $\Delta Np63\alpha$  (Red) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu m$ .



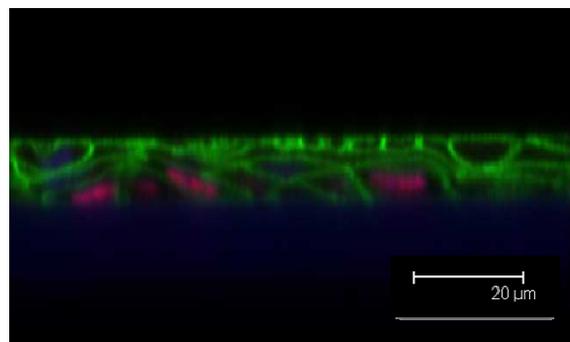
**Figure: 3.6.9** Confocal images of immunofluorescence staining for  $\Delta Np63\alpha$  in condition C Bot-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B-basal),  $\Delta Np63\alpha$  (Red) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu$ m.



**Condition A No-3T3**

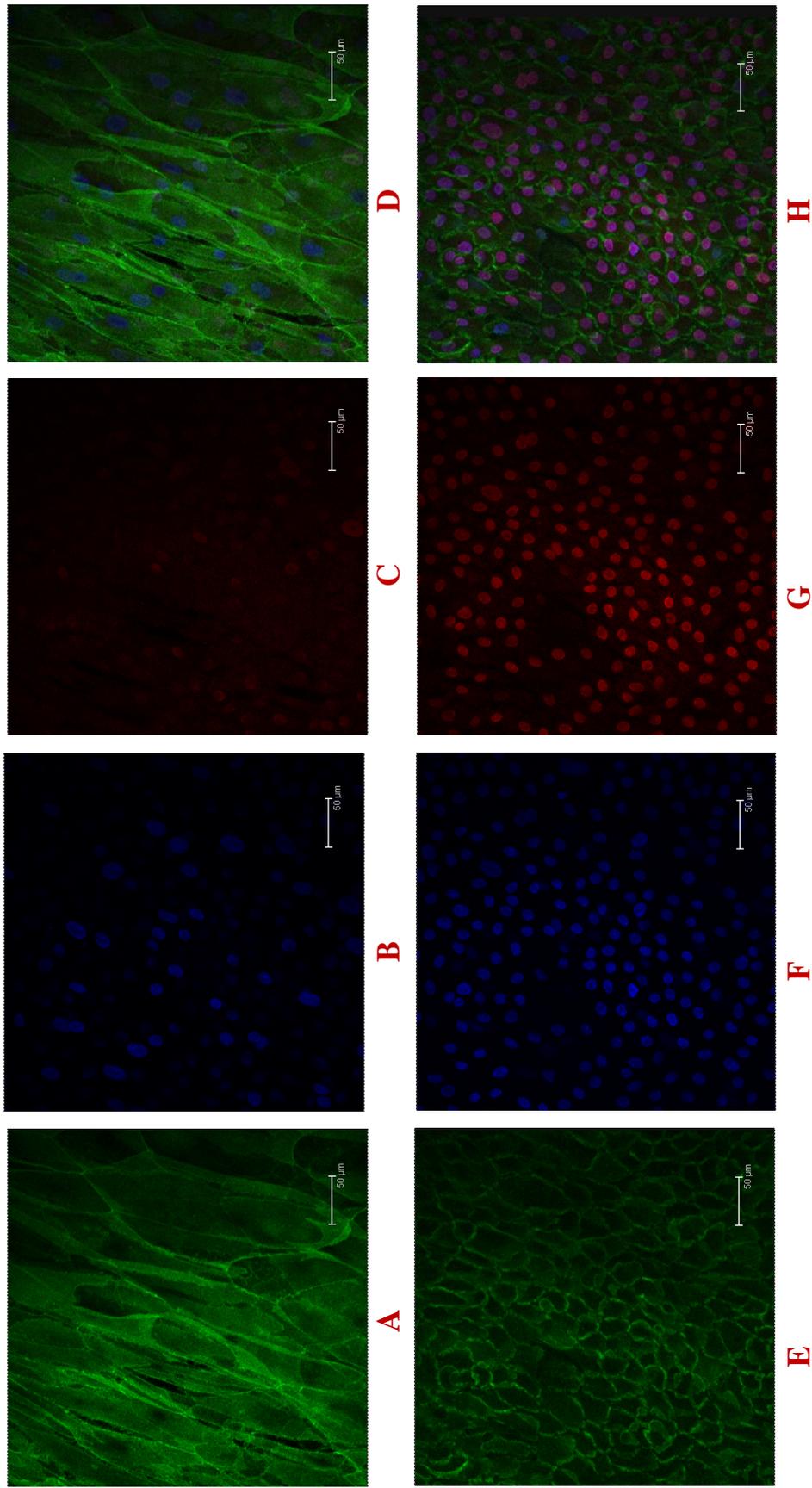


**Condition B Co-3T3**

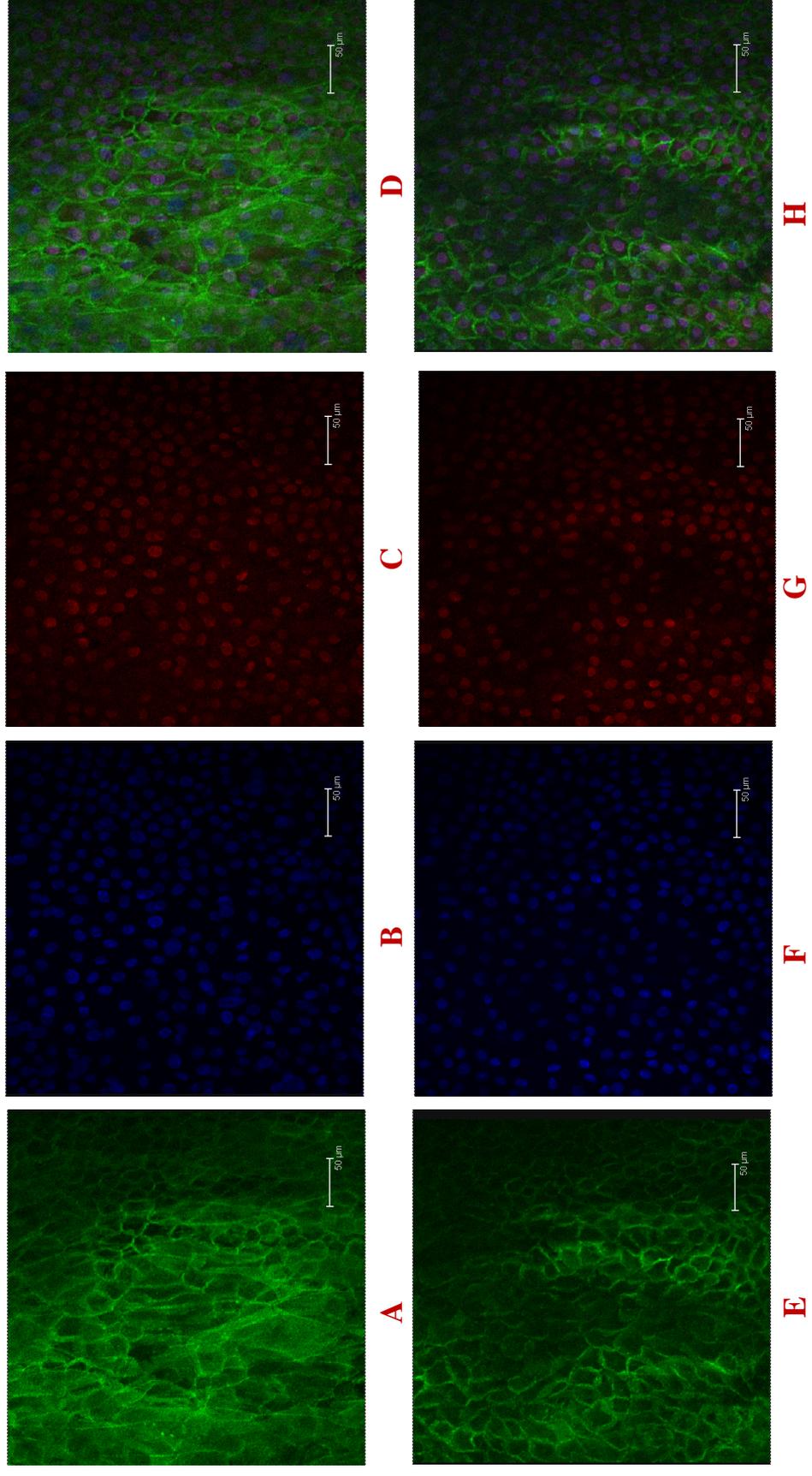


**Condition C Bot-3T3**

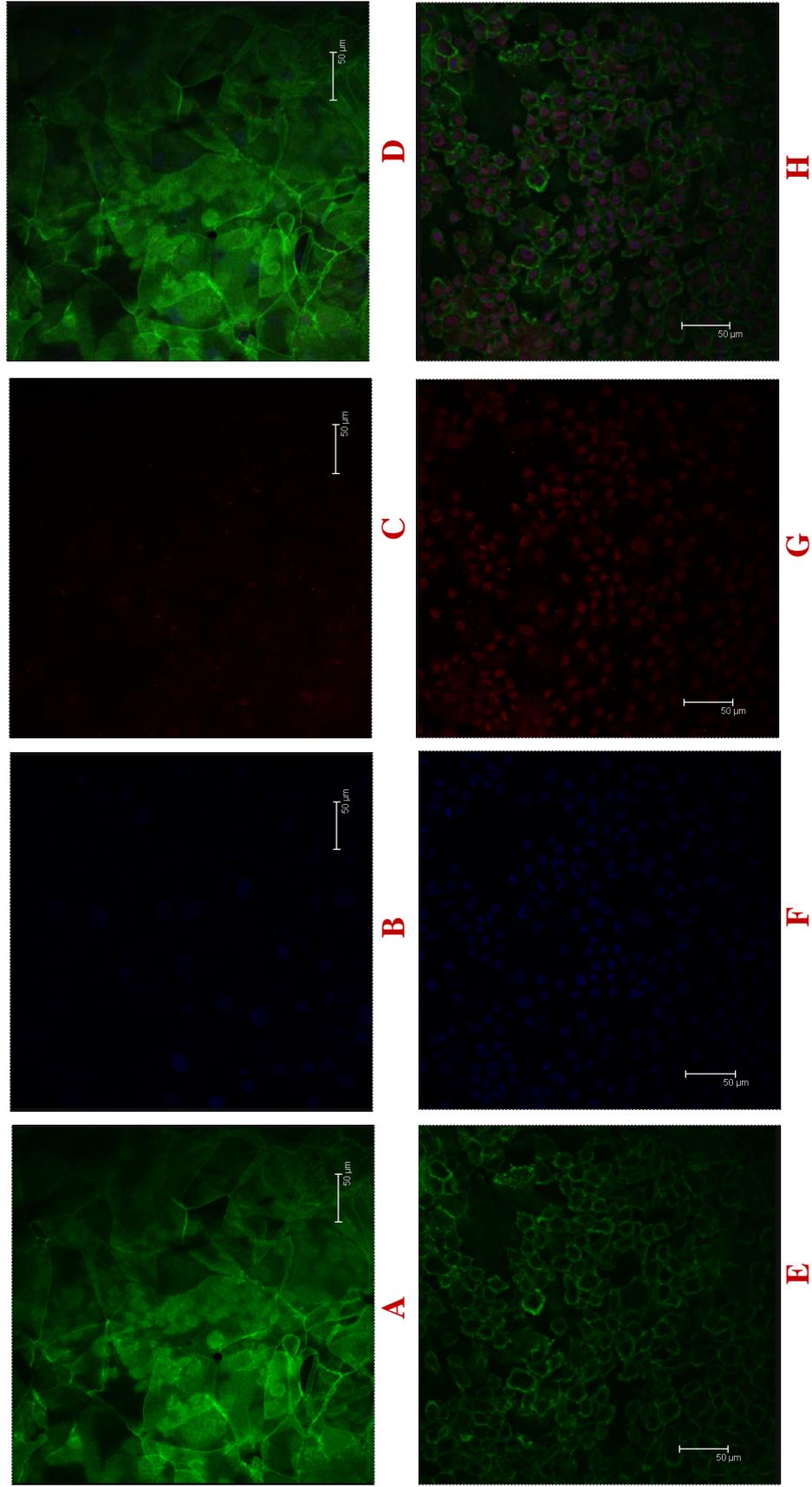
**Figure: 3.6.10** Limbal-corneal epithelial cells on cell culture insert. Cross section  $\Delta$ Np63 $\alpha$  (red) /nuclei (blue)/E-cadherin (green). Scale bar 20 $\mu$ m.



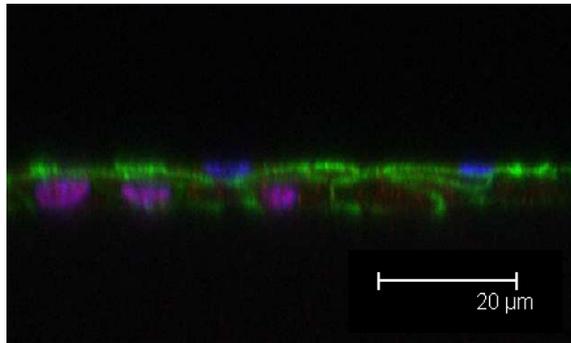
**Figure 3.6.11:** Immunofluorescence staining confocal images of  $\Delta Np63\alpha$  in condition D dHAM no-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu$ m.



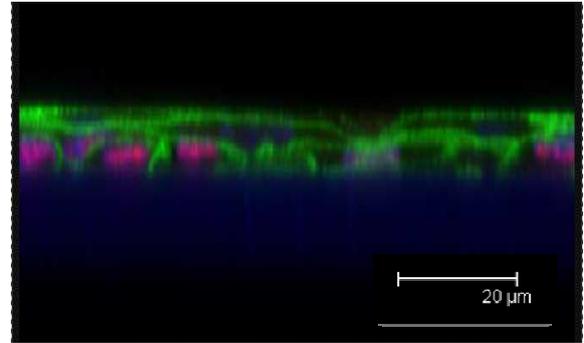
**Figure 3.6.12:** Immunofluorescence staining confocal images of  $\Delta Np63\alpha$  in condition E dHAM Co-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu m$ .



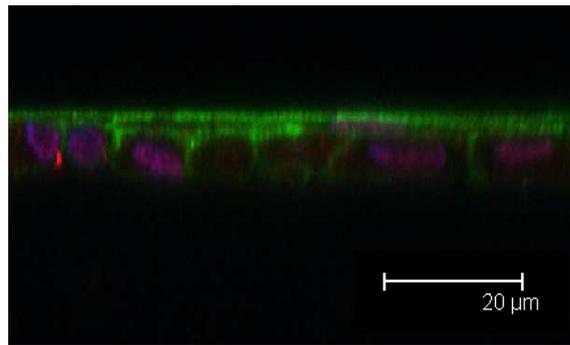
**Figure 3.6.13:** Immunofluorescence staining confocal images of  $\Delta Np63\alpha$  in condition F dHAM Bot-3T3. E-cadherin (green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu$ m.



**Condition D dHAM No-3T3**

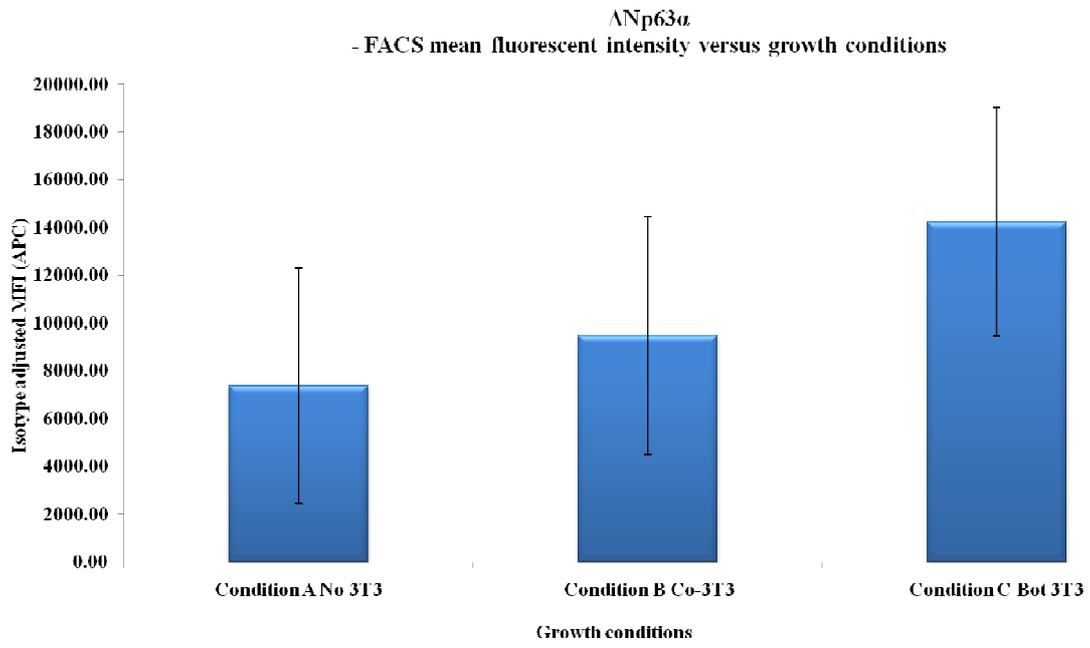


**Condition E dHAM Co-3T3**

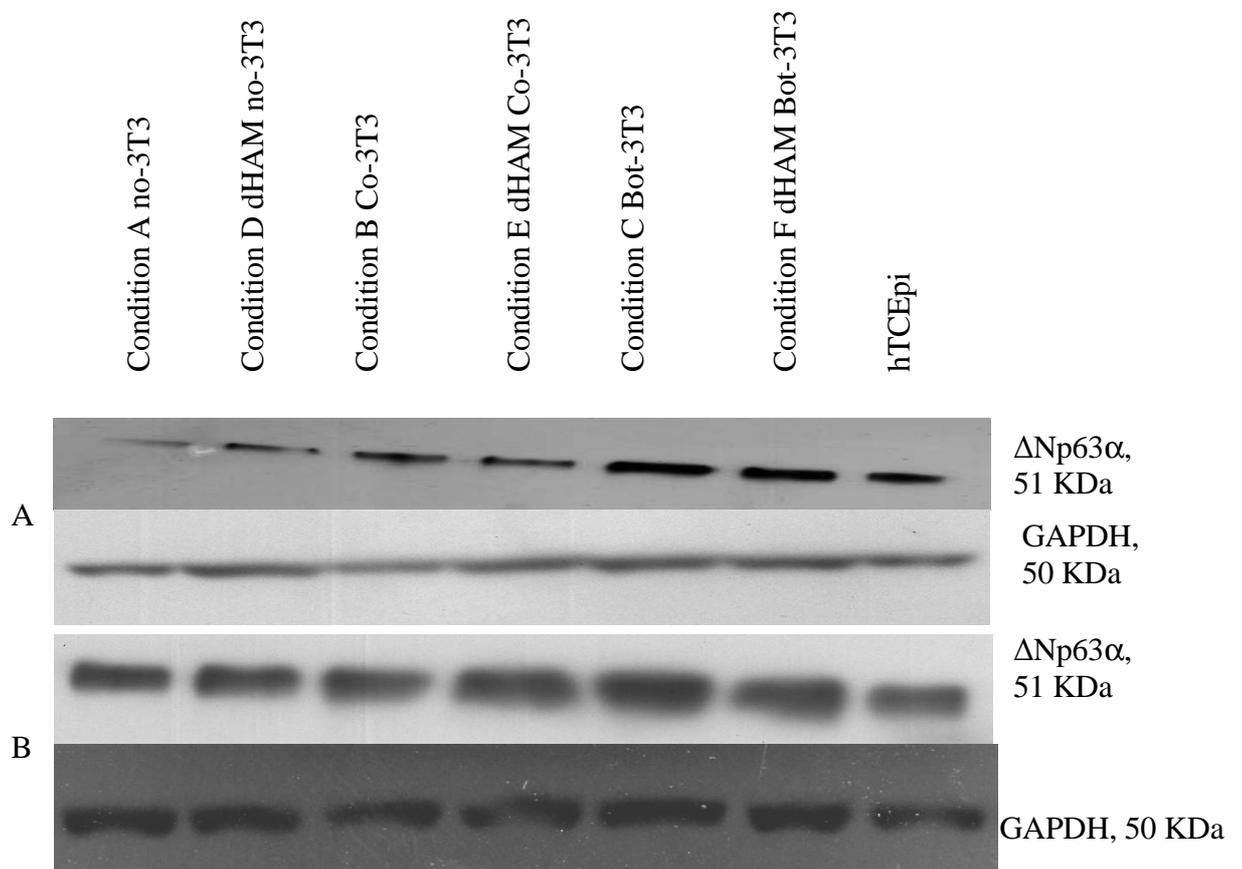


**Condition F dHAM Bot-3T3**

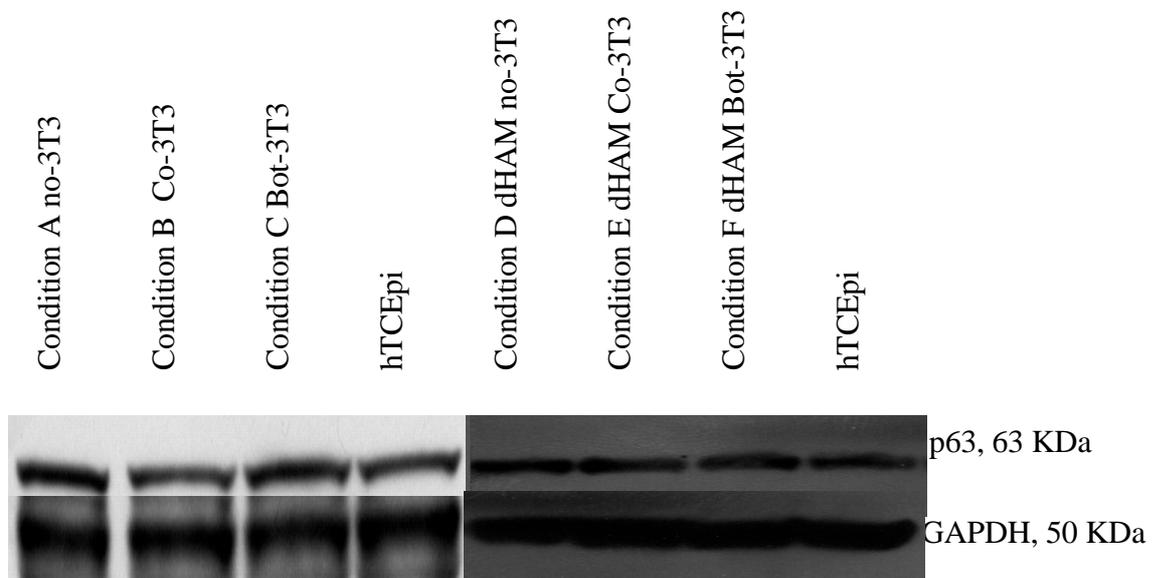
**Figure: 3.6.14** Limbal-corneal epithelial cells on denuded human amniotic membrane (dHAM) cross section  $\Delta Np63\alpha$  (red) nuclei (blue) E-cadherin (green). Scale bars 20 $\mu$ m.



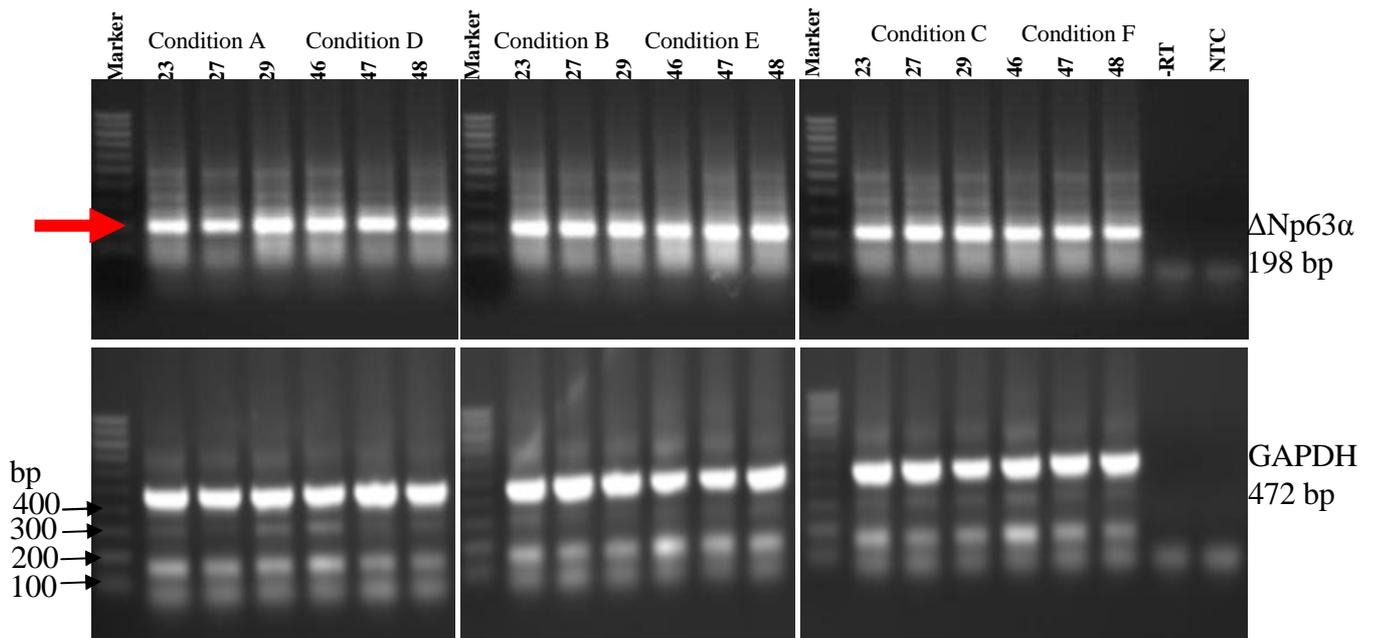
**Figure: 3.6.15** Flow cytometry analysis of  $\Delta Np63\alpha$  in limbal-corneal epithelial cells grown on trans-well inserts with conditions A, B and C. Graphical representation of mean fluorescent intensity versus growth conditions. Mean =  $\pm$  S.D and (n=2; Sample 63 and 65)



**Figure: 3.6.16** Representative western blot profile of  $\Delta Np63\alpha$ , in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 18, and condition D, E & F with sample 48. (B) Condition A, B and C with sample number 19, and condition D, E & F with sample 53. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=5 biological replicates).



**Figure: 3.6.17** Representative western blot profile of p63, in cultured limbal-corneal cells on cell culture inserts and dHAM. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control. Housekeeping protein GAPDH as an internal control (n=3 biological replicates).



**Figure 3.6.18:** RT-PCR of  $\Delta Np63\alpha$  (198 bp arrow) in limbal-corneal epithelial cells grown on cell culture inserts and dHAM, with three biological samples for each condition. Sample 23, 27 and 29 cultured on cell culture inserts with condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 conditions. Samples 46, 47 and 48 cultured on dHAM with condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Internal control gene GAPDH (472 bp).

### **3.6.4 Expression of stem cell marker ABCG2**

In 2004 ATP-binding cassette (ABC) transporters, such as ABCG2 transporter protein have been identified as marker for limbal stem cells. This protein is immunolocalised to the cell membrane and cytoplasm of some human limbal basal epithelial cells, but not in most limbal suprabasal cells and corneal epithelial cells (Chen *et al.*, 2004).

#### **3.6.4.1 Immunofluorescence analysis of ABCG2**

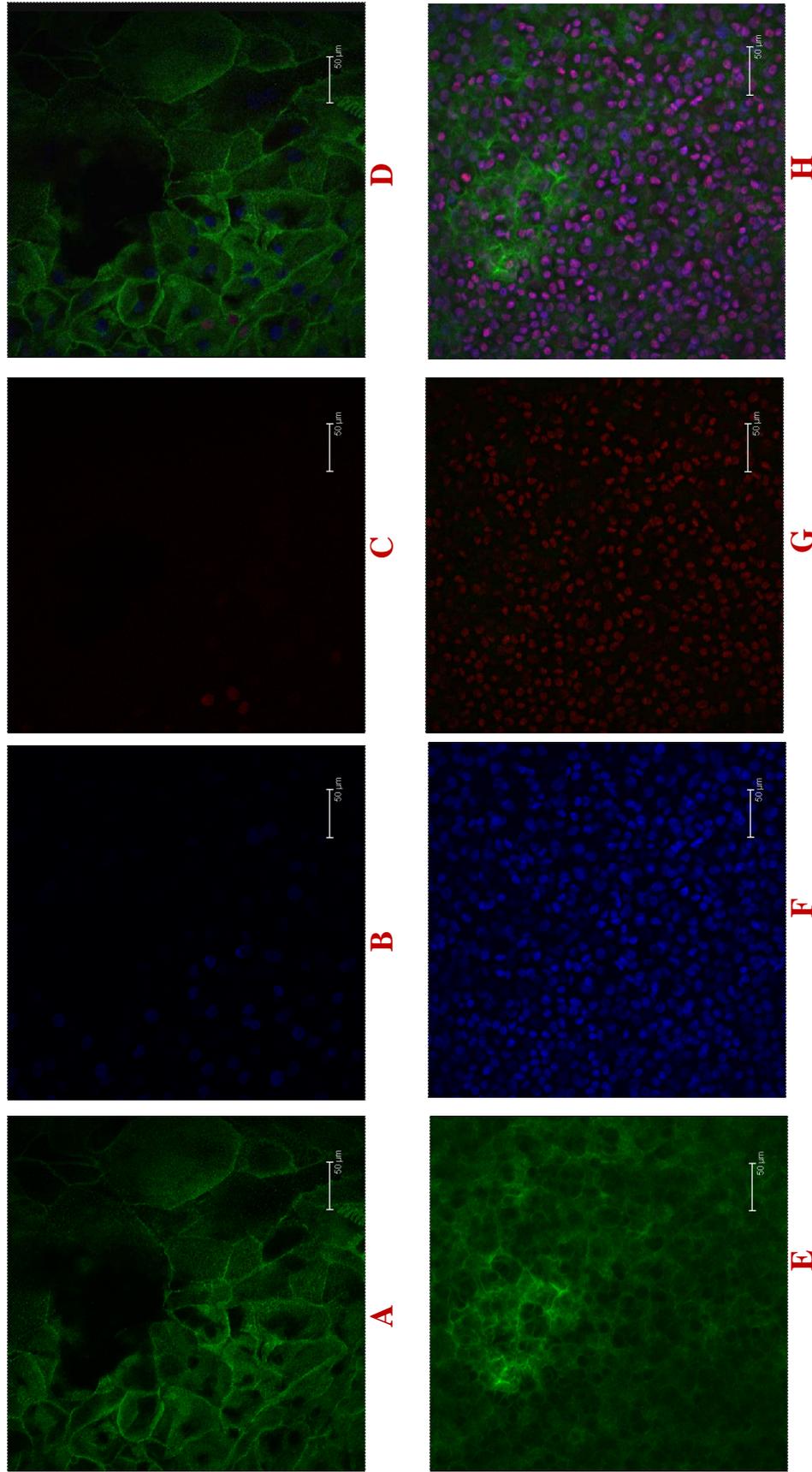
Using mAb clone BXP-21, the ABCG2 transporter protein was primarily immunodetected in the cell membrane and cytoplasm of cultured limbal-corneal epithelial cells at basal and apical layers. Three biological samples were analysed, representative ABCG2 expression shown in **figures 3.6.19 to 3.6.26**.

The staining of ABCG2 in condition A no-3T3 showed slightly lower sporadic membrane stain in basal cells and increased stain at apical cells with mostly cytoplasmic localisation. Similarly in condition B Co-3T3 and condition C Bot-3T3, ABCG2 expression was increased in basal cell membrane stain and in apical cells with cytoplasmic staining (**figures 3.6.19 to 3.6.21**). Cross sections of condition A no-3T3, condition B Co-3T3, condition C Bot-3T3 illustrated in **figure 3.6.22**, where high ABCG2 expression was shown in apical cells.

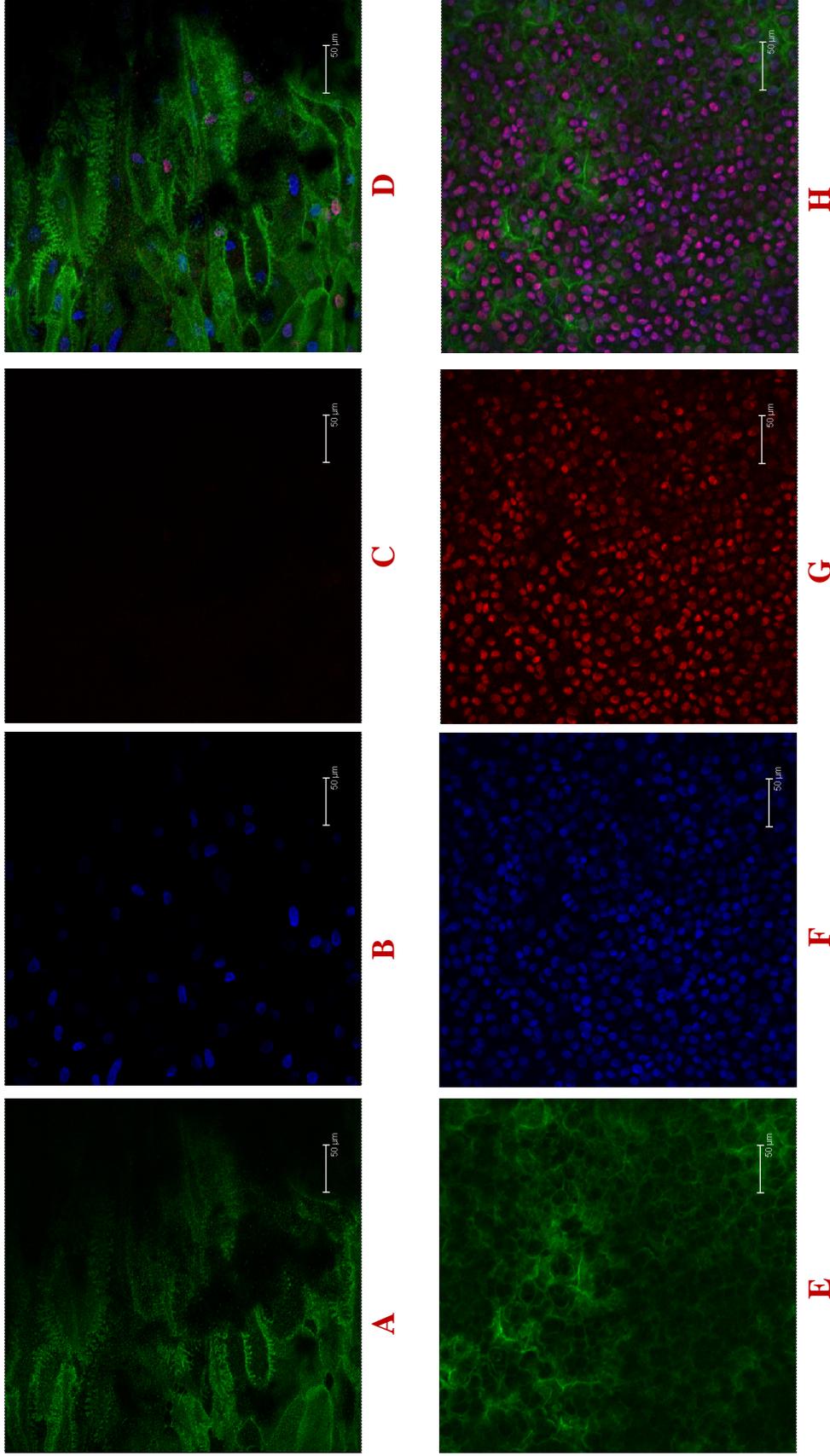
ABCG2 showed increased expression in condition D dHAM no-3T3 in apical and basal cells compared to condition A no-3T3 where only sporadic expression was observed in basal cells

(**figure 3.6.23** and **figure 3.6.19**). The high intense staining of ABCG2 shown in condition F dHAM Bot-3T3 in apical and basal cells is confined to membrane staining. Similar expression was observed in condition E dHAM Co-3T3.

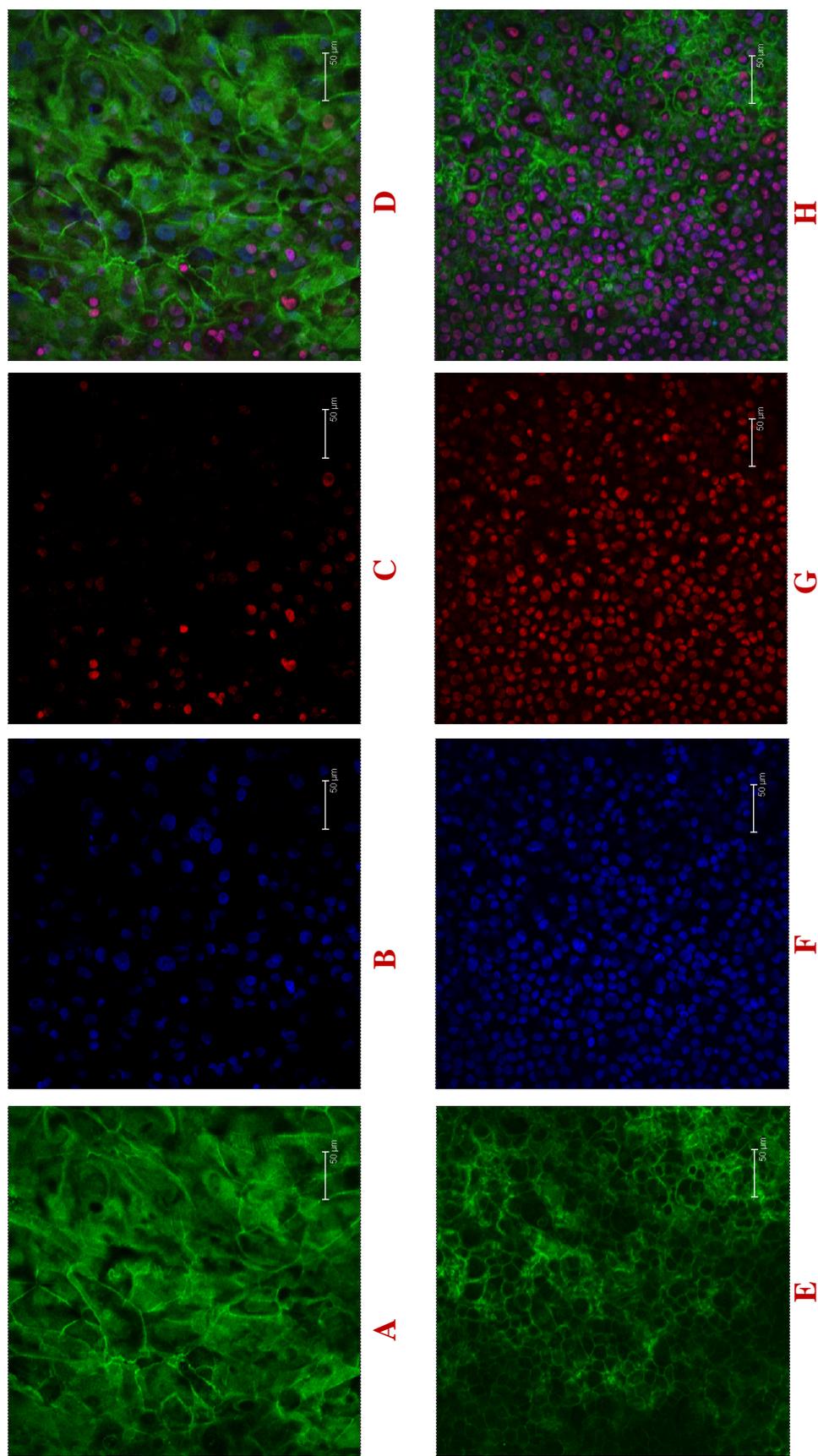
ABCG2 expression was highly intense at basal cells of condition B Co-3T3, condition C Bot-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 cultures. Where ABCG2 negative cells interspersed with the ABCG2 positive cells in the basal layer of culture, and apical cells were expressed most cytoplasmic positive stain of ABCG2.



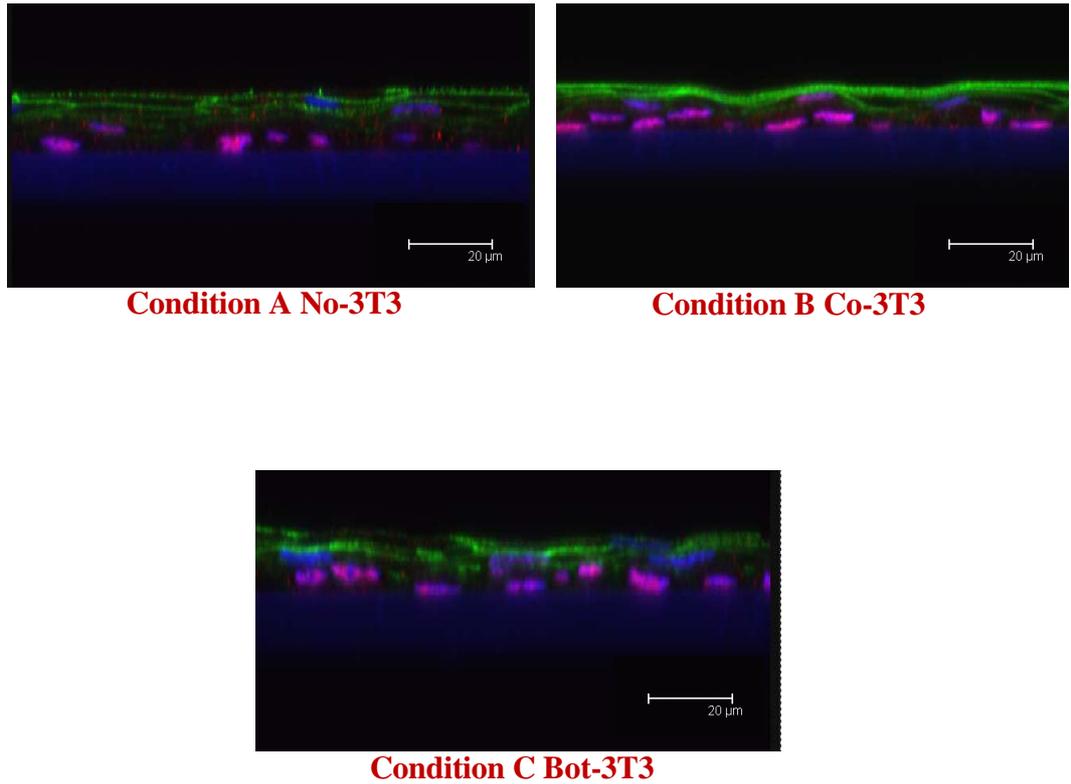
**Figure:3.6.19** Confocal images of immunofluorescence staining for ABCG2 and  $\Delta Np63\alpha$  in Condition A no-3T3, ABCG2 (Green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu m$ .



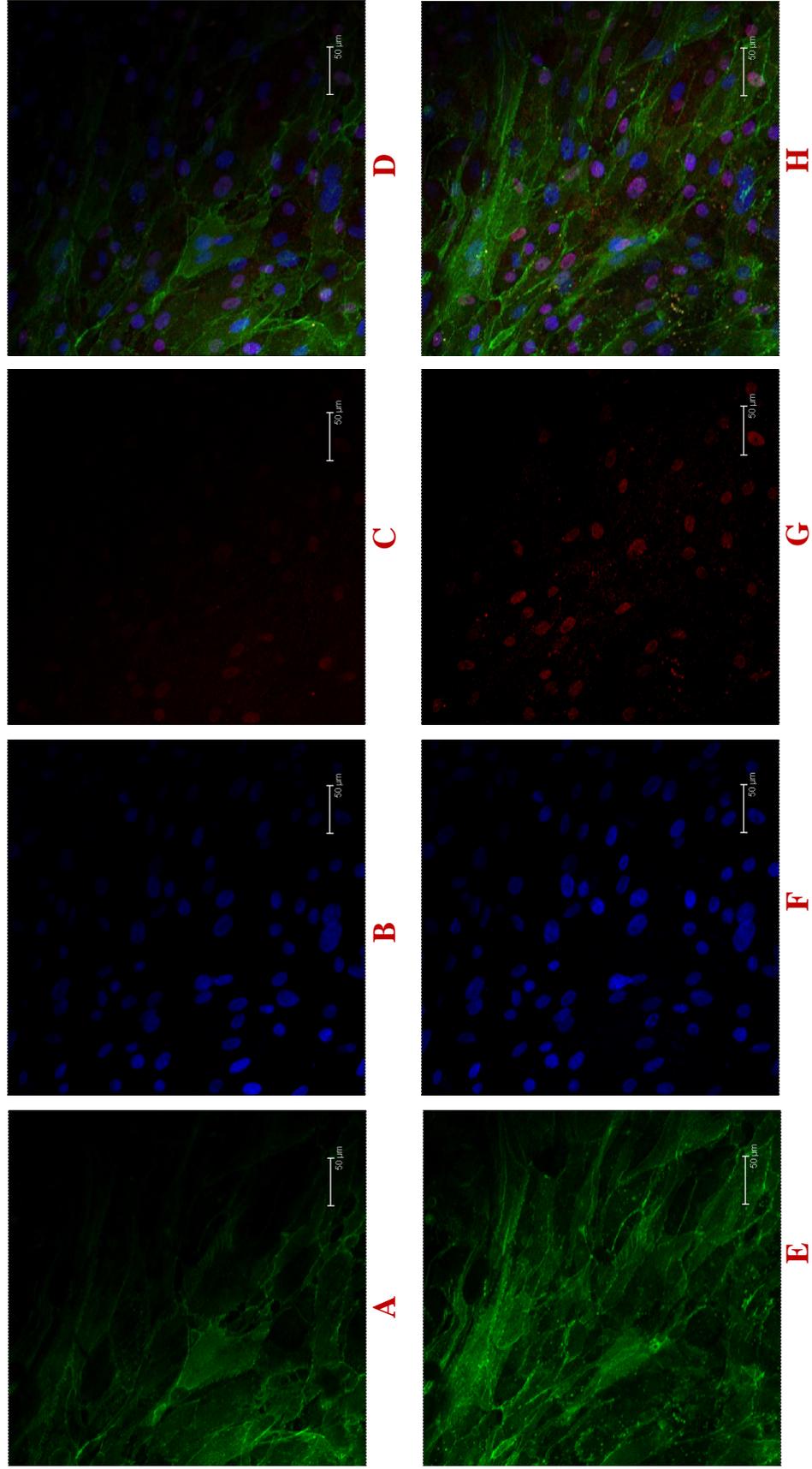
**Figure:3.6.20** Confocal images of immunofluorescence staining for ABCG2 and  $\Delta$ Np63 $\alpha$  in Condition B Co-3T3, ABCG2 (Green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta$ Np63 $\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu$ m.



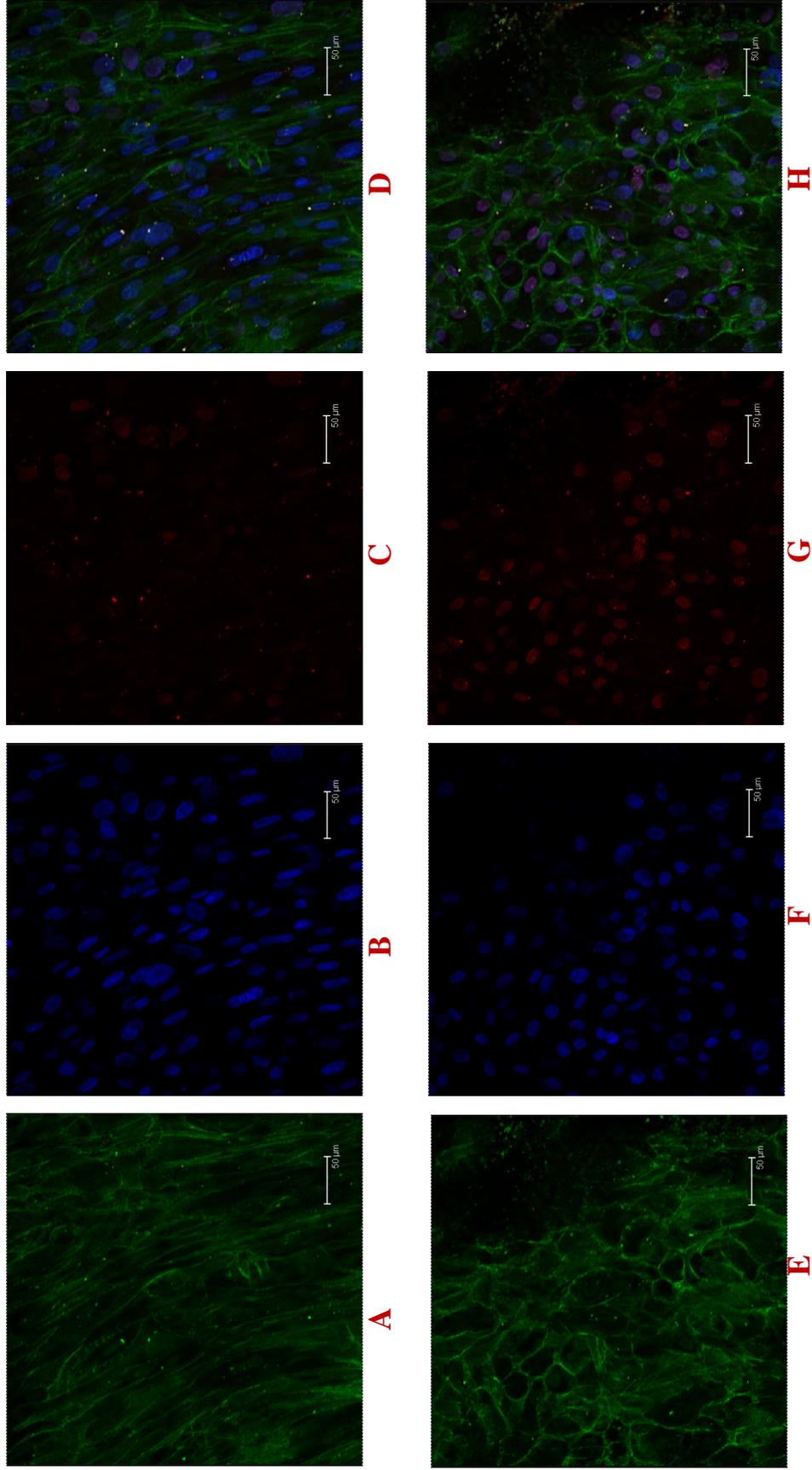
**Figure:3.6.21** Confocal images of immunofluorescence staining for ABCG2 and  $\Delta Np63\alpha$  in Condition C Bot-3T3, ABCG2 (Green) (A) apical, (E) basal, nuclei (blue) (B) apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C) apical, G-basal), and merge images (D) apical, H-basal), Scale bars 50 $\mu$ m.



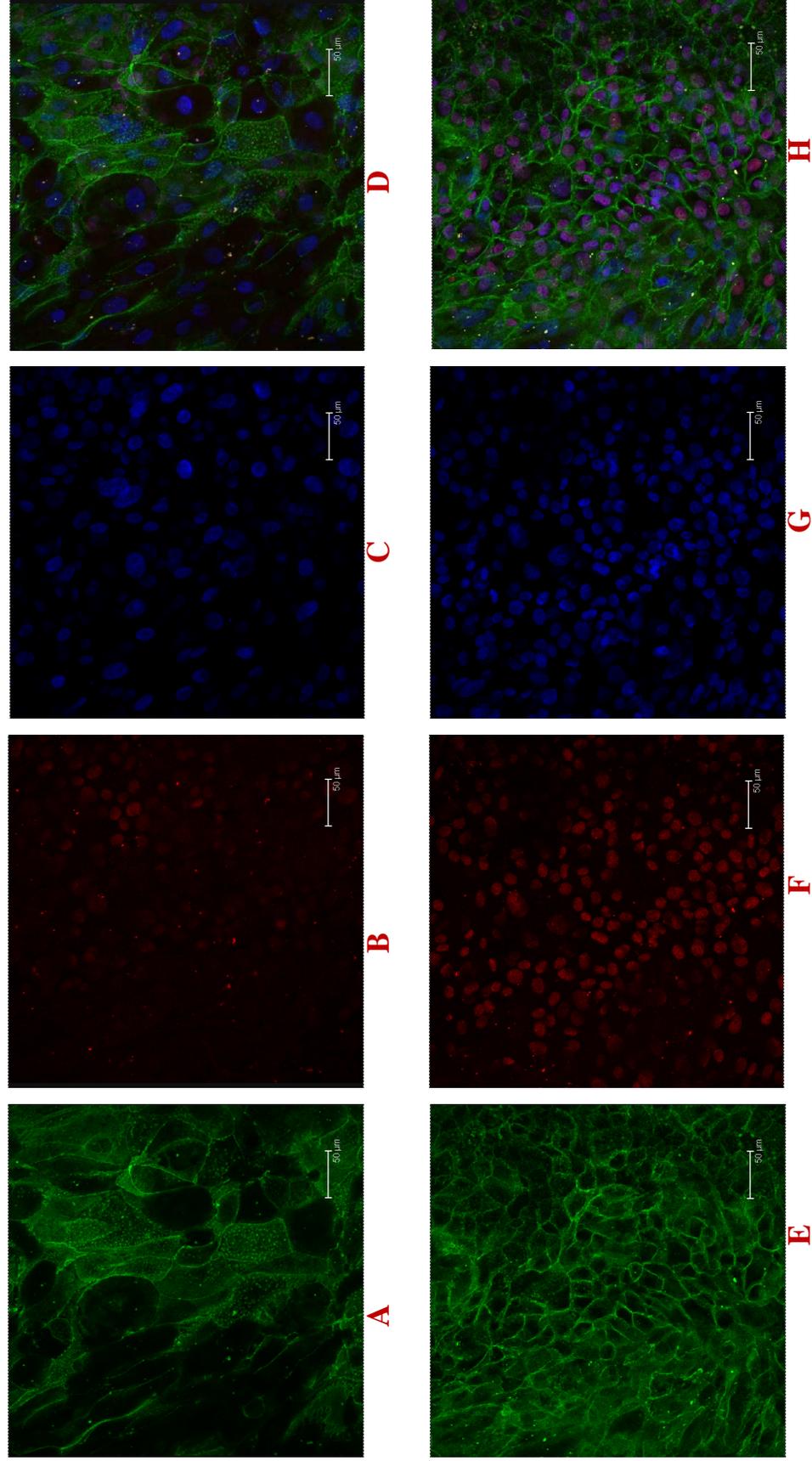
**Figure: 3.6.22** Limbal-corneal epithelial cells on cell culture insert. Cross section ABCG2 (green)/  $\Delta Np63\alpha$  (red)/ nuclei (Blue). Scale bars 20 $\mu m$ .



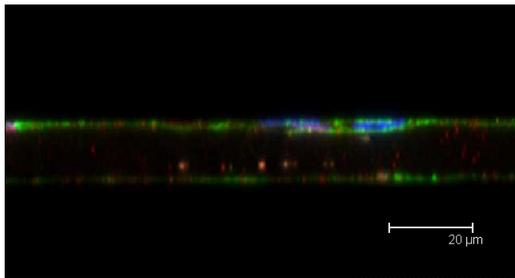
**Figure: 3.6.23** Immunofluorescence staining confocal images of ABCG2 and  $\Delta$ Np63 $\alpha$  in condition D dHAM no-3T3, ABCG2 (Green) (A) apical,(E) basal, nuclei (blue) (B-apical, and F-basal) ,  $\Delta$ Np63 $\alpha$  (Red) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu$ m.



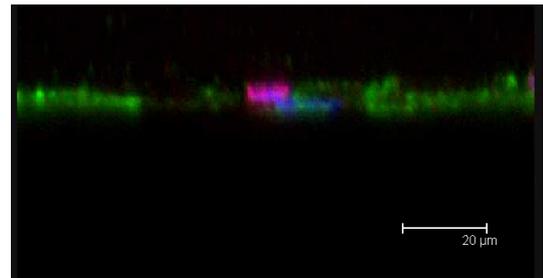
**Figure: 3.6.24** Immunofluorescence staining confocal images of ABCG2 and  $\Delta Np63\alpha$  in condition E dHAM Co-3T3, ABCG2 (Green) (A) apical, (E) basal, nuclei (blue) (B-basal, and F-basal),  $\Delta Np63\alpha$  (Red) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu$ m.



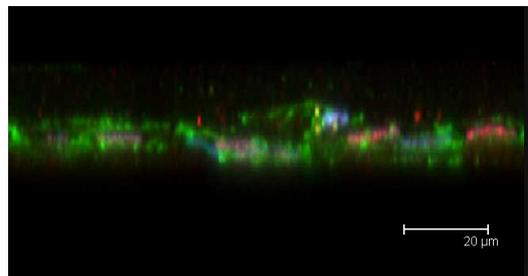
**Figure: 3.6.25** Immunofluorescence staining confocal images of ABCG2 and  $\Delta Np63\alpha$  in condition F dHAM Bot-3T3, ABCG2 (Green) (A) apical, (E) basal, nuclei (blue) (B) (B-apical, and F-basal),  $\Delta Np63\alpha$  (Red) (C) (C-apical, G-basal), and merge images (D-apical, H-basal), Scale bars 50 $\mu$ m.



**Condition D dHAM No-3T3**



**Condition E dHAM Co-3T3**

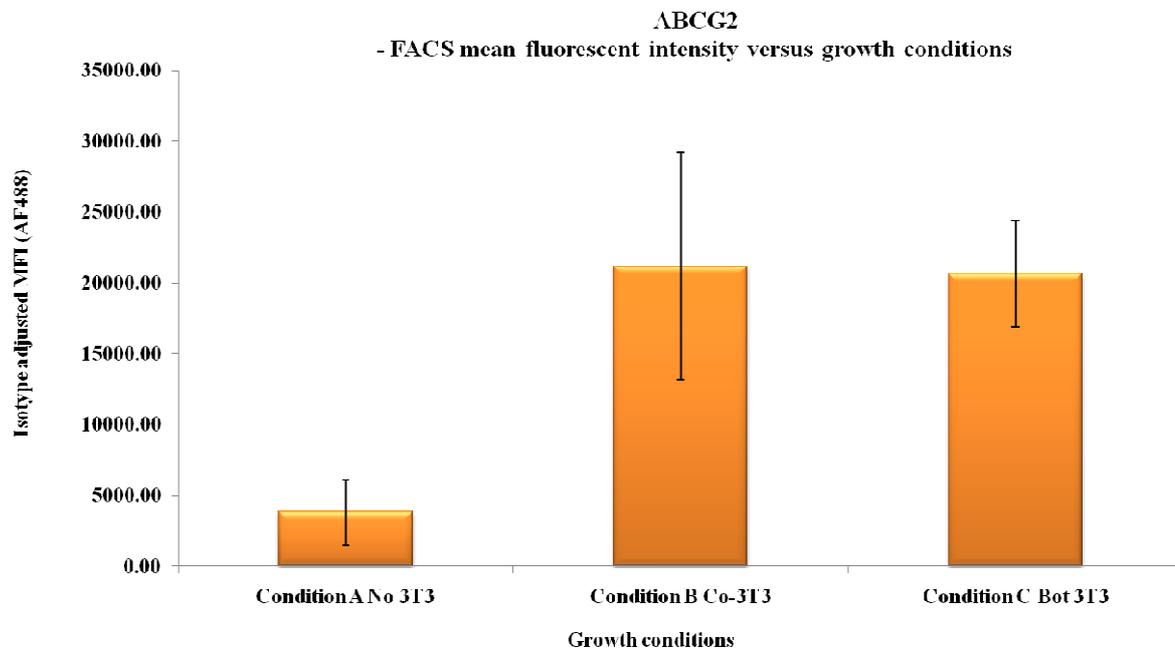


**Condition F dHAM Bot-3T3**

**Figure: 3.6.26** Limbal-corneal epithelial cells on denuded human amniotic membrane (dHAM) cross section ABCG2 (green)/ $\Delta$ Np63 $\alpha$  (red)/ nuclei (Blue). Scale bars 20 $\mu$ m.

#### **3.6.4.2 Flow cytometry analysis of ABCG2**

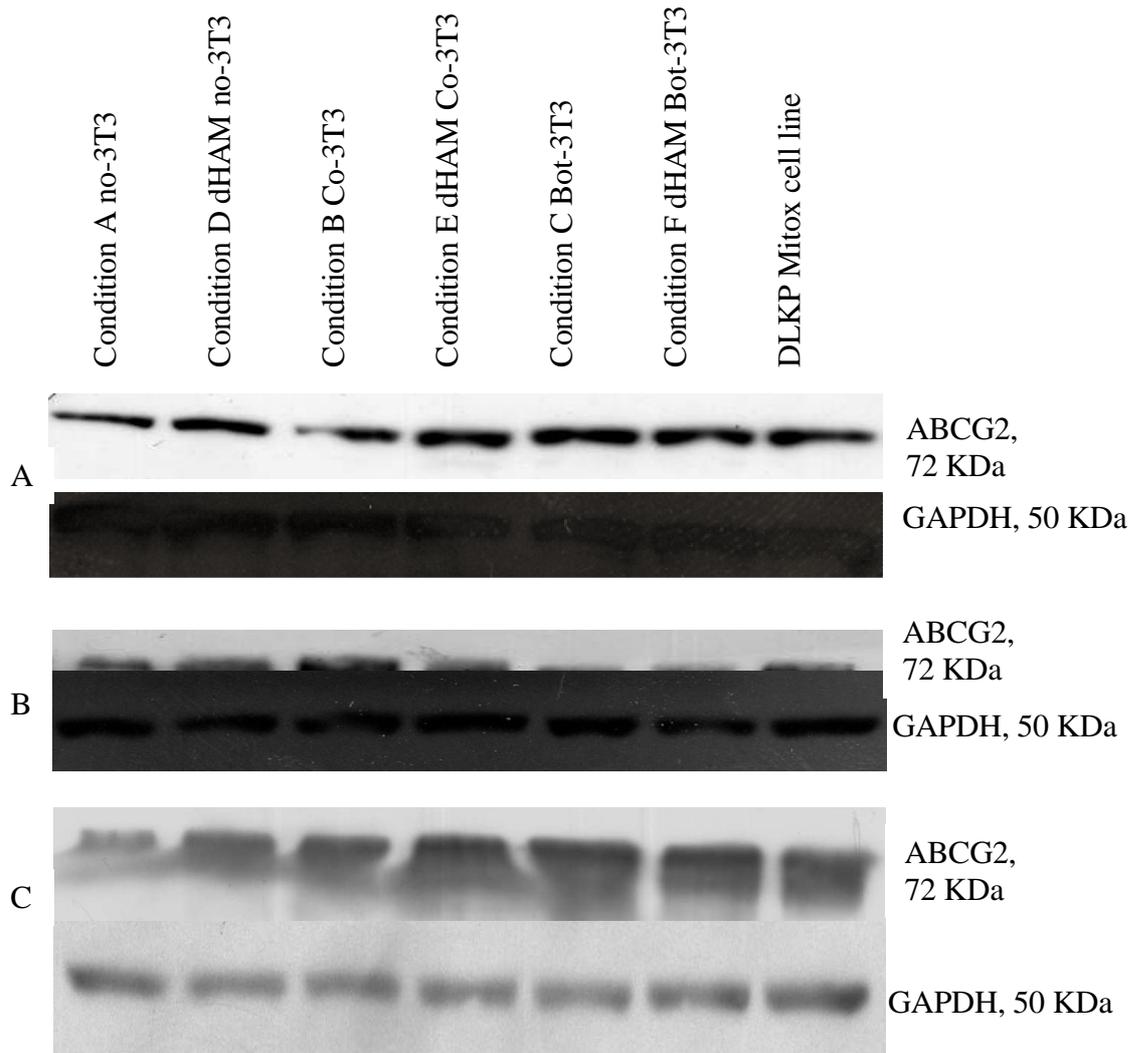
Flow cytometry analysis of ABCG2 showed positive expression in all three conditions, condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 of limbal-corneal epithelial cells grown on trans-well inserts. The mean fluorescent intensity (MFI) indicates differential expression of ABCG2; **figure 3.6.27** illustrates MFI of ABCG2 in three culture conditions with low expression of in condition A no-3T3 compared to condition B Co-3T3 and condition C Bot-3T3. No major difference in MFI was observed between condition B Co-3T3 and condition C Bot-3T3.



**Figure: 3.6.27** Flow cytometry analysis of ABCG2 in limbal-corneal epithelial cells grown on trans-well inserts with conditions A, B and C. Graphical representation of mean fluorescent intensity versus growth conditions. Mean =  $\pm$  S.D and (n=2; Sample 63 and 65)

### 3.6.4.3 Western blot analysis of ABCG2

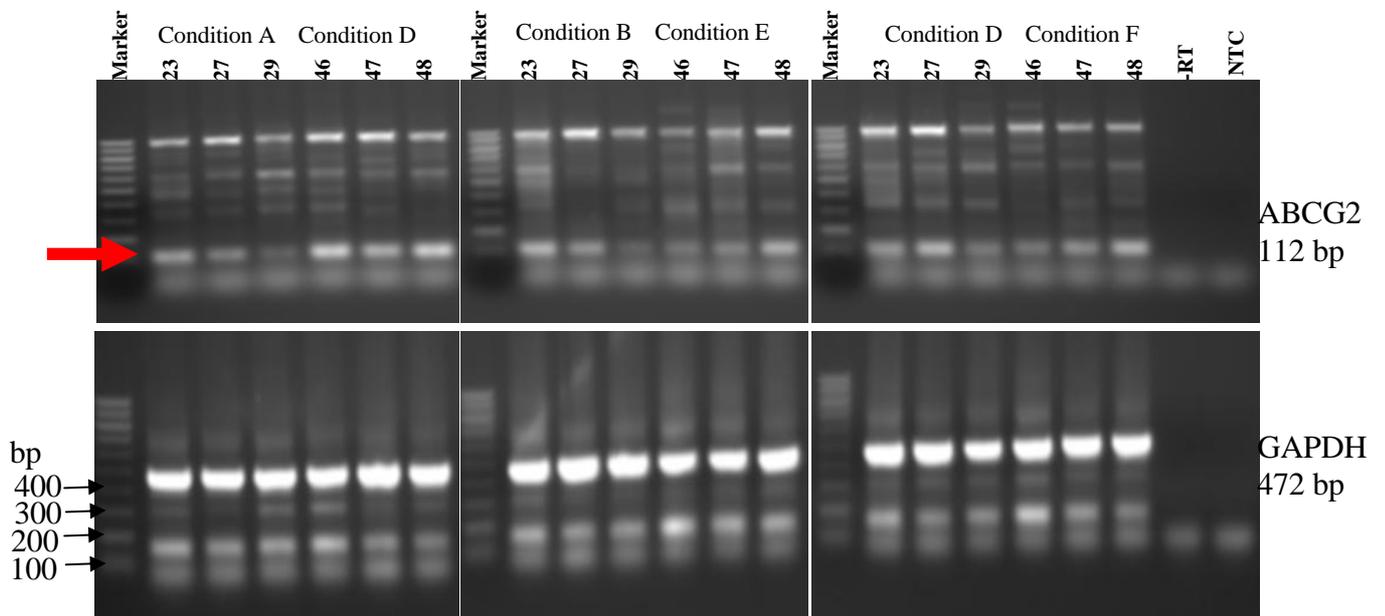
Western blot analysis was performed four technical with four biological repeats; a representative blot is shown in **figure 3.6.28**. Similar to immunofluorescence observations, intense staining of ABCG2 was observed in condition E dHAM Co-3T3, condition C Bot-3T3 and condition F dHAM Bot-3T3. Protein analysis of ABCG2 (72 KDa) showed differential expression in all six conditions, with increased level of protein in condition E dHAM Co-3T3, condition C Bot-3T3 and condition F dHAM Bot-3T3. Increased protein expression was observed in condition D dHAM no-3T3 compared to condition A no-3T3.



**Figure: 3.6.28** Representative western blot profile of ABCG2, in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 18, and condition D, E & F with sample 48. (B) Condition A, B and C with sample number 19, and condition D, E & F with sample 53. (C) Condition A, B and C with sample number 19, and condition D, E & F with sample 69 DLKP-Mitox cell line as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates).

#### **3.6.4.4 RT-PCR analysis of ABCG2**

The end point RT-PCR of ABCG2 (112 bp) for all six conditions has shown differential expression in biological samples (three biological repeats). Expression of ABCG2 is higher in samples 46, 47, 48 in condition D dHAM no-3T3 compared to condition A for three biological samples. Whereas the expression of ABCG2 mRNA shown decreased expression in condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 for three biological samples **figure 3.6.29**.



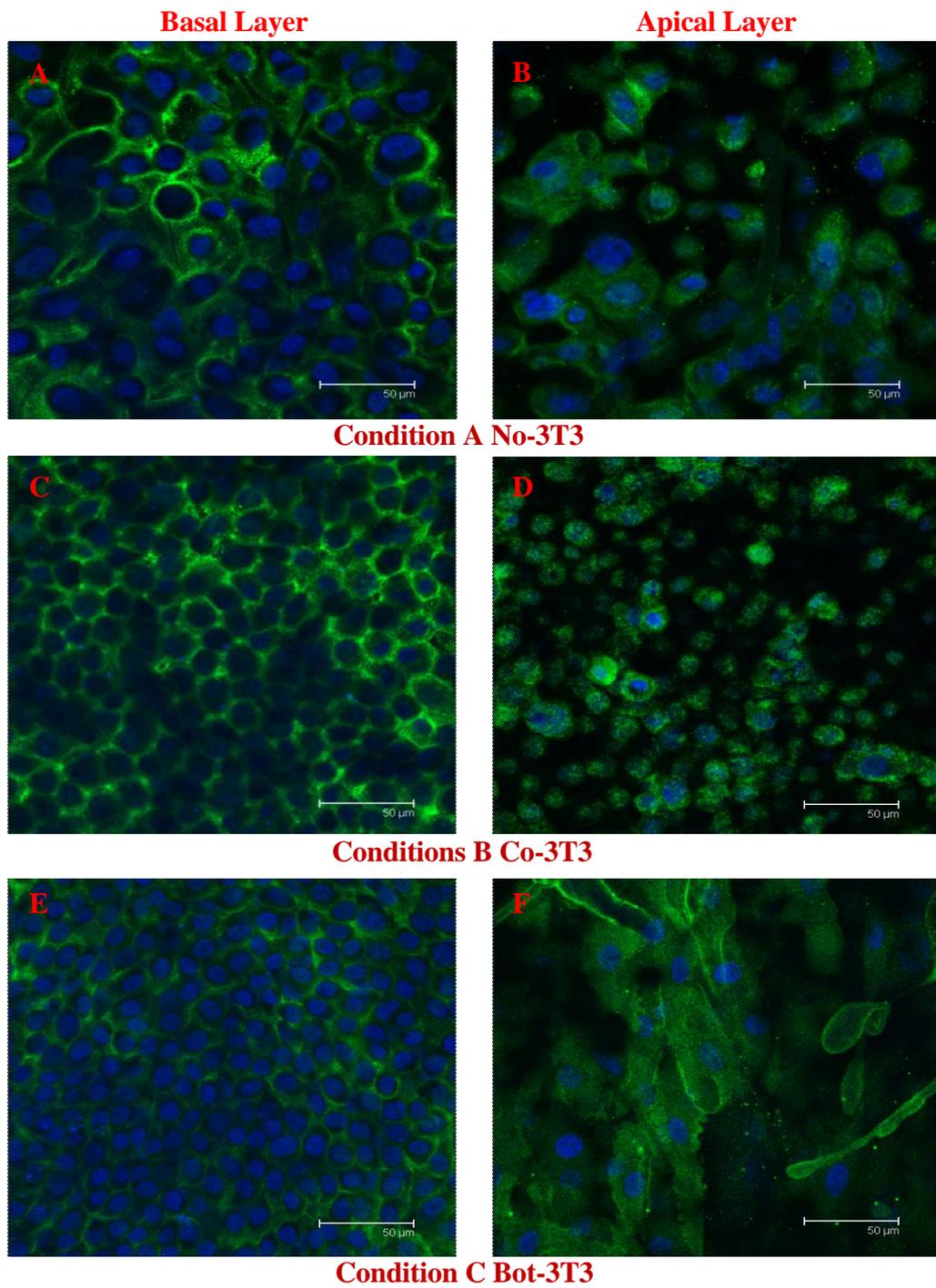
**Figure 3.6.29:** RT-PCR of ABCG2 (112 bp, arrow) on limbal-corneal epithelial cells grown on cell culture inserts and dHAM, with three biological samples for each condition. Sample 23, 27 and 29 cultured on cell culture insets with condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 conditions. Samples 46, 47 and 48 cultured on dHAM with condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Internal control gene GAPDH (472 kbp).

### 3.6.5 Expression of $\alpha$ -2 integrin

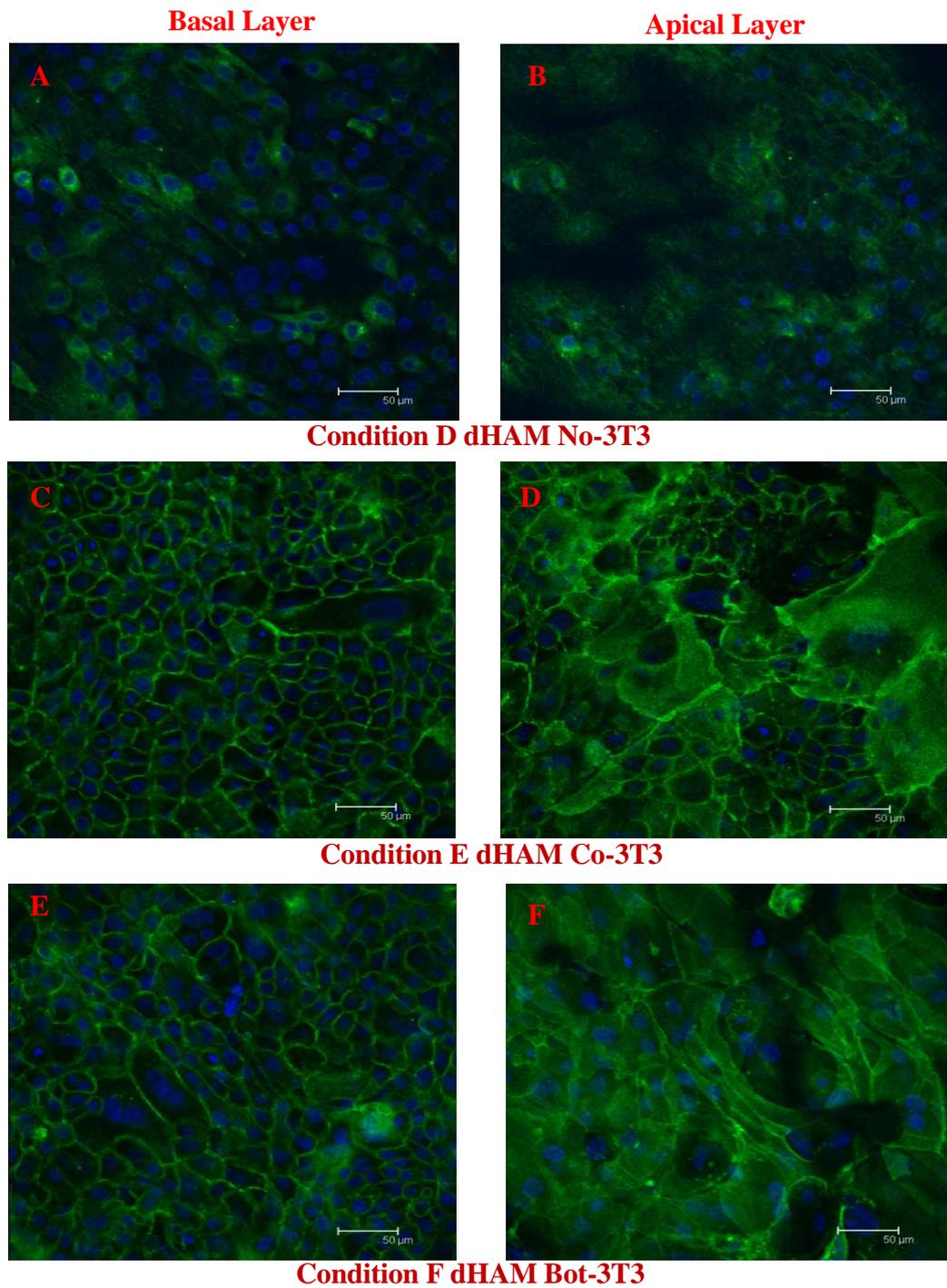
Integrins are a group of trans-membrane proteins that play a pivotal role in cell-to-basement membrane adhesion. *In vivo*  $\alpha$ -2 integrin is present in limbus and basal cells of the cornea. Difference in the composition between basal membranes of the cornea and limbus could at least in part explain the differences in phenotype and in behaviour of the basal cells (Dietlein *et al.*, 1997, Fukuda *et al.*, 1999, Ljubimov *et al.*, 1995)

#### 3.6.5.1 Immunofluorescence analysis of $\alpha$ -2 integrin

Immunofluorescence analysis was performed for  $\alpha$ -2 integrin three times with three biological replicates. Representative results are shown in **figures 3.6.30** and **3.6.31**. For condition A no-3T3, condition B Co-3T3, condition C Bot-3T3, condition E dHAM Co-3T3, and condition F dHAM Bot-3T3  $\alpha$ -2 integrin showed bright staining in basal cells confined to cell-cell margin stain (**figure 3.6.30 a, c, e** and **figure 3.6.31 c, e**) and lower staining pattern in apical cells with cytoplasmic localisation (**figure 3.6.30 b, d, f** and **figure 3.6.31 d, f**). In contrast, expression of  $\alpha$ -2 integrin in condition D dHAM no-3T3 shows low intensity in basal and apical cells **figures 3.6.31 a, b**.



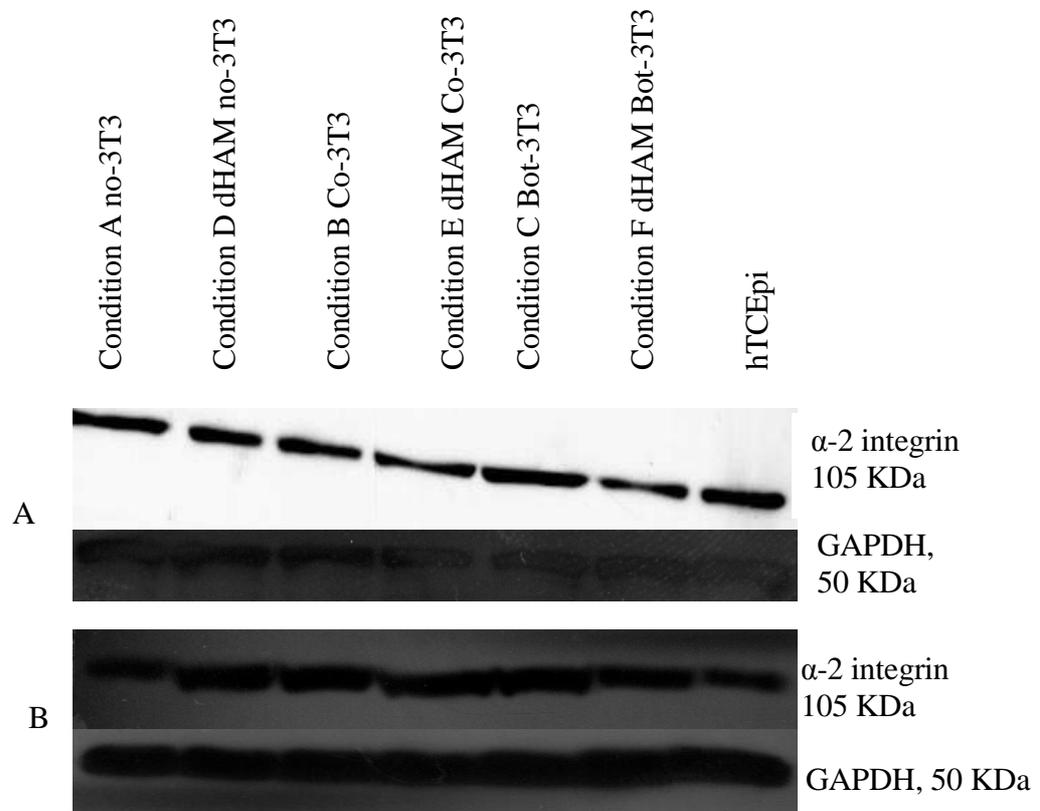
**Figure: 3.6.30** Confocal images of immunofluorescence staining for  $\alpha 2$  integrin in limbal-corneal epithelial cells grown on cell culture inserts with conditions A, B and C in basal and apical layers; nuclei (blue) &  $\alpha 2$  integrin (green); scale bars 50 $\mu$ m.



**Figure: 3.6.31** Confocal images of immunofluorescence staining for  $\alpha 2$  integrin in limbal-corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E and F in basal and apical layers; nuclei (blue) &  $\alpha 2$  integrin (green); scale bars 50 $\mu$ m.

### 3.6.5.2 Western blot analysis of $\alpha$ -2 integrin

Total protein analysis (with four biological and technical replicates) of  $\alpha$ -2 integrin (105 KDa) showed differential expression in all six conditions, with low protein expression in condition A no-3T3 compared to other culture conditions. In contrast to immunofluorescence results, in condition D dHAM no-3T3 protein expression was higher **figure 3.6.32**.



**Figure: 3.6.32:** Representative western blot profile of  $\alpha$ -2 integrin, in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 29, and condition D, E & F with sample 69. (B) Condition A, B and C with sample number 30, and condition D, E & F with sample 84. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates)

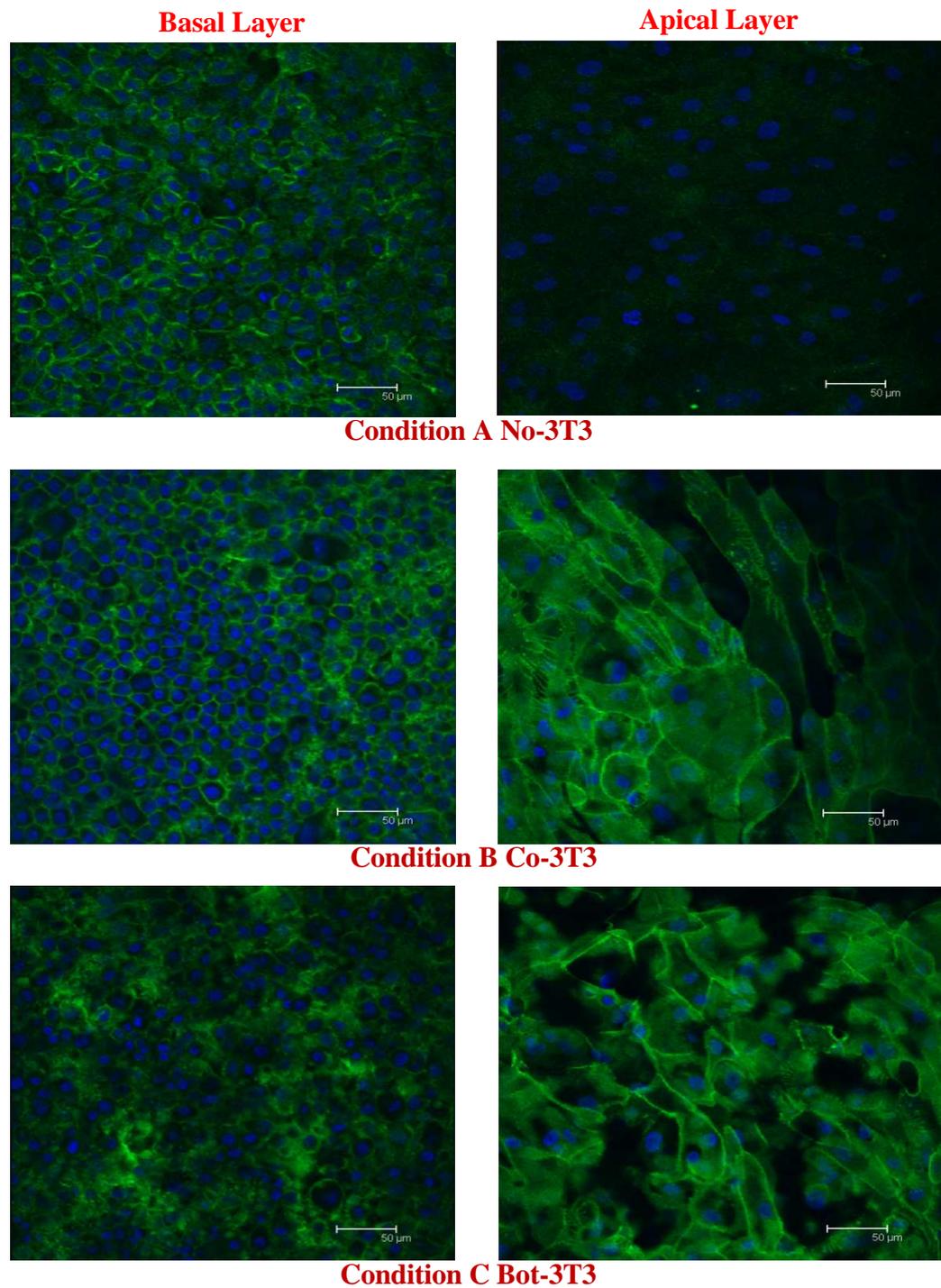
### **3.6.6 Expression of $\beta$ 1 integrin**

$\beta$  1 Integrin was originally suggested to be a keratinocyte marker (Jones and Watt, 1993). Cells that rapidly adhere to the  $\beta$  1 integrin ligand, collagen IV also display limbal epithelial stem cell properties (Li and Lu, 2005).

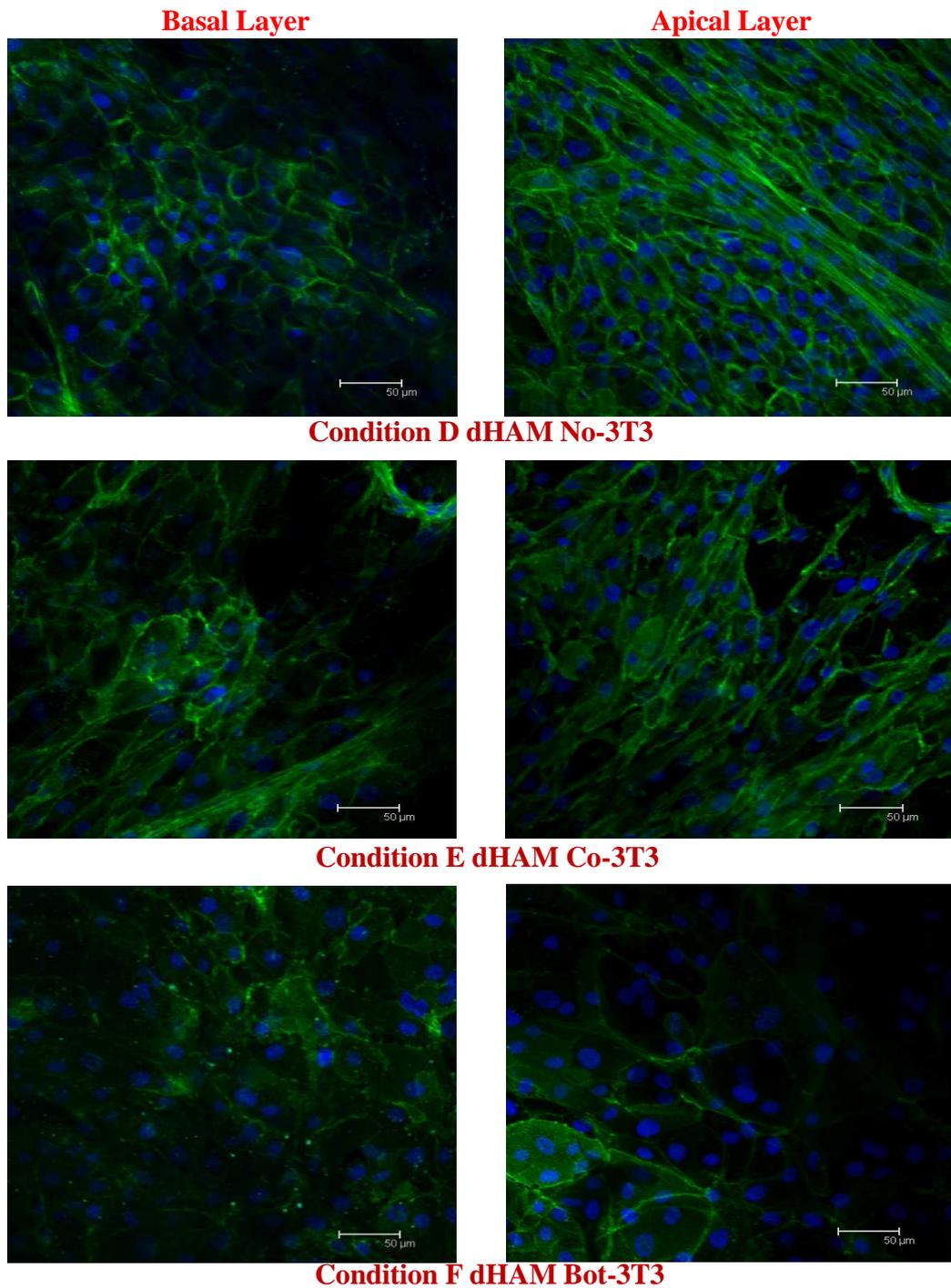
#### **3.6.6.1 Immunofluorescence analysis of $\beta$ 1 integrin**

Immunofluorescence analysis was performed for  $\beta$  1 integrin three times, representative results are shown in **figure 3.6.33** and **3.6.34** limbal-corneal epithelial cells grown in condition A no-3T3 showed bright staining of  $\beta$  1 integrin in basal cells, with reduced staining in apical cells (**figure 3.6.33 a, b**). Condition B Co-3T3 and condition C Bot-3T3 showed bright staining of  $\beta$  1 integrin in basal cells with membrane localisation (**figure 3.6.33 c, e**), in apical cells cytoplasmic staining is seen (**figure 3.6.33 c, e**). In contrast to condition B Co-3T3 and condition C Bot-3T3, limbal corneal epithelial cells in condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 showed lower staining in basal cells and apical cells (**figure 3.6.34 c, d, e, and f**). Condition E dHAM Bot-3T3 showed lower staining of  $\beta$  1 integrin at basal and apical cells.

In contrast to the condition A no-3T3, condition D dHAM no-3T3 showed low staining of basal cells with increased staining intensity in apical cells with clear membrane localisation (**figure 3.6.33 a, b** and **figure 3.6.34 a, b**).



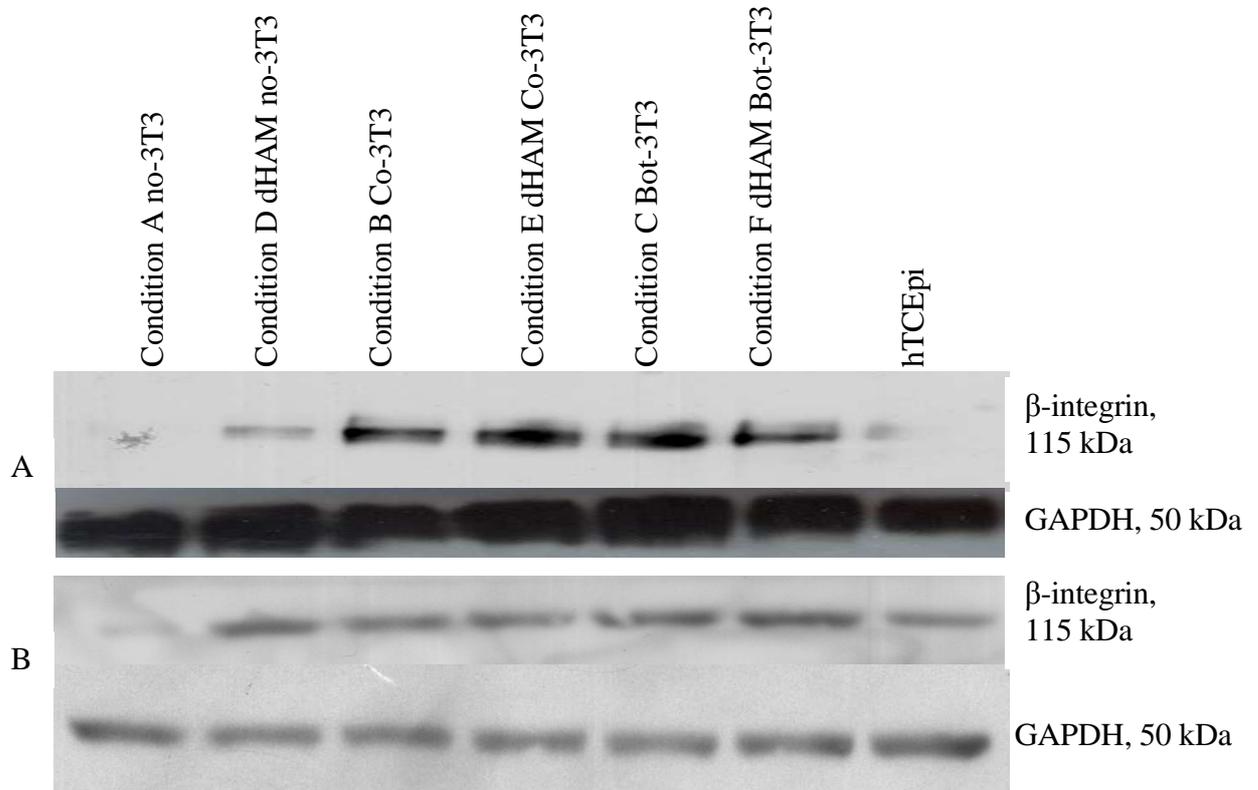
**Figure: 3.6.33** Confocal images of immunofluorescence staining for  $\beta 1$  integrin in limbal-corneal epithelial cells grown on Cell culture inserts with conditions A, B and C in basal and apical layers; Nuclei (blue) &  $\alpha 2$  integrin (green); Scale bars 50 $\mu$ m.



**Figure: 3.6.34** Confocal images of immunofluorescence staining for  $\beta 1$  integrin in limbal-corneal epithelial cells grown on denuded human amniotic membrane (dHAM) with conditions D, E and F in basal and apical layers; Nuclei (blue) &  $\alpha 2$  integrin (green); Scale bars 50 $\mu$ m.

### **3.6.6.2 Western blot analysis of $\beta$ 1 integrin**

Western blot analysis was performed four times with four biological repeats; a representative blot is shown in **figure 3.6.35**. Low  $\beta$  1 integrin protein was observed in condition A no-3T3 compared to other five conditions. Slightly higher levels of total protein were observed in condition F dHAM Bot-3T3, with equal levels in condition D dHAM no-3T3, condition B Co-3T3, condition E dHAM Co-3T3 and condition C Bot-3T3.



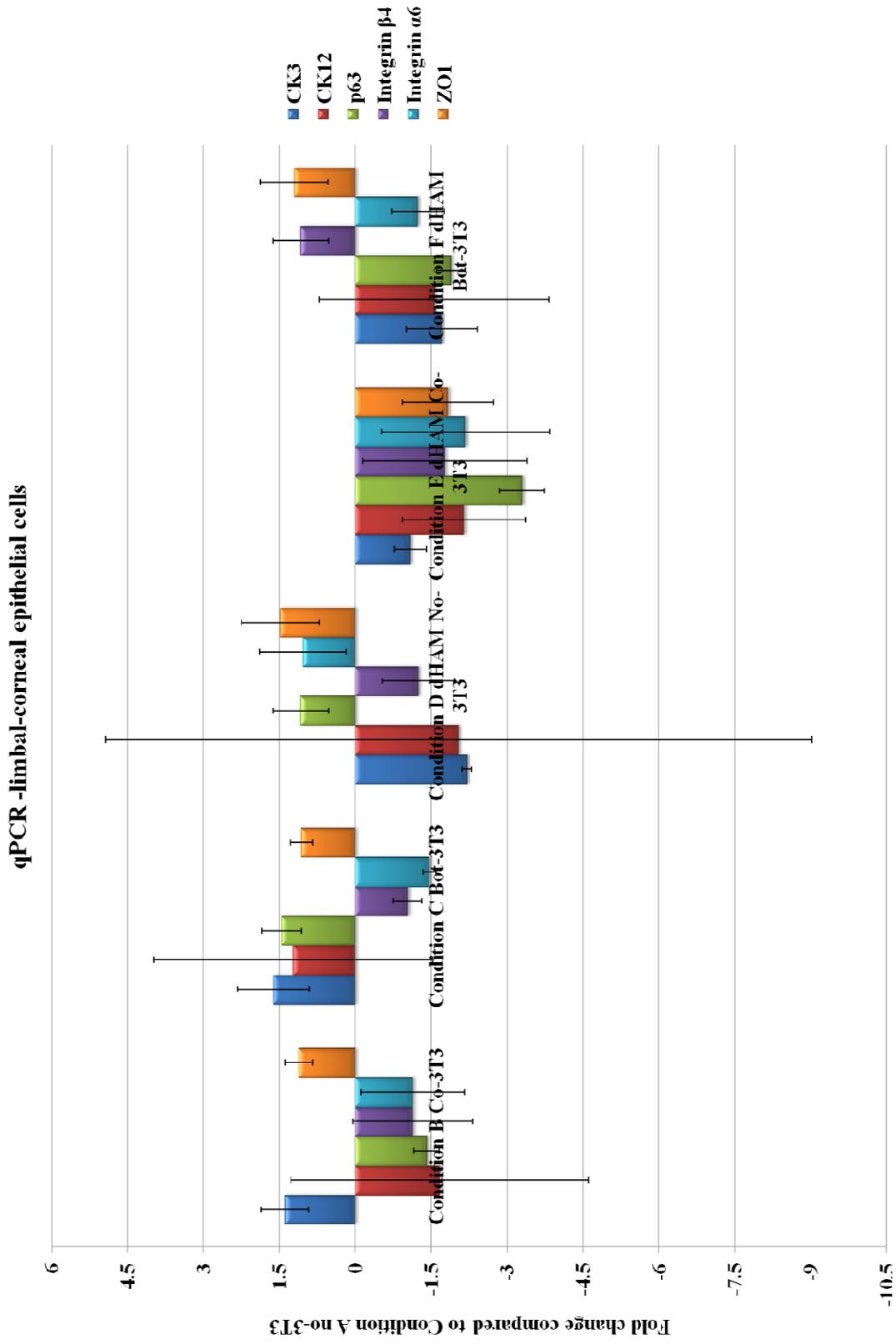
**Figure: 3.6.35** Representative western blot profile of  $\beta$ 1- integrin, in cultured limbal-corneal cells. (A) Condition A, B and C with sample number 29, and condition D, E & F with sample 69. (B) Condition A, B and C with sample number 30, and condition D, E & F with sample 84. Telomerase-immortalized human corneal epithelial cell line (hTCEpi) used as positive control, with the housekeeping protein GAPDH as an internal control (n=4 biological replicates).

### 3.7 qRT-PCR analysis of limbal-corneal epithelial cells

Limbal corneal epithelial cells were subject to six different growth conditions. Quantitative real-time PCR was performed on cultured limbal- corneal cells under the different conditions for relative quantification of expression of cytokeratin 3, 12, p63,  $\beta$  1 integrin,  $\alpha$  6 integrin, tight junction protein ZO1 and endogenous control gene  $\beta$ -actin (**table 2.20**). PCR data was analysed as described in **section 2.20.7** for three biological samples in each condition. Average gene expression fold change of biological replicates was calculated relative to calibrator sample- condition A no-3T3 and displayed in **figure 3.7**. Fold change values less than +1.5, and -1.5 considered as non-significant.

The differential fold change was observed in all genes that were analysed; the most important observation was that condition E dHAM Co-3T3 had shown down regulation of CK3, CK12, p63, integrin  $\beta$ 4, $\alpha$ 6 and ZO1, whereas the down regulation of CK3 is not significant, and CK12, integrin  $\beta$ 4, $\alpha$ 6 and ZO1 are marginally significant. The down regulation of p63 is significant in condition E dHAM Co-3T3.

The raw values and calculations of three biological replicates of all six conditions are available in appendices. Any gene with  $C_T$  value over 35 was excluded from analysis.



**Figure: 3.7** Graphical representation of qPCR analysis for limbal-corneal epithelial cells, with relative fold change.

### 3.8 Colony forming efficiency of limbal-corneal epithelial cells

The ability of cells to generate colonies can be measured as colony-forming efficiency (CFE).

This can be calculated as a percentage using the formula:

$$\text{CFE} = \frac{\text{Number of colonies formed}}{\text{number of cells plated}} \times 100$$

CFE is a means by which to measure the number of progenitor cells in culture, in other words, stem cells and transient amplifying cells that proliferate and generate colonies as opposed to terminally differentiated cells that are unable to proliferate. For example, limbal epithelial cells have an increased ability to generate colonies (i.e., have a higher CFE than those of the cornea). These findings are to be expected as limbal stem cells reside in the limbus, while the central cornea in particular is mainly composed of terminally differentiated corneal epithelial cells (Barrandon and Green, 1987, Barrandon and Green, 1985).

To evaluate the effect of 3T3 feeder layers in maintenance of stem cell niche in limbal-corneal epithelial cultures we performed a colony forming efficiency analysis in all 6 culture conditions. Six biological samples used for condition A no-3T3, condition B Co-3T3, and condition C Bot-3T3 and five biological samples were used for conditions D, E and F. **Figure 3.8.1 to 3.8.1c** are a graphical representations of colony forming efficacy of limbal-corneal epithelial cells and respective conditions colony stained with 0.25% crystal violet. Six biological samples for conditions A, B and C (sample number- 19, 20, 29, 30, 33 and 35), and five biological samples for conditions D, E and F (sample number-39, 49, 50, 77 and 78). P

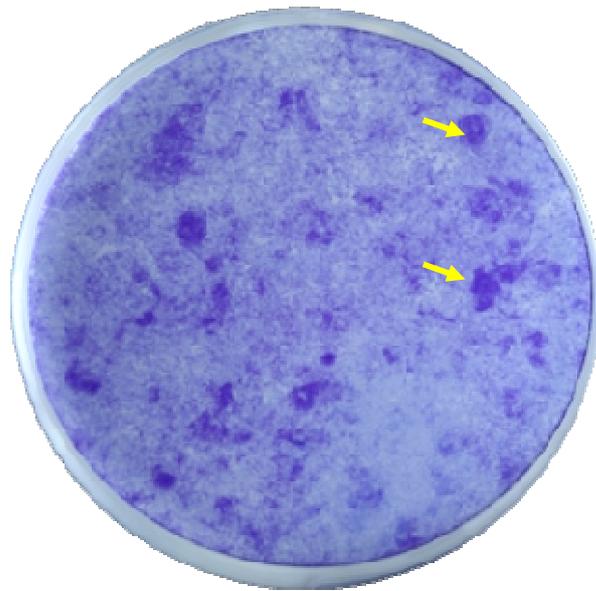
values are derived with reference to condition A no-3T3 in cell culture inserts, and condition D dHAM no-3T3 for dHAM cultures.

Colony Forming Efficacy (CFE) of condition A no-3T3 showed low rate of 0.42% with less than 3-4 colonies per plate, whereas condition D dHAM no-3T3 has shown high number of CFE rate ( $5.25 \pm 0.19\%$ ) with small compact colonies. The percentage of CFE is increased to  $1.17 \pm 0.24\%$  in condition B Co-3T3 and  $2.32 \pm 0.3\%$  in condition C Bot-3T3 with clear prominent colonies.

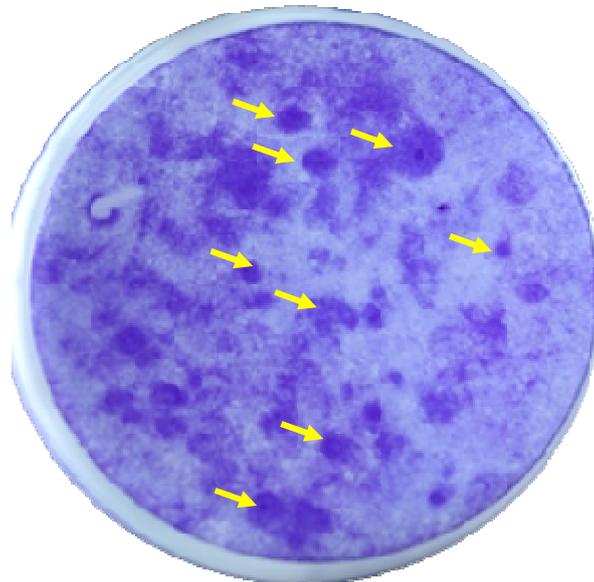
The highest CFE rate was observed in condition E dHAM Co-3T3  $8.92 \pm 2.18\%$ , with small compact colonies compared to large colony size in condition B Co-3T3 and condition C Bot-3T3. In contrast to condition E dHAM Co-3T3, condition F dHAM Bot-3T3 has shown slightly less CFE rate ( $7.2 \pm 1.96\%$ ), whereas the morphology and size of the colonies appeared to be the same as in condition E dHAM Co-3T3 and condition D dHAM no-3T3.

The statistical significance of CFE was assessed with biological samples used for first three conditions (condition A, B and C) *p* values are as follows with mean =  $\pm$  S.D and *n*=6. *p*= 0.007 condition A no-3T3 Vs condition C Bot-3T3, *p*= 0.019 condition A no-3T3 Vs condition B Co-3T3, *p*= 0.006 condition B Co-3T3 Vs condition C Bot-3T3.

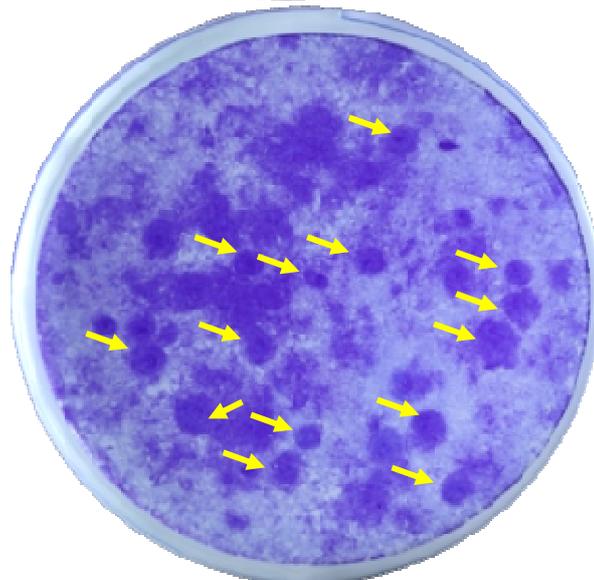
Similarly, the significance of CFE was calculated with biological samples used for second three conditions (condition D, E and F) *p* values are as follows with Mean =  $\pm$  S.D and *n*=5. *p*= 0.0046 condition D dHAM no-3T3 Vs condition F dHAM Bot-3T3, *p*= 0.005 condition D dHAM no-3T3 Vs condition E dHAM Co-3T3, *p*= 0.057 condition E dHAM Co-3T3 Vs condition F dHAM Bot-3T3.



Condition A no-3T3

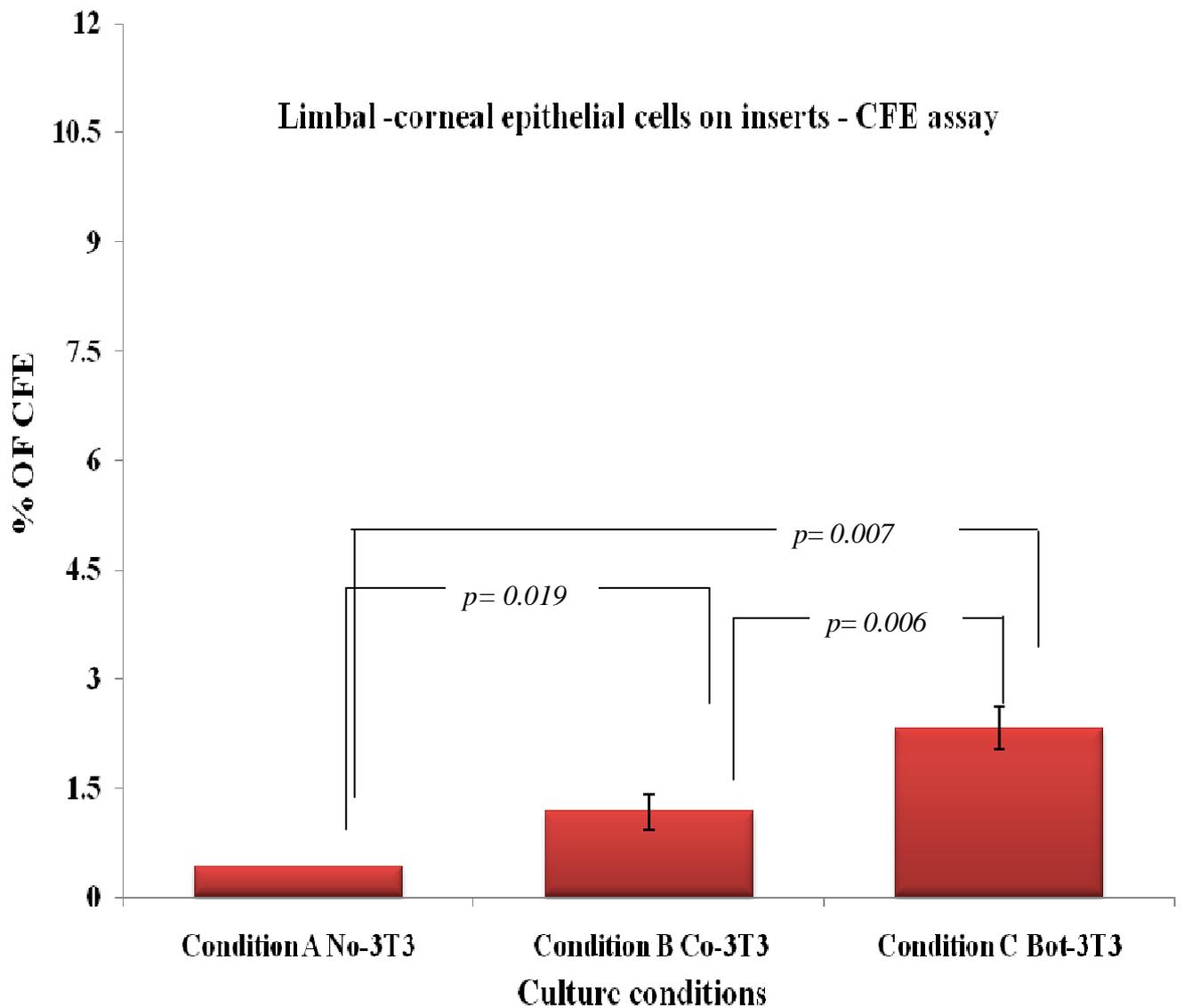


Condition B Co-3T3

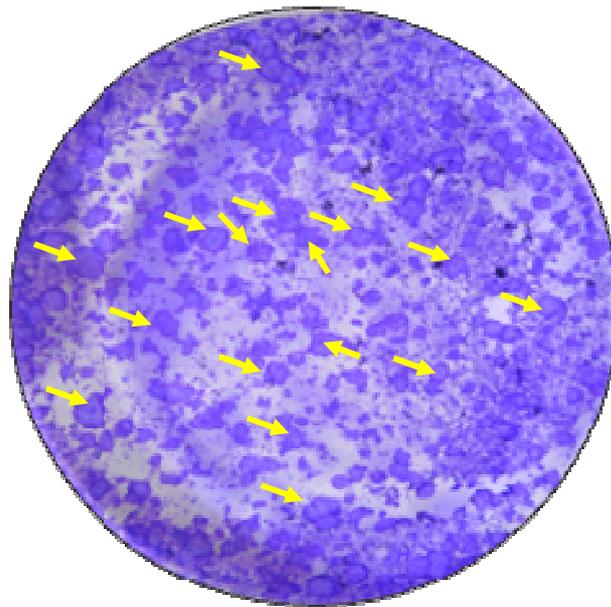


Condition C Bot-3T3

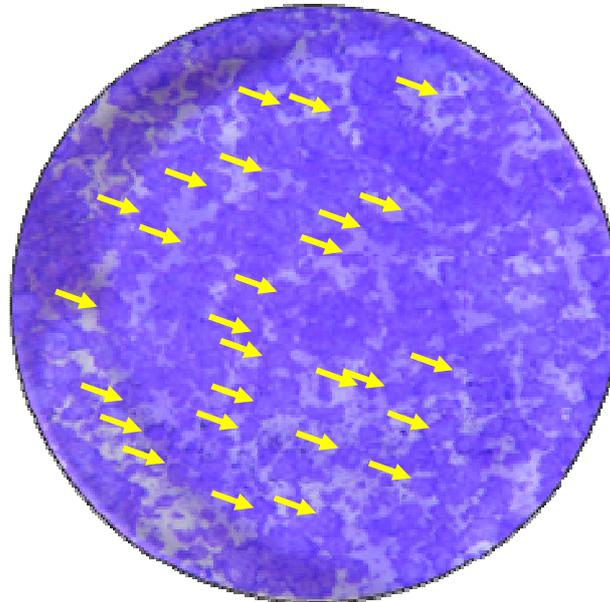
**Figure: 3.8.1:** Colony forming efficacy (CFE) of limbal-corneal epithelial cells grown on cell culture inserts. Representative yellow arrows are indication of counted colonies (2-10mm<sup>2</sup>).



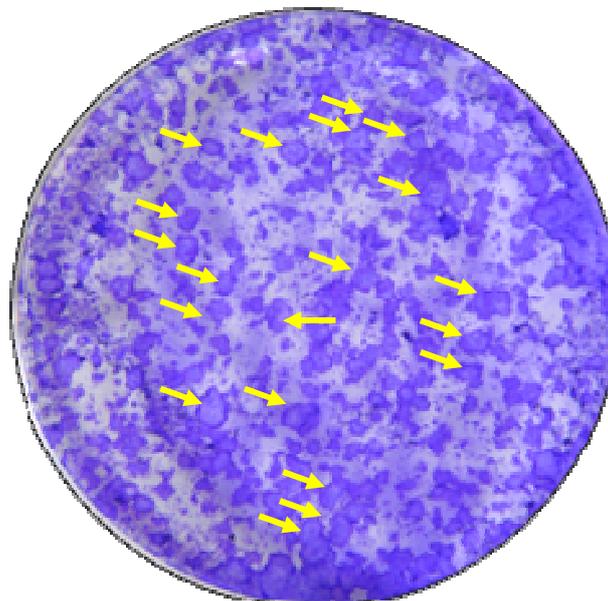
**Figure: 3.8.1a:** Graphical representation of colony forming efficacy (CFE) of limbal-corneal epithelial cells grown on denuded human amniotic membrane (dHAM). Mean=±S.D and n=5 (n= biological replicates).



Condition D dHAM no-3T3

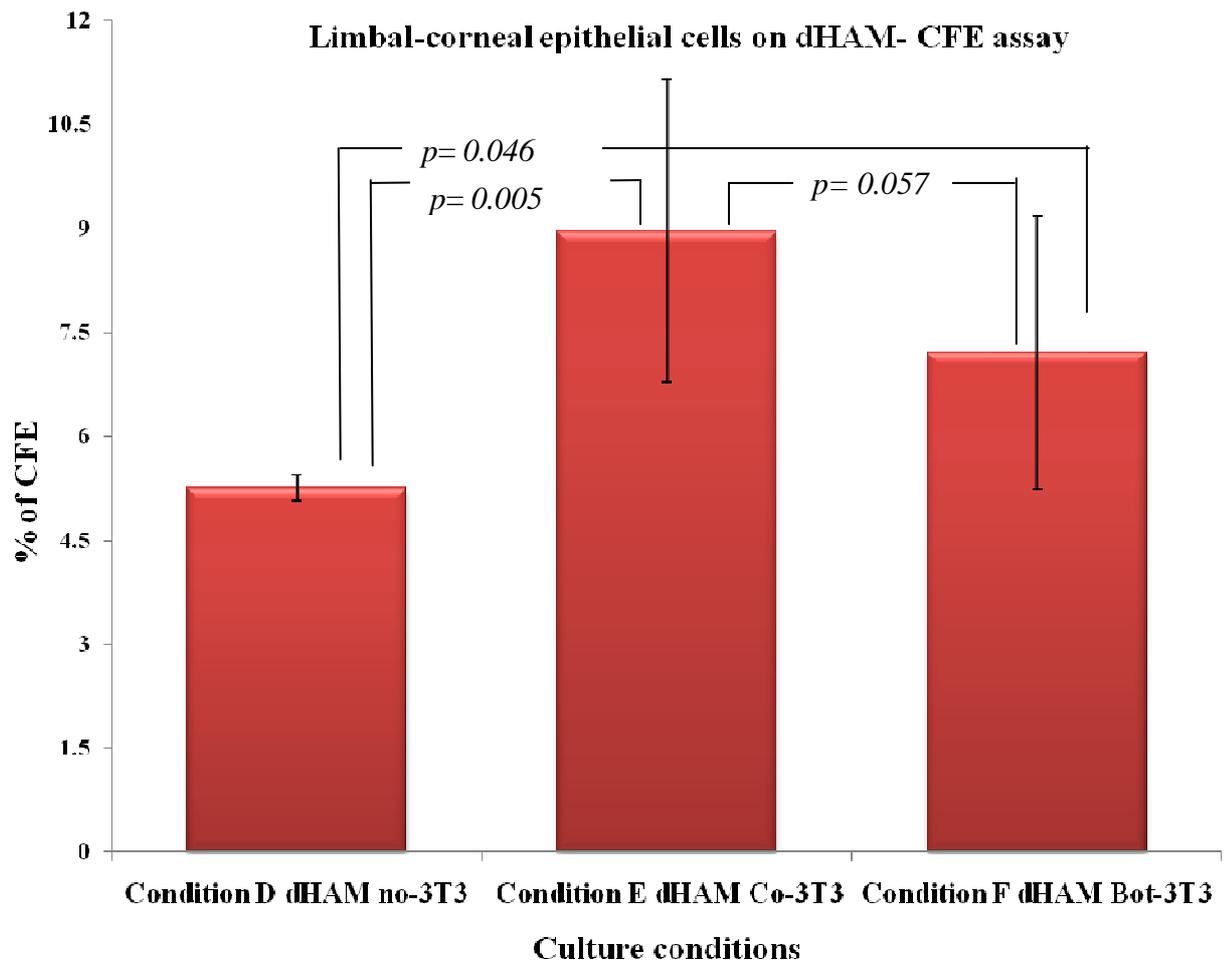


Condition E dHAM Co-3T3



Condition F dHAM Bot-3T3

**Figure: 3.8.1b:** Colony-forming efficacy (CFE) of limbal-corneal epithelial cells grown on denuded human amniotic membrane. Representative yellow arrows are indication of counted colonies (2-10mm<sup>2</sup>).



**Figure: 3.8.1c:** Graphical representation of colony forming efficacy (CFE) of limbal-corneal epithelial cells grown on denuded human amniotic membrane (dHAM). Mean $\pm$ S.D and n=5 (n= biological replicates)

### **3.9 The effect of conditioned media from 3T3 cells on limbal-corneal epithelial growth**

Condition C Bot-3T3 indicated that soluble factors from 3T3 feeder cells could support the growth of limbal-corneal epithelial cells. Further investigation was done with conditioned media from irradiated 3T3 feeder to culture limbal-corneal epithelial cells on trans-well inserts.

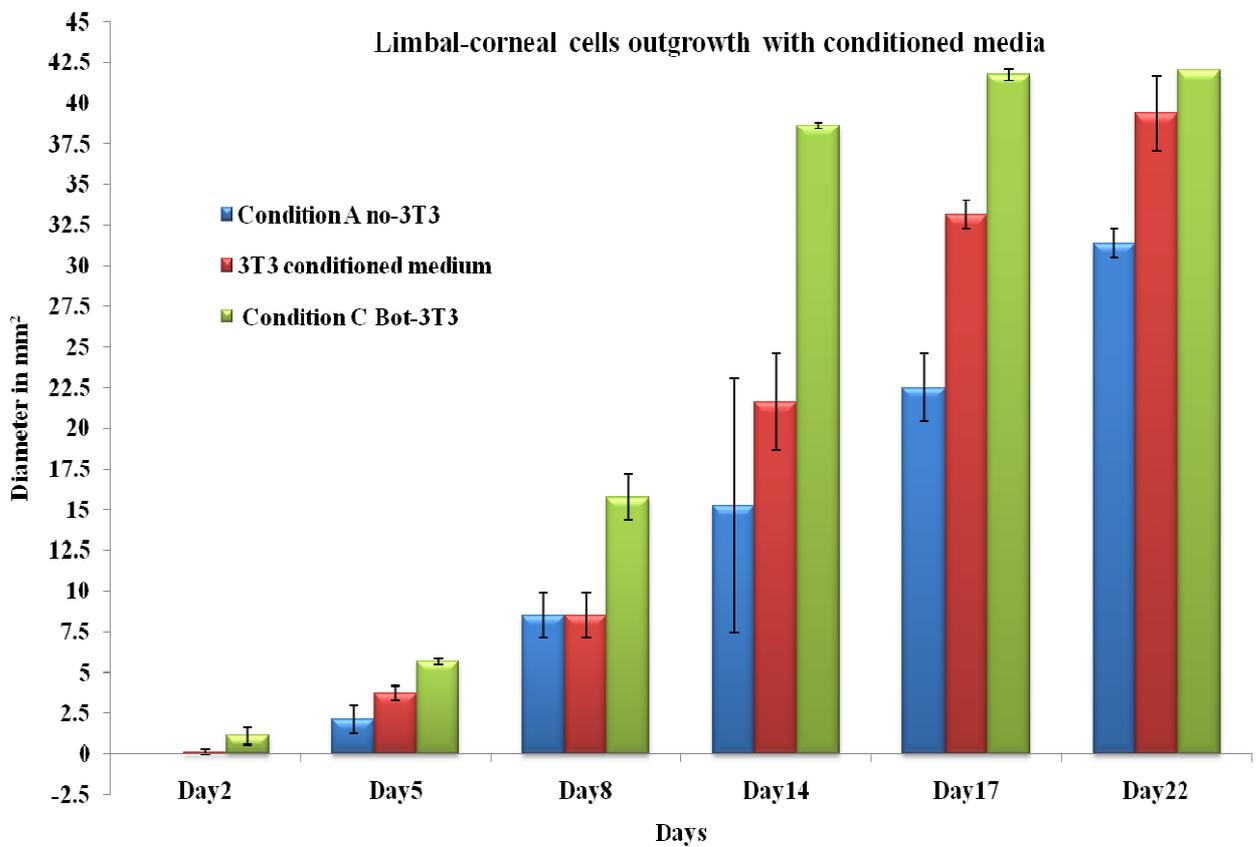
The conditioned media was placed in the below chamber and replaced every two days. Feeder cells provide a suitable environment in co-culture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interaction (Ehmann *et al.*, 1998), production of soluble growth factors and removal of toxicants from the culture medium.

This conditioned media culture was compared to condition A no-3T3 and condition C Bot-3T3 for outgrowth, morphology and colony forming efficacy (CFE).

**Figure 3.9.1** illustrates the outgrowth of limbal-corneal epithelial cells with 3T3 conditioned medium, the initial outgrowth of epithelial cells are higher than the condition A no-3T3 with  $2.1 \pm 0.88\text{mm}^2$  at day 5, in conditioned medium with average of  $3.6 \pm 0.44\text{mm}^2$  at day 5. Whereas, the outgrowth rate was slow compared to condition C Bot-3T3. With average outgrowth, rate  $5.8 \pm 0.17\text{mm}^2$  at day 5.

The final outgrowth attained was on average  $31.2 \pm 0.88\text{mm}^2$  by day 22 in condition A no-3T3, higher growth rate in conditioned medium showed average outgrowth of  $39.3 \pm 2.22\text{mm}^2$  by day 22, and  $42\text{mm}^2$  in condition C Bot-3T3 by day 22.

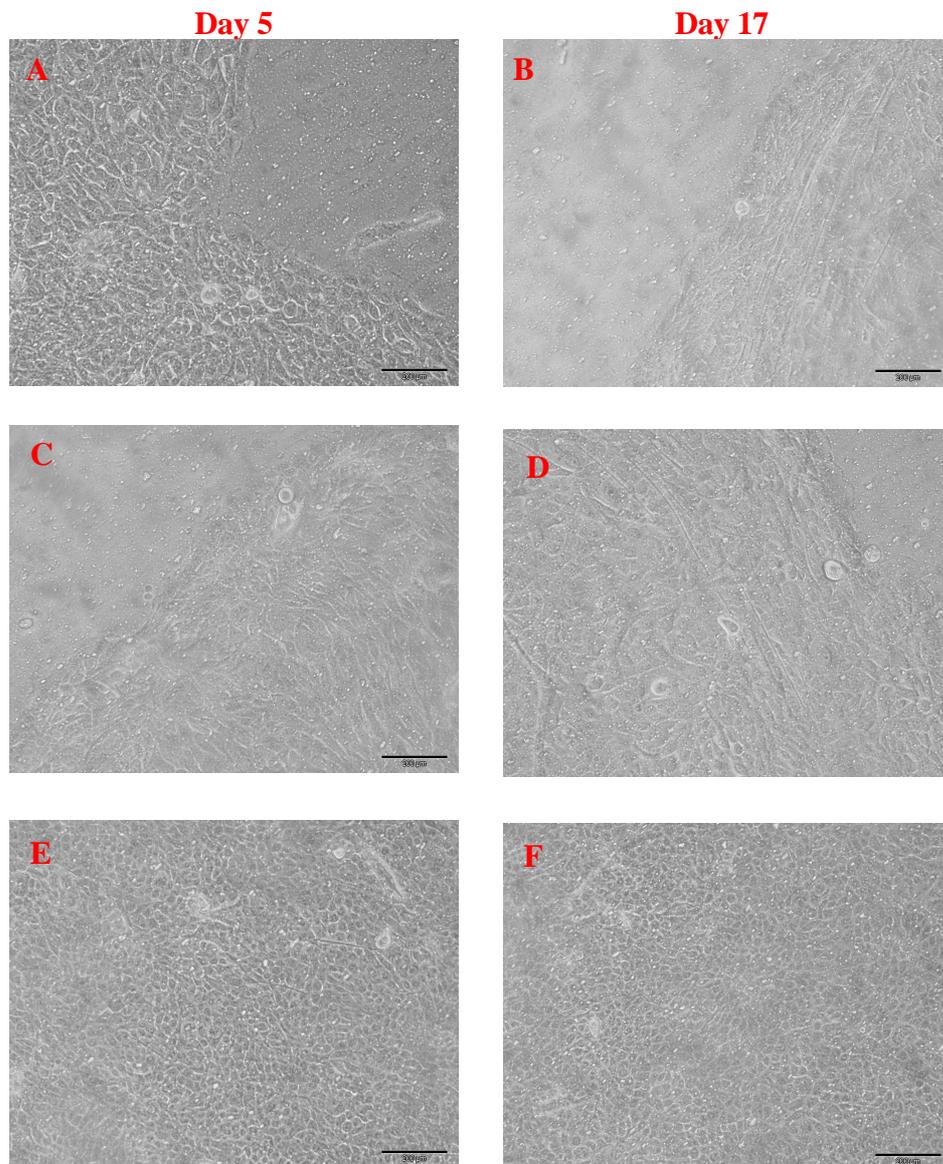
The culture grown in conditioned media showed a greater growth rate than condition A no-3T3. However, it did not perform as well as condition C Bot-3T3.



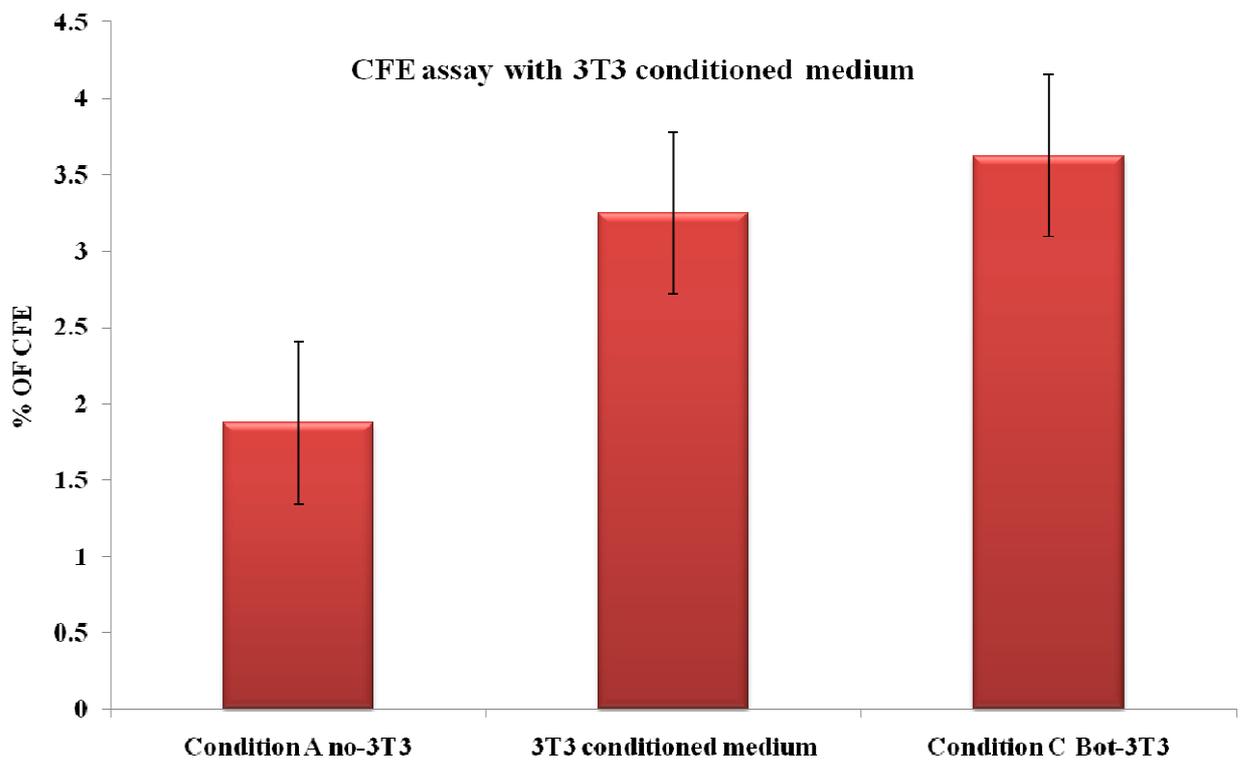
**Figure: 3.9.1:** Graphical representation of limbal-corneal epithelial cell outgrowth with condition A no-3T3, 3T3 conditioned medium and condition C Bot-3T3. Mean values, and n=2 (sample number 81 and 82).

Morphological assessment of limbal-corneal epithelial cells grown with 3T3 feeder cell conditioned medium was performed during culture period. Compact round epithelial cell morphology was observed at day 5, which is similar to the condition C Bot-3T3 cell morphology. Monolayer formation was observed by day 8. At the time of culture termination morphology was similar to that of condition C Bot-3T3 with compact tight cell-cell contact morphology with 1-2 cell thick (**figure 3.9.2**).

Limbal-corneal epithelial cells grown with conditioned media showed higher colony-forming efficacy compared to condition A no-3T3, whereas this condition is not greater than condition C Bot-3T3 condition (**figure: 3.9.3**).



**Figure: 3.9.2:** Phase contrast microscopic assessment of explant growth on trans-well inserts with no-3T3, conditioned medium and Bot-3T3 conditions; no 3T3 day 5 (A), day 17 (B); conditioned medium day 5 (C), and day 17 (D); condition C Bot-3T3 day 5 (E) and day 17 (F). Scale bar 200μm.



**Figure: 3.9.3:** Colony forming efficacy of limbal-corneal epithelial cells grown in trans-well inserts with no-3T3, conditioned medium and Bot-3T3 conditions. Mean values, and n=2 (sample number 81 and 82).

### **3.10 Limbal-corneal epithelial cultures translation to clinic**

*In vitro* cultivated limbal stem cell transplantation is promising as a treatment for limbal stem cell deficiency caused by chemical or thermal burns, ocular cicatrizing pemphigoid, Steven-Johnson syndrome, advanced microbial keratitis and aniridia. Limbal-corneal epithelial cell cultivation is not currently available in Ireland as a treatment option for patients. In collaboration with the Irish Blood Transfusion Service (IBTS), the National Eye Bank (NEB), and the Royal Victoria Eye and Ear Hospital (RVEEH), work on transferring and adopting the culture process to a suitable Good Manufacturing Process (GMP) was initiated.

Human amniotic membrane (HAM) supports the growth and expansion of limbal-corneal epithelial cells as demonstrated in this thesis and by other groups (Nakamura *et al.*, 2004b, Sangwan *et al.*, 2003b, Sangwan *et al.*, 2007, Kolli *et al.*, 2008). However the sourcing of HAM suitable for GMP process is problematic as issues over source (i.e. country of origin) sterility (i.e. potential infections risk) and logistic problems (transportation) exist.

As a part of the transfer to a GMP process, a number of commercially available HAM (**table 3.10**) preparations were investigated for their ability to support limbal-corneal epithelial growth compared to fresh frozen human amniotic membrane.

Trade Name	Properties
<b>Acelagraft™</b>	Amniotic membrane with decellularized, leaving a product composed of a complex collagen-based matrix, with 25 micron thick.
<b>AmbioDry2™</b>	Human amniotic membrane consists of collagen layers including basement membrane, and stromal matrix. 40µm thick.
<b>Ambio5™</b>	Processed for full thickness by RETAINING additional collagen matrices from the placental interface. Cells are devitalized and Nominal thickness: 110+ microns.
<b>EpiFix™</b>	The multi-layer structure of EpiFix™ is comprised of an epithelial layer and two fibrous connective tissue layers.

**Table: 3.10** List of commercially available HAM used in this study

### 3.10.1 Acelagraft™

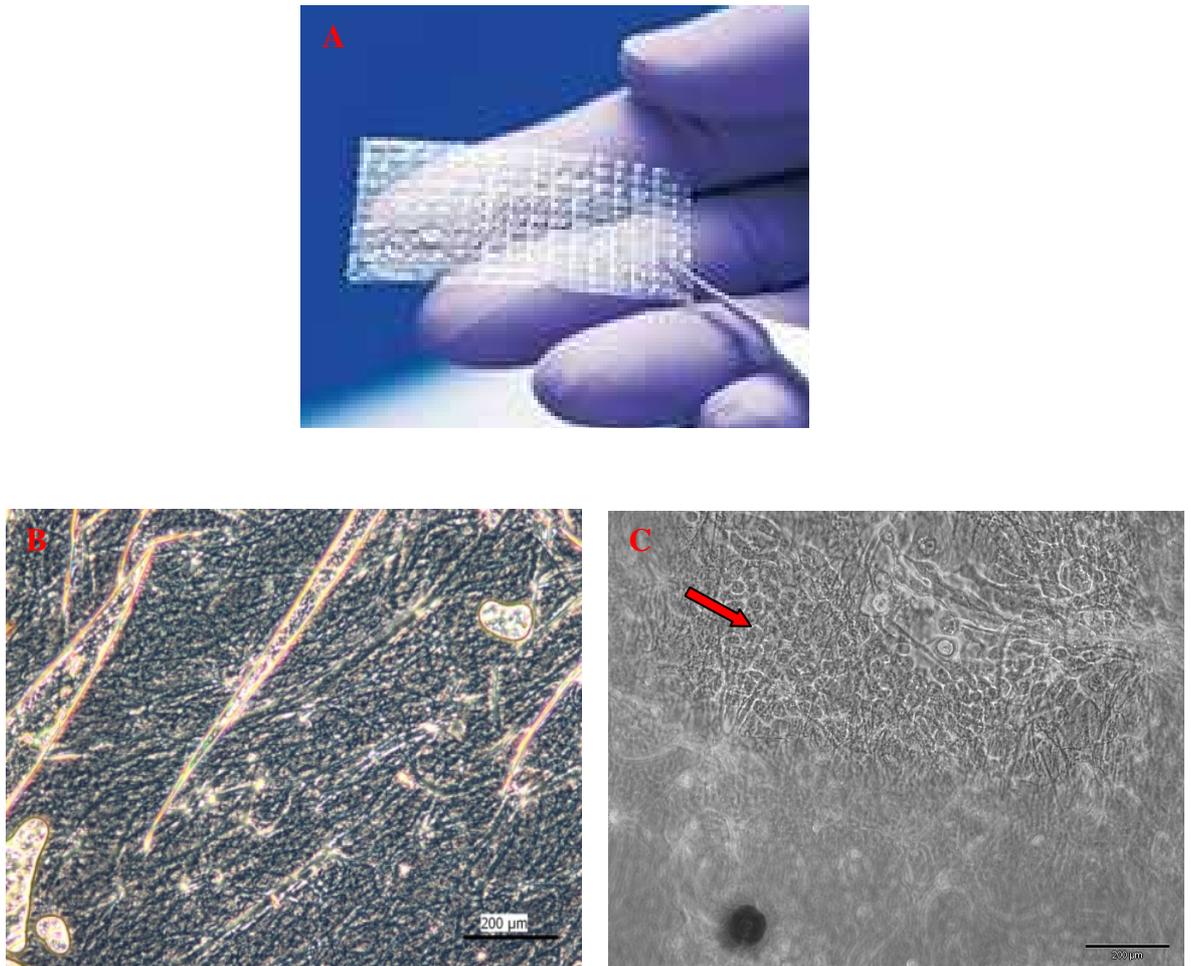
Acelagraft™ (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey) is a commercially available de-cellularised and dehydrated HAM (DDHAM) product. The tissue is sterilized by  $\gamma$ -irradiation and is acellular and freeze dried, with thickness of 25 microns, it can thus be stored and shipped at room temperature, making handling much easier. We used explants culture method (developed in this thesis) to grow limbal-corneal epithelial cells on Acelagraft™ after hydrating membrane with culture media for 15 minutes.

### 3.10.1.1 limbal-corneal epithelial cells grown on Acelagraft™

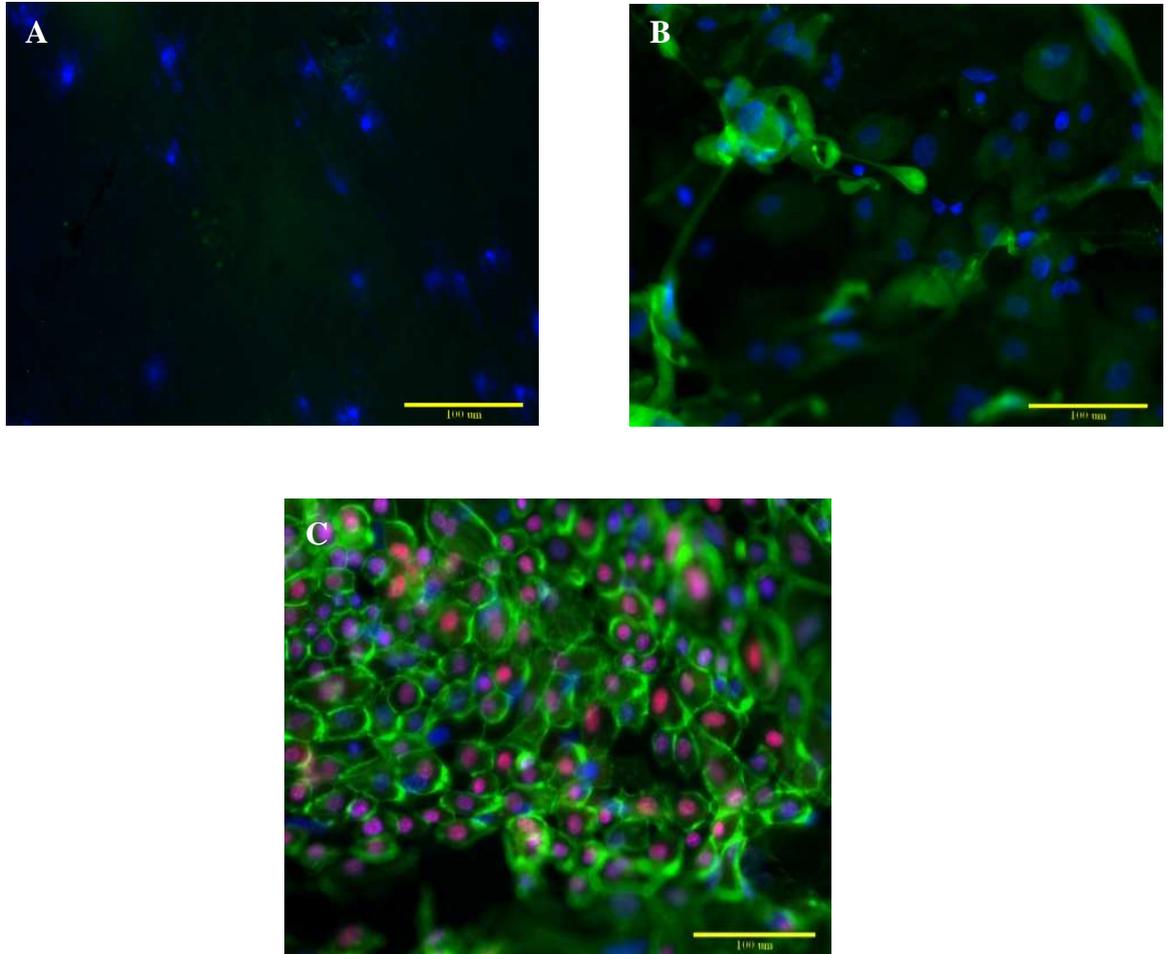
Limbal-corneal epithelial cells grown on Acelagraft™ is illustrated in **figure 3.10**. Acelagraft™ show membrane process marks leaving uneven surface **figure 3.10 a**. **Figure 3.10 b** illustrates the trypan blue stained Acelagraft™ membrane where amniotic epithelial cells were not seen.

A single layer of cuboidal limbal-corneal epithelial cells were grown on Acelagraft™ membrane with explants culture method **figure 3.10c**. The major technical issue with these cultures is that observation of epithelial cells is difficult due to undulations on membrane, leftover from to freeze-dry process.

Limbal-corneal epithelial cells grown on Acelagraft™ were stained for corneal differentiation marker cytokeratin 3 (CK3) and stem cells marker  $\Delta$ Np63 $\alpha$ , (**figure 3.10.1**) illustrates the representative of two biological replicates. The intact Acelagraft™ membrane without limbal-epithelial cell growth was stained for E-cadherin to rule out amniotic epithelial growth (**figure 3.10.1a**) illustrates E-cadherin stain of Acelagraft™ where E-cadherin staining was negative. Limbal-corneal epithelial cells grown on Acelagraft™ show positive staining for CK3 and  $\Delta$ Np63 $\alpha$  (**figure 3.10.1 b, c**).



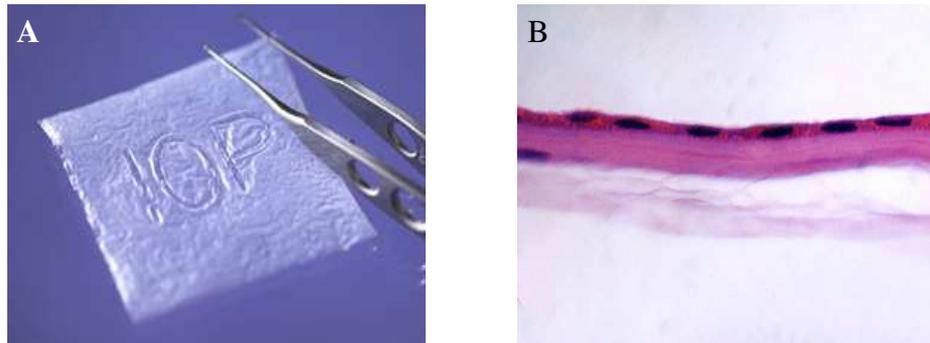
**Figure 3.10:** Acelagraft™ (A) Physical appearance of Acelagraft™ (B) Trypan blue staining of Acelagraft™ demonstrating a lack of amniotic epithelial cells , (C) Phase contrast image of limbal-corneal epithelial outgrowth on Acelagraft™, arrow indicating epithelial cells .Scale bar 200μm.



**Figure 3.10.1:** Immunofluorescence staining of Acelagraft<sup>TM</sup>: (A) Acelagraft<sup>TM</sup> alone stained for E-cadherin (green) and nuclei (blue). Cultured limbal-corneal epithelial cells on Acelagraft<sup>TM</sup> (B) Cytokeratin 3 (green) and nuclei (blue). (C)  $\Delta$ NP63 $\alpha$  (red), nuclei (blue) and E-cadherin. Scale bar 100 $\mu$ m.

### 3.10.2 AmbioDry2™

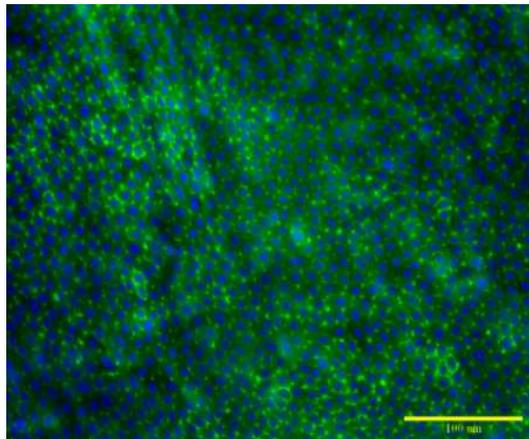
AmbioDry2™ is a dried amniotic membrane with 40µm thickness. The production process removes bioburden and potential virulency, though the mechanism of how they do this is not specified. The membrane still contains intact but devitalised epithelial and fibroblast cells.



**Figure 3.10.2:** (A) Physical appearance of AmbioDry2™ and (B) photomicrograph shows intact epithelial and fibroblast cell components with dense extracellular matrix. (Source-company website, <http://www.iopinc.com> )

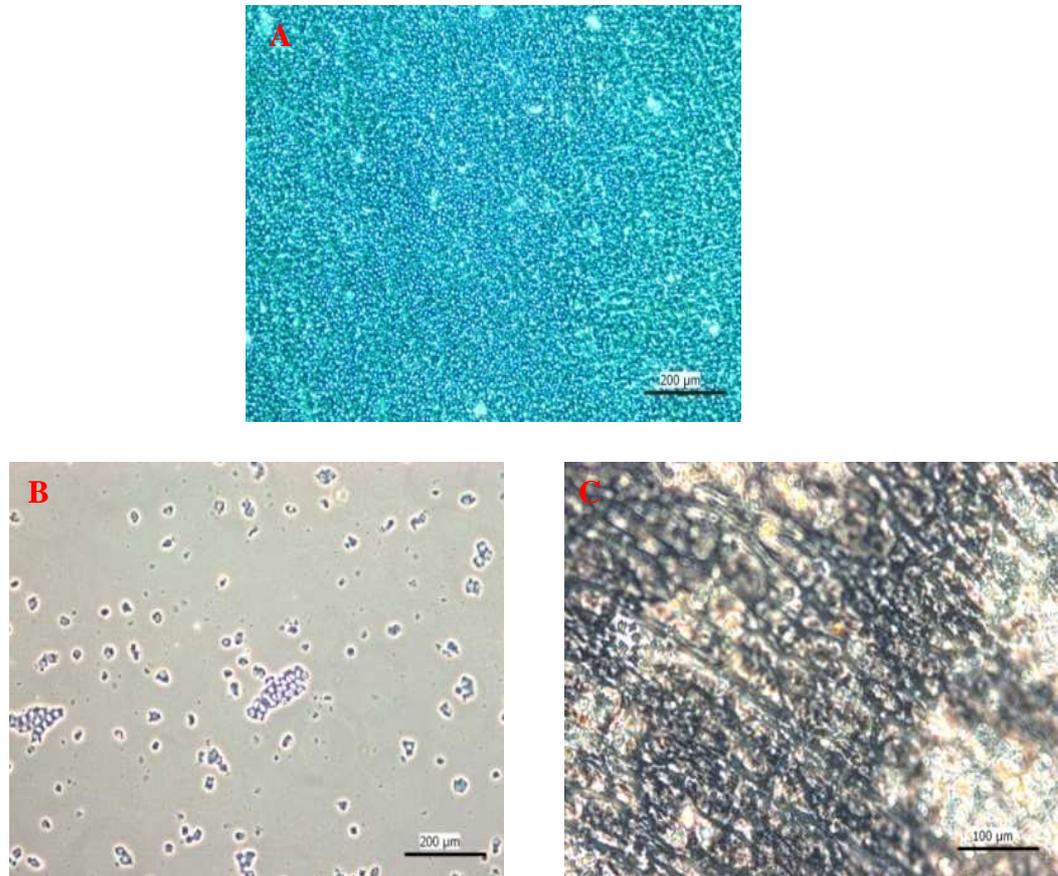
**Figure 3.10.2a** illustrates the physical appearance of AmbioDry2™ membrane, this membrane has intact amniotic epithelial and fibroblast cells, which are not removed with freeze-dried process (**figure 3.10.2b**).

The initial test run was with an intact AmbioDry2™ that was rehydrated in growth media. No obvious growth was obtained. There is a complete epithelial layer present on AmbioDry2™; which was confirmed by in house staining of membrane with E-cadherin (**figure 3.10.2.1**). This could be the possible reason for lack of growth of limbal-corneal epithelial cells on AmbioDry2™. Intact amniotic membrane epithelial cells could be removed using trypsin EDTA.



**Figure 3.10.2.1:** Immunofluorescence staining of AmbioDry2™ for E-cadherin (green) and Nuclei (blue) without limbal -corneal epithelial cells. Scale bar 100μm

To remove intact epithelial cells AmbioDry2™ membrane was treated with 0.25% trypsin EDTA for 5 minutes at 37<sup>0</sup>C and cells were scraped off with cell scraper (**figure 3.10.2.2 b**). Trypsinised membrane was stained with trypan blue and observed under bright field microscope (**figure 3.10.2.2 c**).



**Figure 3.10.2.2:** (A) Trypan blue staining of AmbioDry2<sup>TM</sup> demonstrating presence of epithelial cell layer. (B) Material removed from AmbioDry2<sup>TM</sup> by treatment with Trypsin EDTA. (C) Trypan blue staining of AmbioDry2<sup>TM</sup> membrane post-trypsin EDTA treatment demonstrating removal of epithelial layer. Scale bar 200μm.

### 3.10.2.1 Limbal-corneal epithelial cells grown on AmbioDry2™

The trypsinisation process was able to remove intact epithelial cells from AmbioDry2 membrane. A second experiment was performed in which AmbioDry2 was rehydrated in 0.9% saline and rehydrated in growth media. Both rehydrated conditions were further subdivided to yield four culture conditions

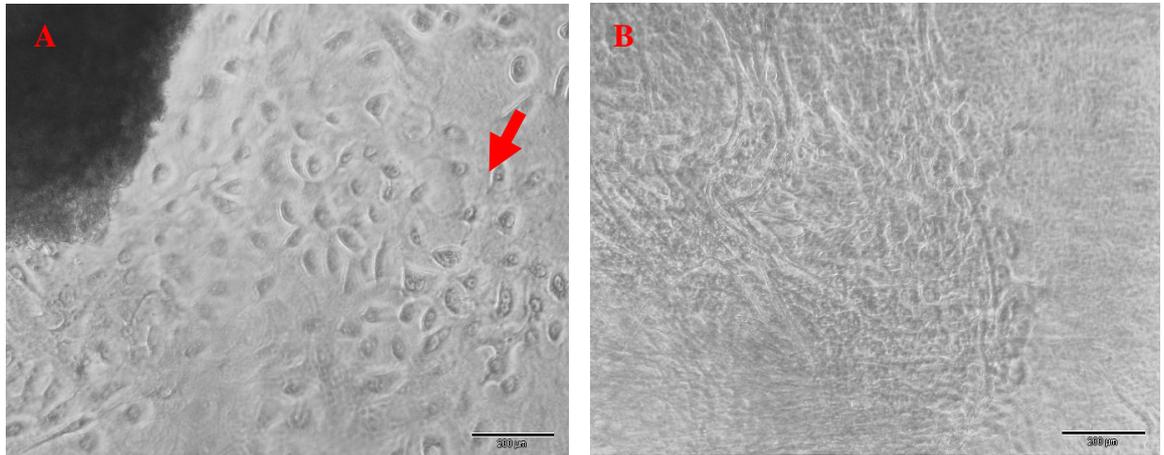
1. Saline rehydrated
2. Saline rehydrated de-epithelialised with trypsin-EDTA
3. Growth media rehydrated
4. Growth media rehydrated de-epithelialised with trypsin-EDTA

No growth was observed in condition 1 saline rehydrated, and condition 3 growth media rehydrated. Limbal-cornea epithelial cell growth was observed in condition 2, saline rehydrated de-epithelialised with trypsin-EDTA, epithelial cells growing from explants show round epithelial morphology, with irregular margins (arrow) and slow growth (**figure 3.10.2.3 a**).

Condition 4, growth media rehydrated de-epithelialised with trypsin-EDTA, shows epithelial growth from explants in monolayer format (**figure 3.10.2.3 b**). Thus the presence of the epithelial cells on the AmbioDry2 product was deemed to be inhibitory to limbal-cornea epithelial outgrowth from the explants.

However, trypsinised AmbioDry2 membrane was very thin and fragile/delicate and increased chance of tearing during processing. Limbal-corneal epithelial cells grown on AmbioDry2 with trypsinisation showed slow growth and poor, irregular epithelial monolayer morphology

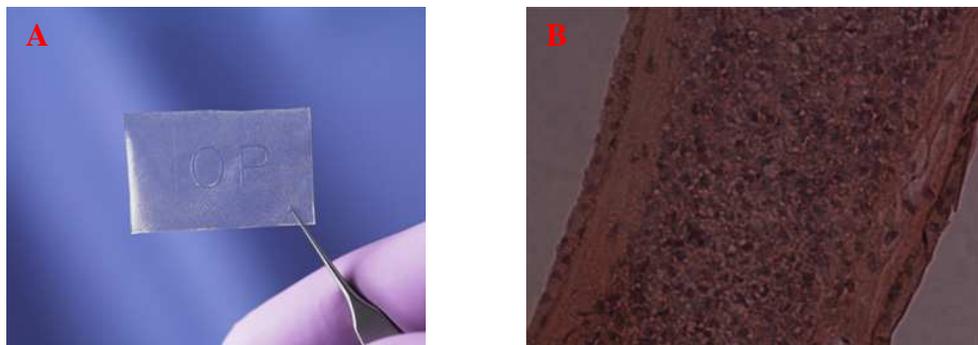
and did not cover the entire membrane. Poor and irregular growth of epithelial cells are not suitable for transplantation.



**Figure 3.10.2.3:** Phase contrast images of corneal- limbal epithelial outgrowth on: (A) AmbioDry2™ rehydrated with saline and treated with trypsin-EDTA. (B) AmbioDry2™ rehydrated with growth media and treated with trypsin-EDTA. Scale bar 200µm.

### 3.10.3 Ambio5™

Due to the reduced thickness of AmbioDry2 membrane after trypsinisation, Ambio5 membrane was used which is substantially thicker (110+ microns), more intact, native amniotic membrane allograft than AmbioDry2. The Ambio5 process removes bioburden and potential virulency, but retains the devitalized, cellular components (**figure 3.10.3**).

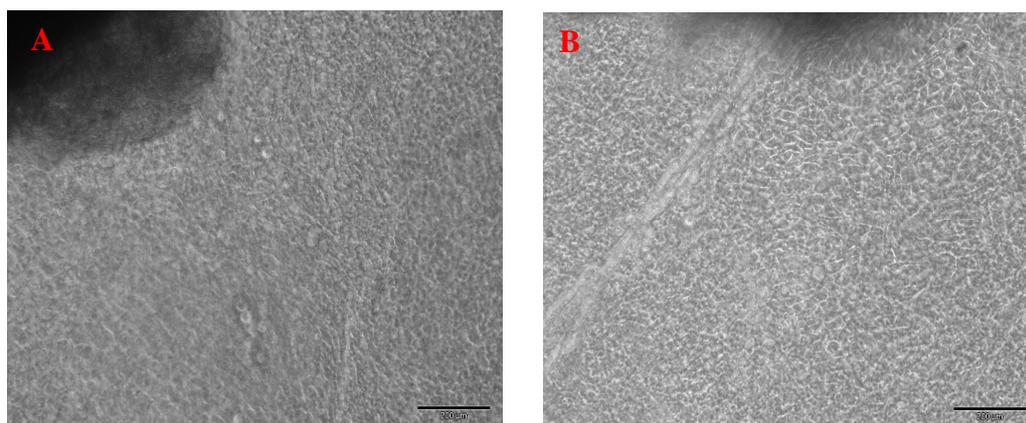


**Figure 3.10.3:** (A) Physical appearance of Ambio5™ and (B) Cross section of Ambio5™ an intact columnar epithelial cell layer on the membrane's surface; an intact, dense basement membrane; the presence of a loose collagen layer with fibroblasts; and an additional, thick layer of retained collagen from the placental interface. (Source-company website, <http://www.iopinc.com> )

### 3.10.3.1 limbal-corneal epithelial cells grown on Ambio5™

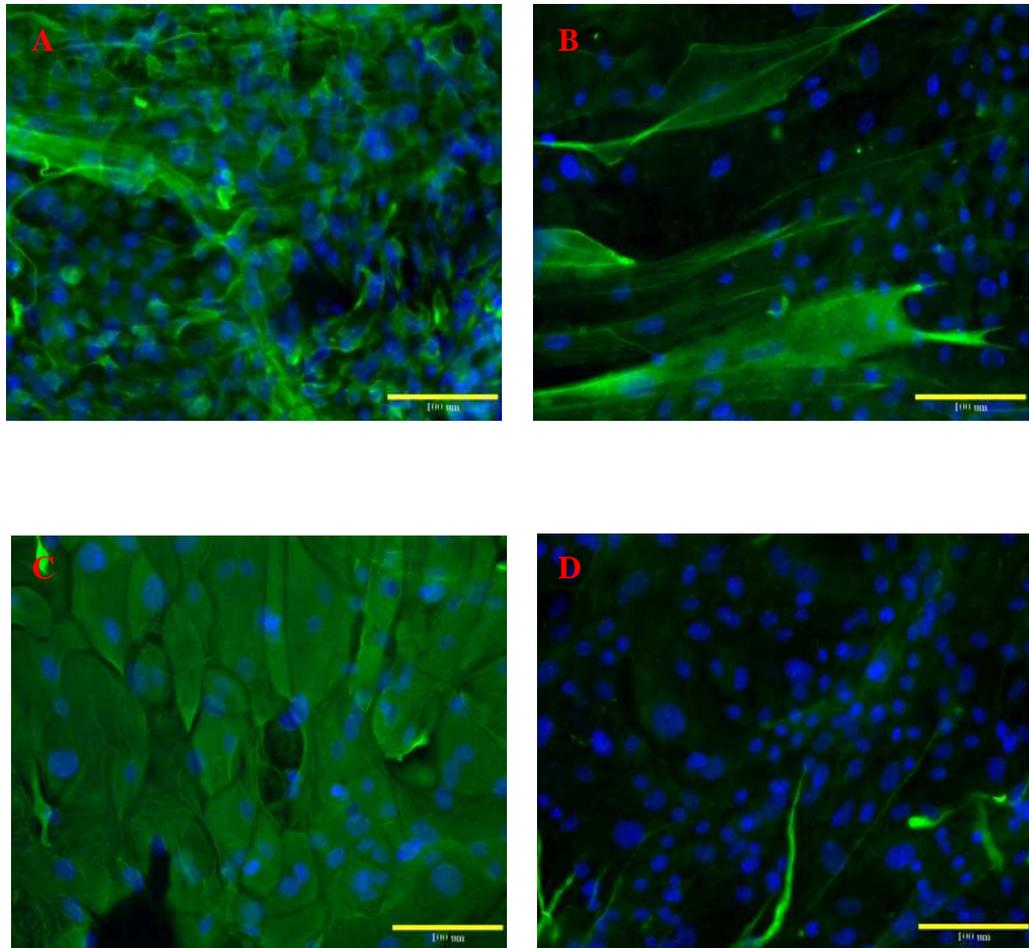
Due to the presence of thick epithelial sheet and collagen layer with fibroblasts on Ambio5™ **figure 3.10.3**, the membrane was processed using 0.25% trypsin EDTA to remove epithelial cells, and then followed the same procedure that described in above **section 3.10.2.1**.

AmbioDry5™ rehydrated with saline and growth media and de-epithelialised with trypsin-EDTA showed favourable limbal-epithelial cell growth from explants, with cobble shaped morphology and compact monolayer (**figure 3.10.3.1**).

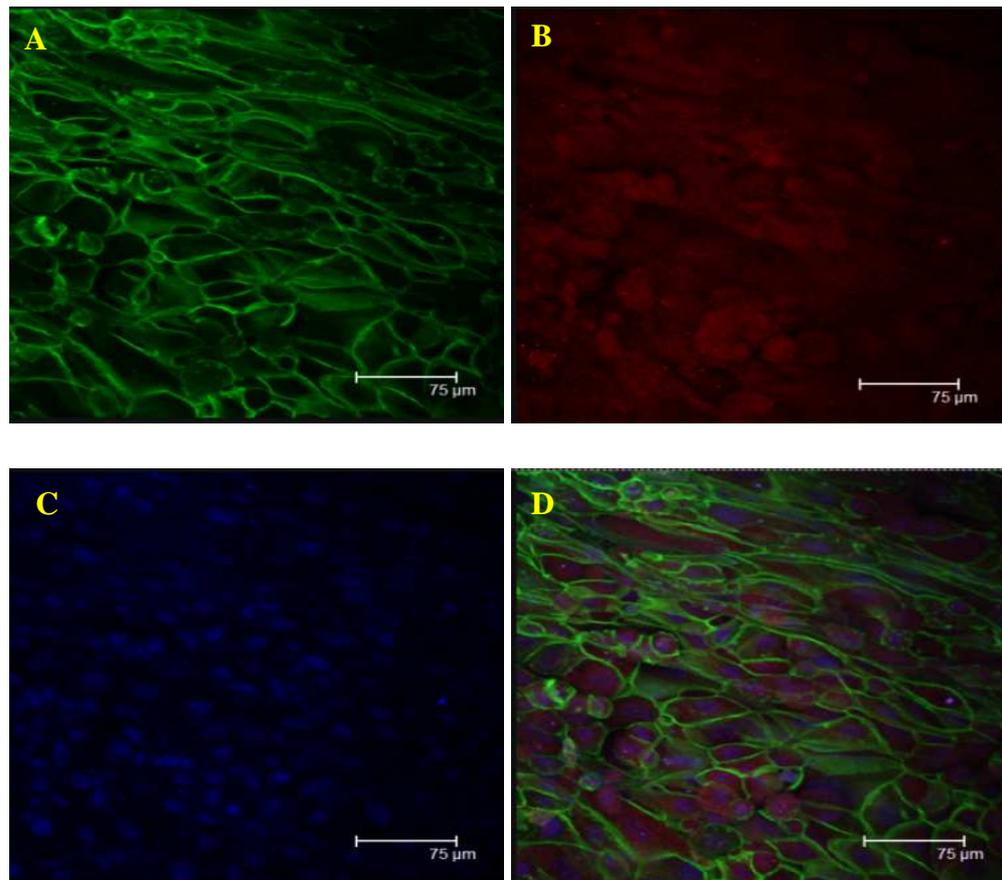


**Figure 3.10.3.1:** Bright field images of limbal-corneal epithelial cell outgrowth on: (A) AmbioDry5™ rehydrated with saline and treated with trypsin-EDTA. (B) AmbioDry5™ rehydrated with growth media and treated with trypsin-EDTA. Scale bar 200µm.

Limbal-corneal epithelial cells grown on AmbioDry5<sup>TM</sup> with above methods were analysed for corneal epithelial markers CK3, CK12 and stem cell marker  $\Delta$ Np63 $\alpha$  as illustrated in **figures 3.10.3.2** and **3.10.3.3**. Limbal-corneal epithelial cells grown on AmbioDry5<sup>TM</sup> membrane with treatment of saline hydration and trypsinisation has shown bright staining of CK3 (**figure 3.10.3.2 a**), whereas expression of CK12 is slightly lower in basal cells (**figure 3.10.3.2 b**). Similarly the expression of CK3 is higher in growth media rehydrated and trypsinised cells, compared to lower staining in (**figure 3.10.3.2 c, d**). AmbioDry5<sup>TM</sup> treated with growth media and trypsinised showed bright staining of stem cell marker  $\Delta$ Np63 $\alpha$ , with compact epithelial cell sheet, observed with E-cadherin stain (**figure 3.10.3.3**).



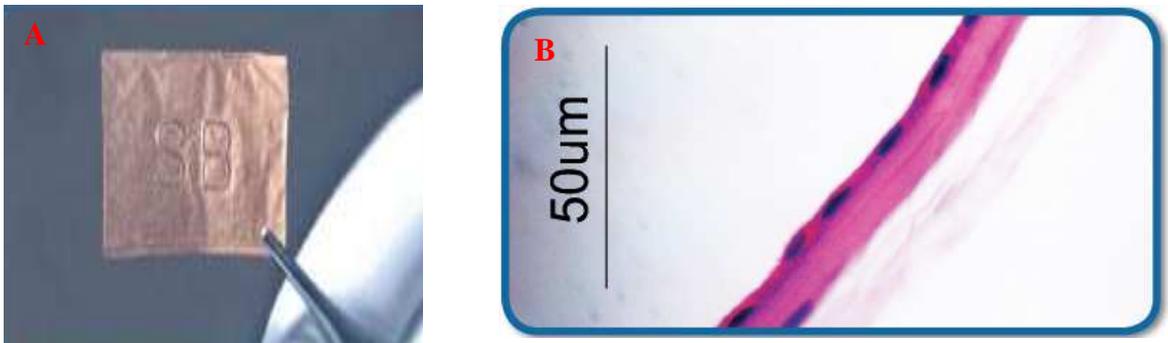
**Figure 3.10.3.2:** Immunofluorescence staining of limbal-corneal epithelial cells grown on AmbioDry5 rehydrated in saline treated with trypsin-EDTA. (A) Cytokeratin 3 (green) and nuclei (blue) and (B) CK12 (green) and nuclei (blue). AmbioDry5 rehydrated in growth media treated with trypsin EDTA for (C) CK3 (green) and nuclei (blue) and (D) CK12 (green) and nuclei (blue). Scale bar 100 $\mu$ m.



**Figure 3.10.3.3:** Immunofluorescence staining of limbal-corneal epithelial cells grown on AmbioDry5 rehydrated in growth media treated with trypsin-EDTA. (A) E-cadherin (green), (B)  $\Delta$ Np63 $\alpha$  (red) (C) nuclei (blue) and (D) Merge image. Scale bar 75 $\mu$ m.

### 3.10.4 EpiFix™

The multi-layer structure of EpiFix™ is comprised of an epithelial layer and two fibrous connective tissue layers. The Purion<sup>SM</sup> process used in this process of making this membrane uses minimal manipulation to maintain the structural integrity of the membrane, resulting in the unique characteristic features.



**Figure 3.10.4:** EpiFix™: (A) Physical appearance of EpiFix™ membrane, (B) Microscopic view shows the amniotic epithelium layer, the dense basement membrane layer, and a layer of compact fibroblast and collagen. (Source- [www.surgicalbio.com](http://www.surgicalbio.com) )

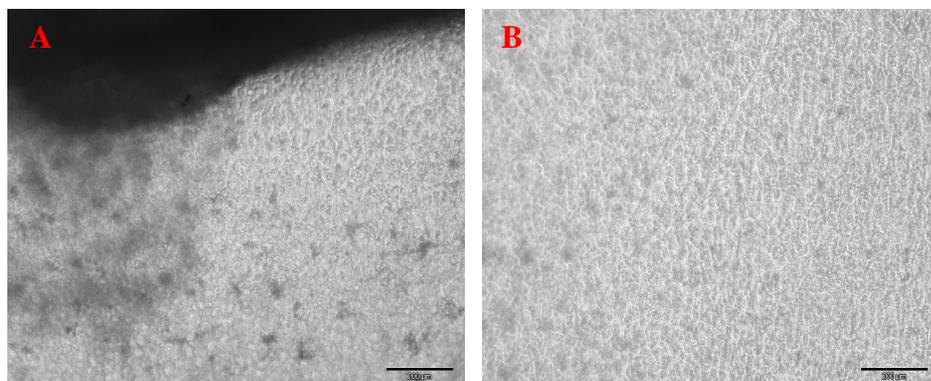
EpiFix™ membrane appeared to contain amniotic epithelial cells (**figure 3.10.4**). This membrane also requires the treatment of trypsinisation to remove epithelial cells after rehydration.

### 3.10.4.1 Limbal-corneal epithelial cells grown on EpiFix™

The initial experiment using EpiFix™ : Membrane was divided in two to yield two culture conditions.

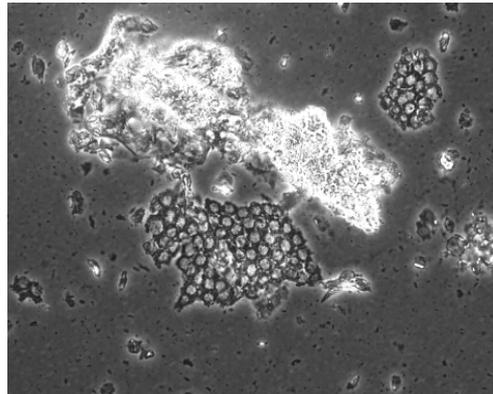
1. Rehydrated with saline and treated with trypsin EDTA
2. Rehydrated with growth media and treated with trypsin EDTA.

After trypsinisation and removal of intact amniotic epithelial cells, explants culture method was used to grow limbal-corneal epithelial cells. Limbal-corneal epithelial cell growth was obtained in both conditions, membrane treated with saline and trypsinised showed epithelial cell growth in monolayer from explants (**figure 3.10.4.1 a**). Whereas membrane rehydrated with growth media and trypsinised showed thick multilayered cells (**figure 3.10.4.1b**).



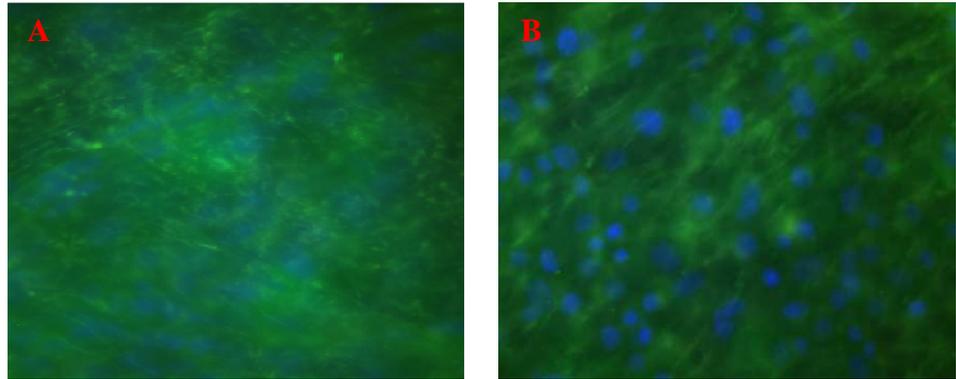
**Figure 3.10.4.1:** Phase contrast images of limbal-corneal epithelial outgrowth at day 7 on: (A) EpiFix™ rehydrated with saline and treated with trypsin-EDTA. (B) EpiFix™ rehydrated with growth media and treated with trypsin-EDTA. Scale bar 200µm.

It was noted that EpiFix™ did appear to contain cellular material and on the reverse side a yellowish layer was present. The presence of this yellow layer made observing the cultures under microscope very difficult. Treating the EpiFix™ membrane with trypsin resulted in the removal of the cellular type material (**figure 3.10.4.2**).

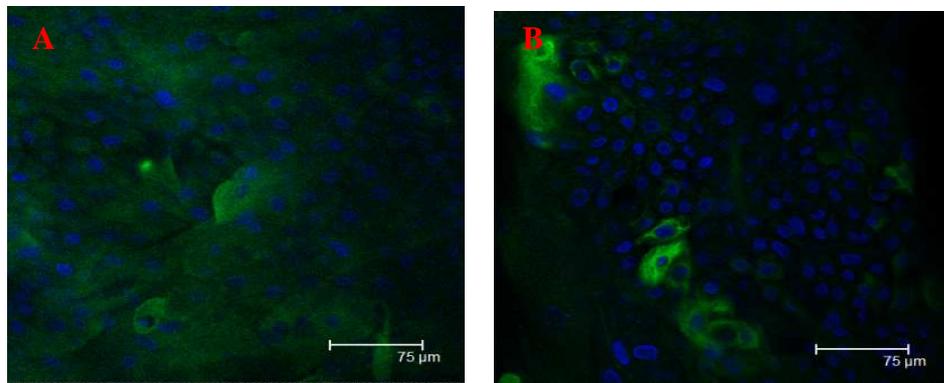


**Figure 3.10.4.2:** Phase contrast image of material; removed from EpiFix™ by Trypsin-EDTA treatment. 20x Magnification

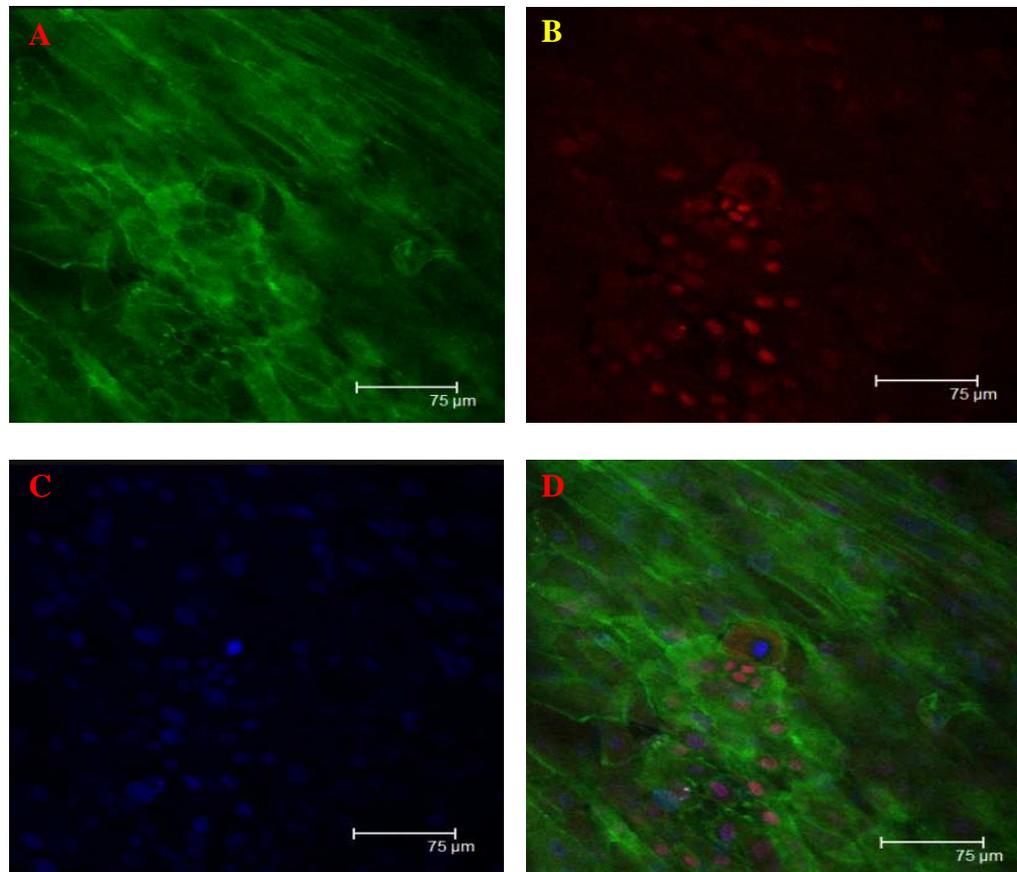
Limbal-corneal epithelial cells grown on EpiFix™ with both treatments were analysed for corneal markers CK3 and CK12 and the stem cell marker  $\Delta$ Np63 $\alpha$  (**figures 3.10.4.3 to 3.10.4.6**). EpiFix™ treated with saline and trypsinisation showed bright staining for CK3 and CK12, whereas  $\Delta$ Np63 $\alpha$  showed brighter staining with thick epithelial cell sheet (**figure 3.10.4.3 and 3.10.4.5**). EpiFix™ membrane rehydrated with growth media and treated with trypsin showed lower staining of CK3, CK12 (**figure 3.10.4.4**), whereas a bright stain of  $\Delta$ Np63 $\alpha$  was observed with thick epithelial sheet (**figure 3.10.4.6**).



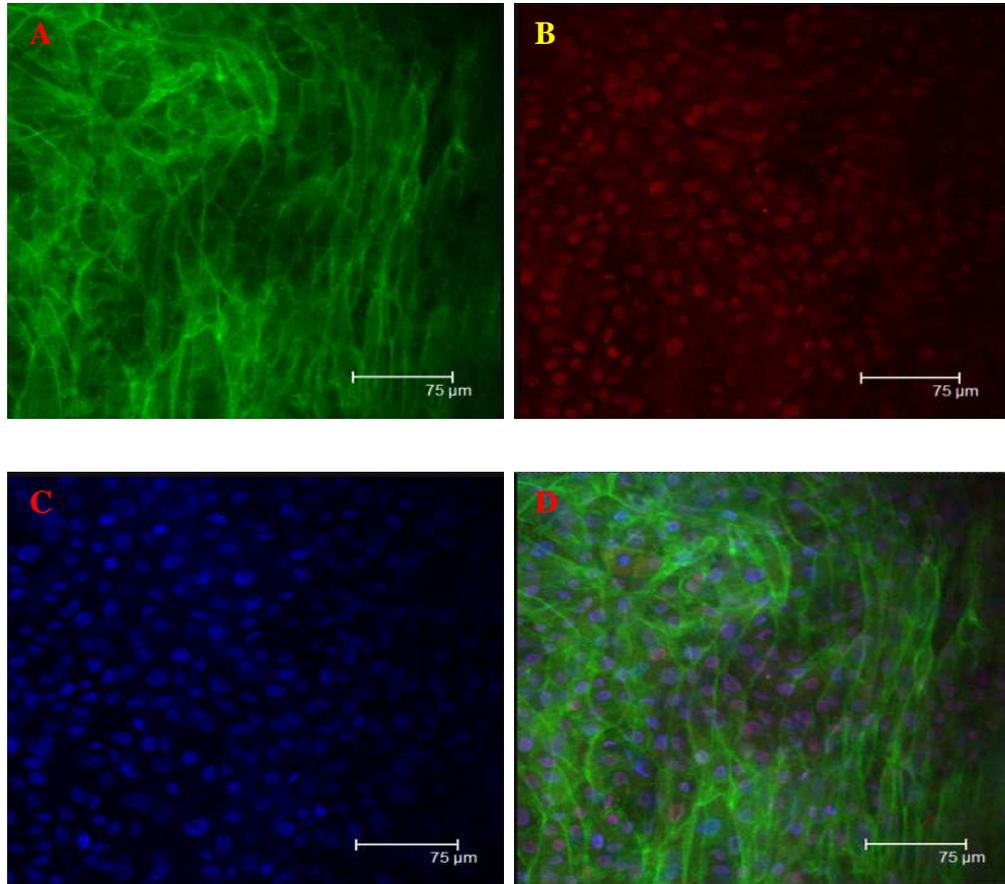
**Figure 3.10.4.3:** Immunofluorescence staining of limbal-corneal epithelial cells grown on EpiFix rehydrated with saline and treated with trypsin-EDTA. (A) Cytokeratin 3 (green), nuclei (blue), (B) Cytokeratin 12 (green), nuclei (blue) 20x Magnification.



**Figure 3.10.4.4:** Immunofluorescence staining of limbal-corneal epithelial cells grown on EpiFix™ rehydrated with growth media and treated with trypsin-EDTA (A) Cytokeratin 12 (green), Nuclei (Blue). (B) Cytokeratin 3 (green), Nuclei (Blue). Scale bar 75μm.



**Figure 3.10.4.5:** Immunofluorescence staining of limbal-corneal epithelial cells grown on EpiFix™ rehydrated with saline and treated with trypsin-EDTA. (A) E-cadherin (Green), (B)  $\Delta$ Np63 $\alpha$  (Red), (C) Nuclei (Blue) and (D) Merge image. Scale bar 75 $\mu$ m.

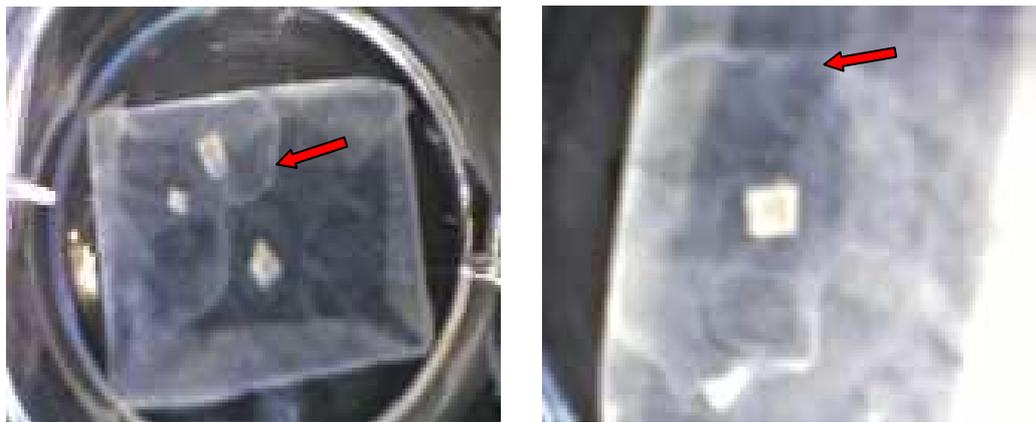


**Figure 3.10.4.6:** Immunofluorescence staining of limbal-corneal epithelial cells grown on EpiFix™ rehydrated with growth media and treated with trypsin-EDTA. (A) E-Cadherin green. (B)  $\Delta Np63\alpha$  (Red) (C) Nuclei (Blue) and (D) Merge image. Scale bar 75 $\mu$ m.

A third repeat was performed with EpiFix™ membrane in two culture conditions.

1. Saline rehydrated
2. Saline rehydrated treated with trypsin-EDTA.

Limbal-corneal epithelial cell growth was observed in both conditions, whereas the growth rate was very slow and did not progress far into the membrane (**figure 3.10.4.7**), an arrow indicates the edge of epithelial cell growth, and growth of limbal-corneal epithelial cells was inconsistent and unsatisfactory.



**Figure 3.10.4.7:** Outgrowth of limbal-corneal epithelial cells on EpiFix™ (A) EpiFix™ treated with saline rehydration and without trypsinisation, (B) EpiFix™ rehydrated with saline and de-epithelialised.

### **3.11 Characterization of limbal fibroblast-like cells (LFLc)**

In human cornea, the corneal stroma makes up about 80% of the corneal thickness and consists of a densely packed yet transparent connective tissue, the transparency of which is thought to originate from its regularly ordered and equally spaced collagen bundles, produced by the corneal fibroblasts called keratinocytes.

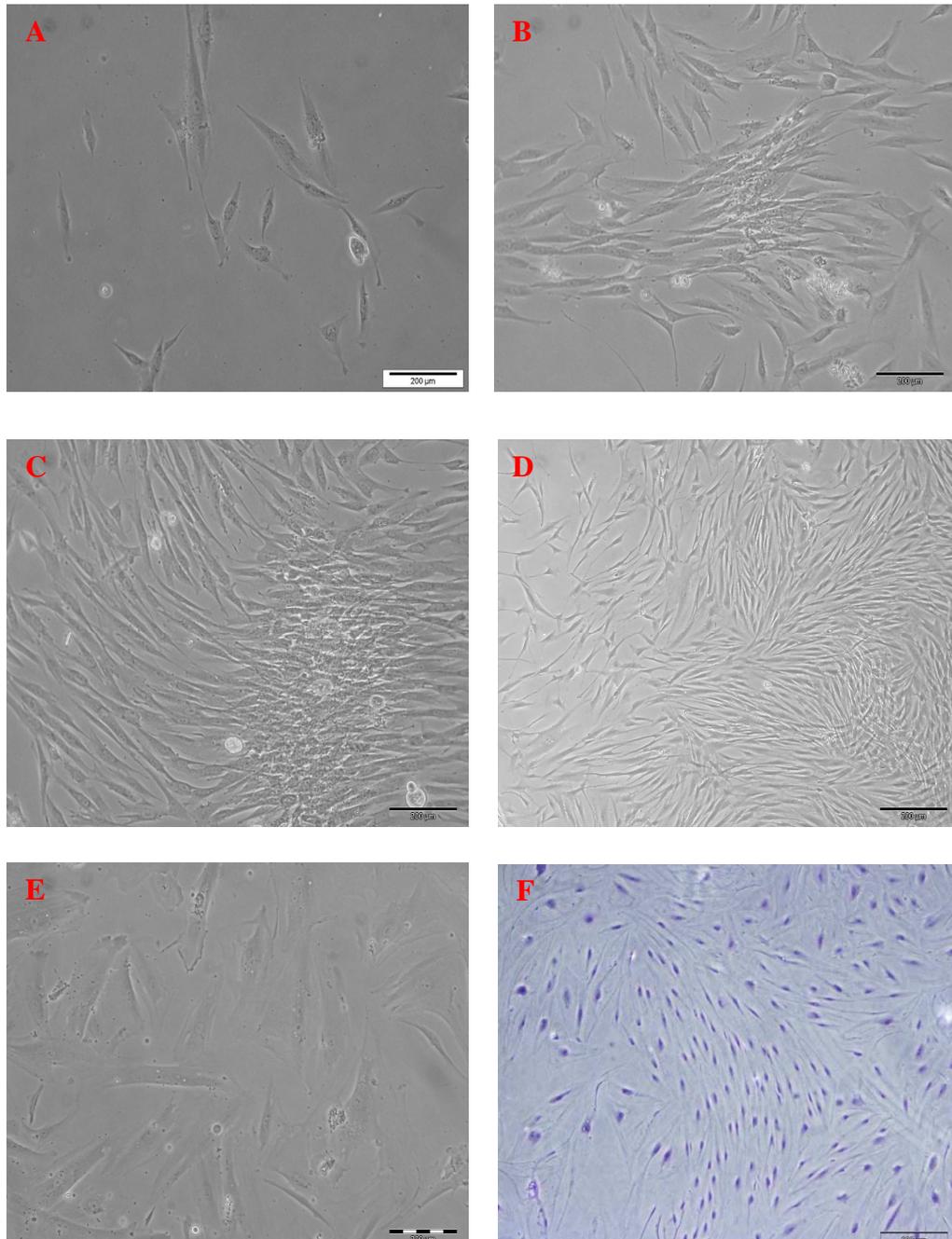
Corneal keratinocytes are easily obtained and grown by any standard culture methods (Masur *et al.*, 1993, Beales *et al.*, 1999). Enzyme action will release stromal cells as well as the desired epithelial cells and both cell types, whereas, explanted full-thickness corneal pieces give rise to stromal cells in culture (Eggl *et al.*, 1989, He and McCulley, 1991, Majo *et al.*, 2008).

#### **3.11.1 Morphology of limbal fibroblast-like cells (LFLc)**

Limbal fibroblast-like cells (LFLc) were derived as described in materials and methods section **2.11**. Initially no cells were observed attached to culture surface of a 6 well plate. However by day 7 (**figure 3.11 a**) 8-9 cells are observed attached to culture surface.

The morphology of these isolated attached cells at day 7 was an elongated spindle shape, similar to what would be expected of fibroblast cells (**figure 3.11 a**). These cells proliferate rapidly (**figure 3.11 b**), with confluency observed at day 6-10 of the culture. The cells maintain the elongated spindle shaped morphology (**figure 3.11 c**).

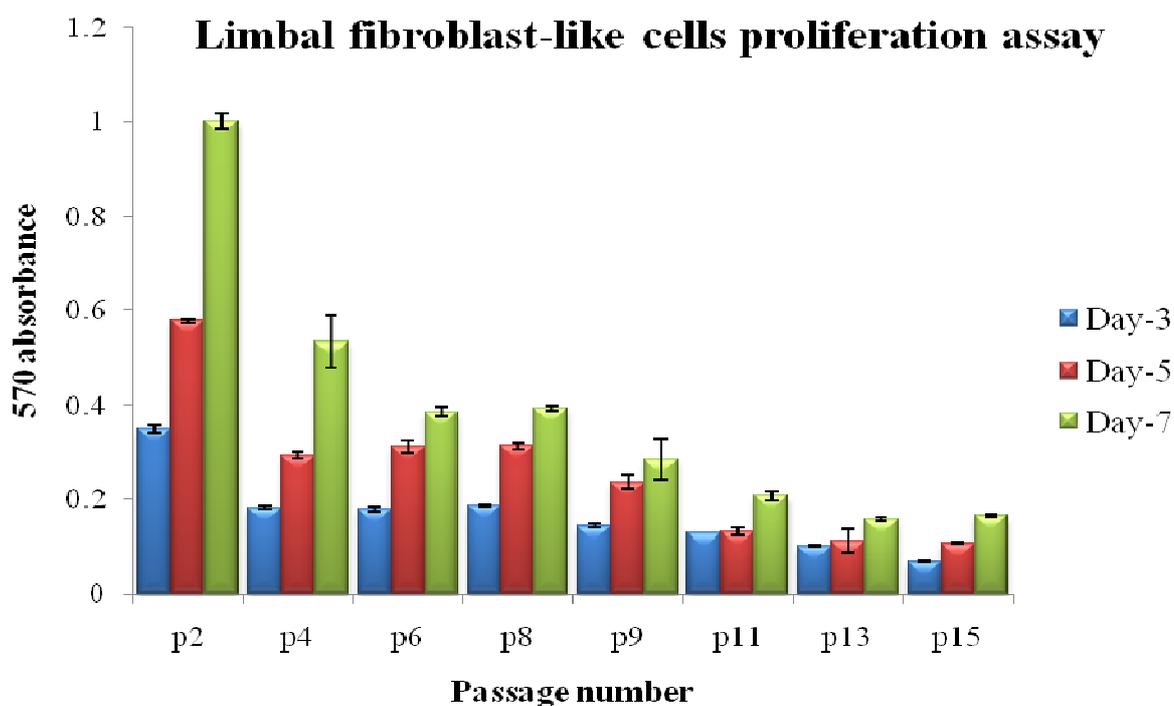
Cells that are passaged maintain the observed elongated spindle shaped morphology associated with fibroblast cells (**figure 3.11 d**). However, at later passages, up to passage 10 to 15, the cell morphology alters with the loss of elongated spindle shape and becoming large and more spread out (**figure 3.11 e**). The spindle-shaped, fibroblast morphology was observed with Wright-Giemsa staining (**figure 3.11 f**).



**Figure 3.11 :** Bright field microscopic assessment of limbal fibroblast-like cells (LFLC) (A) Early stage initial growth day 7 (B), Day 10, (C) Day 15, (D) passage 1, (E) passage 15, and (F) Giemsa stained cells. Scale bars 200μm.

### 3.12 Proliferation of limbal fibroblast-like cell (LFLc)

The observed change in morphology with passage number appeared to be associated with a change in proliferation. To test this, a proliferation assay was performed with cells at different passages. The results presented in (figure 3.12), show that an increase in passage number is accompanied by a decrease in proliferation. With a significant decrease observed between passage 2 and passage 4.

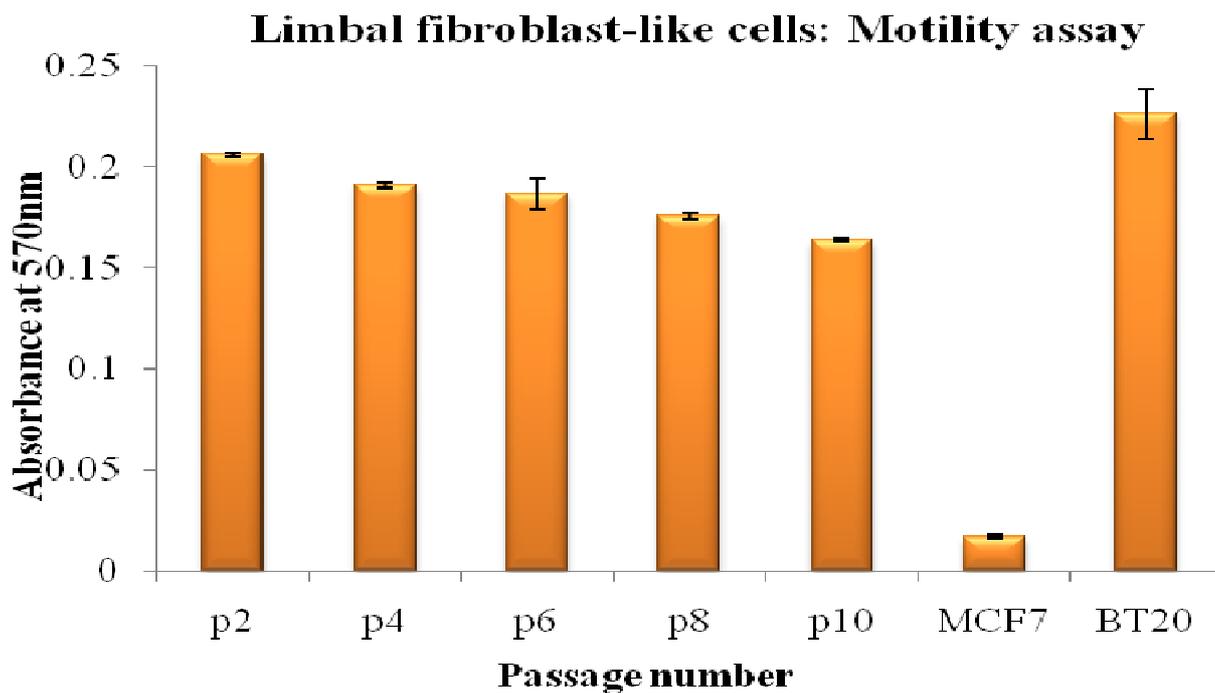


**Figure: 3.12** Proliferation assays of limbal ‘Fibroblast-like’ cells at different passage number terminated on day 3, 5 and 7 cultures. Decreased proliferation rate observed with higher passage number. Mean =  $\pm$  S.D and n=6 (sample number 17, 19, 20, 22, 25, 56).

### 3.13 Limbal fibroblast-like cells (LFLC) motility assay

Different passage numbers of limbal fibroblast-like cells were grown for 24 hours in culture prior to the motility assay. The motility assay were carried out by the addition of 100 $\mu$ L of media containing  $1 \times 10^5$  cells into non-coated inserts with pore size 8.0 $\mu$ m (BD bioscience 353097) and were incubated for 24 hours at 37<sup>0</sup> C, 5% CO<sub>2</sub> atmosphere. **Figure 3.13** illustrates the motility of LFLC in relation to passage number.

Limbal fibroblast-like cells are motile as compared to a non-motile cell line MCF-7, and motility rate was decreasing slightly as passage number increased



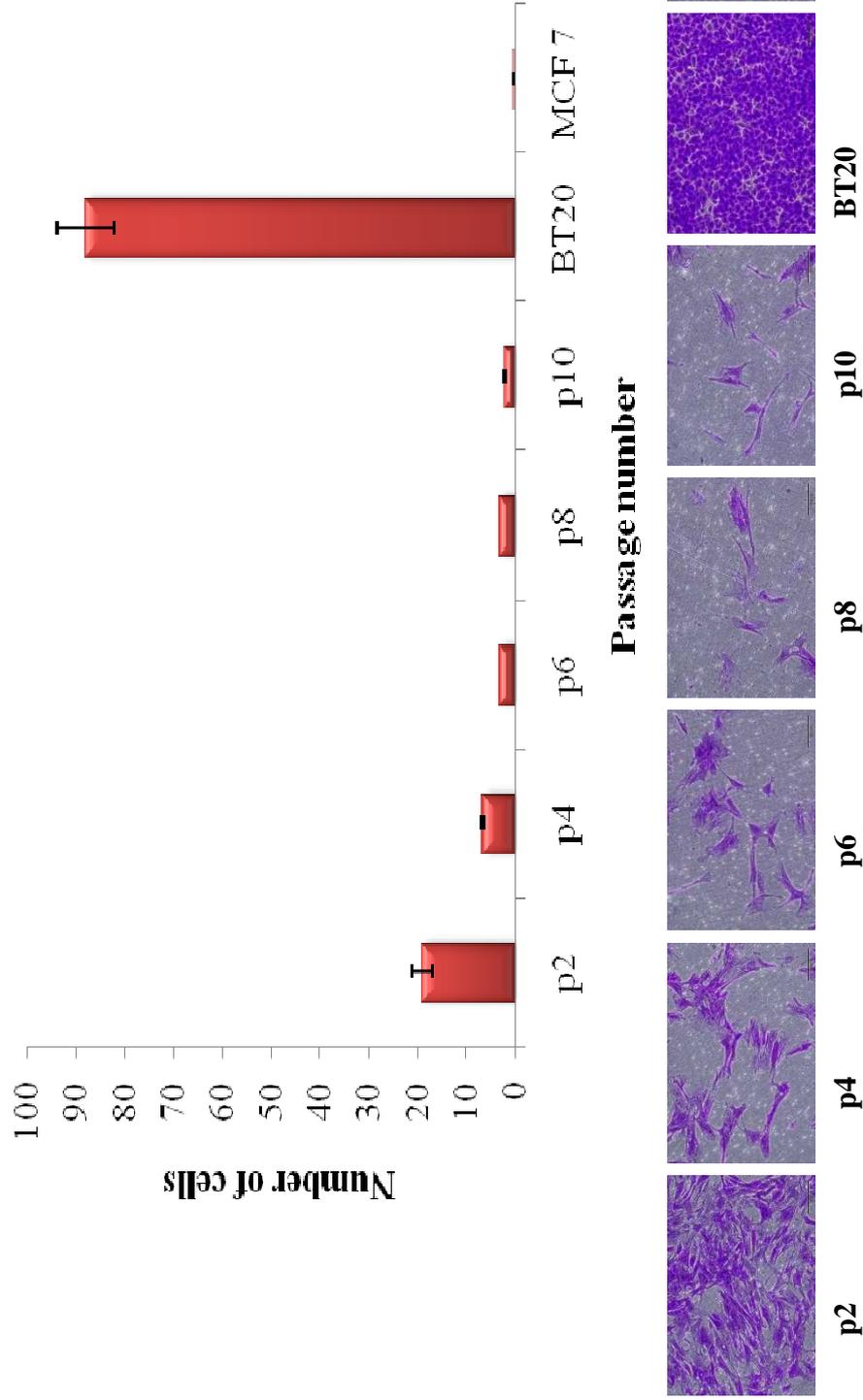
**Figure: 3.13** Limbal-fibroblast like cells motility assay, limbal fibroblast-like cells are motile compared to control non motile cells MCF7, Mean =  $\pm$  S.D and n=4 (sample number 17, 19, 20, 22).

### **3.14 Limbal fibroblast-like cells (LFLc) invasion assay:**

We examined the invasion properties of LFLc *in vitro*, Invasion chambers and cells were prepared as discussed in material and methods **section 2.12** and incubated at 37<sup>0</sup> C, 5% CO<sub>2</sub> for 24 hours. **Figure 3.14** illustrates the invasion properties of LFLc in relation to passage number for three biological replicates.

The limbal fibroblast-like cells were found to be invasive, with decrease in invasiveness with increasing passage number.

### Limbal fibroblast-like cells: Invasion assay

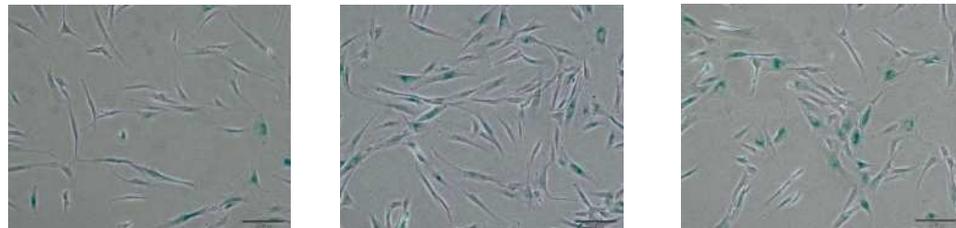
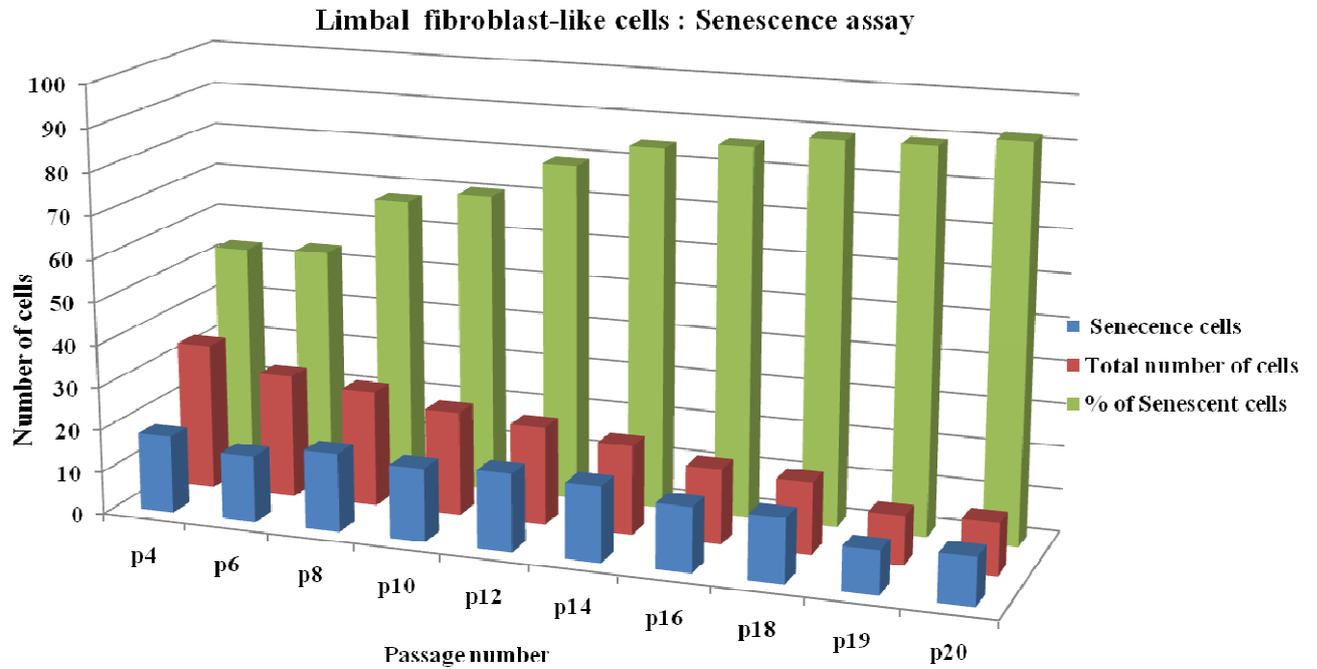


**Figure 3.14:** Limbal fibroblast-like cells Invasion assay, graph showing invasion of limbal fibroblast-like cells at different passage number, light microscopy pictures of cells after invasion, stained with 0.5% crystal violet, (magnification 10 x) Mean =  $\pm$  S.D and n=3 (sample number 17, 19, 20)

### **3.15 Senescence-associated $\beta$ -Galactosidase assay**

Proliferation assay for limbal fibroblast-like cells indicated that, in *in vitro* conditions these cells have decreased proliferation level compared to early passage cells, proliferation assays also show significant decreases in invasion with increasing passage number and slightly decreased motility rate with higher passage number. The morphology of limbal fibroblast-like cells changed from long stretched fibroblasts to large cell body as passage number increase **figure 3.11 e**.

A senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) assay was performed as described in materials and method **section 2.14**, to monitor the senescence stage of LFLc at different passages. The percentage of senescence cells increased as the passage number increased in limbal fibroblast-like cells **figure 3.15**.

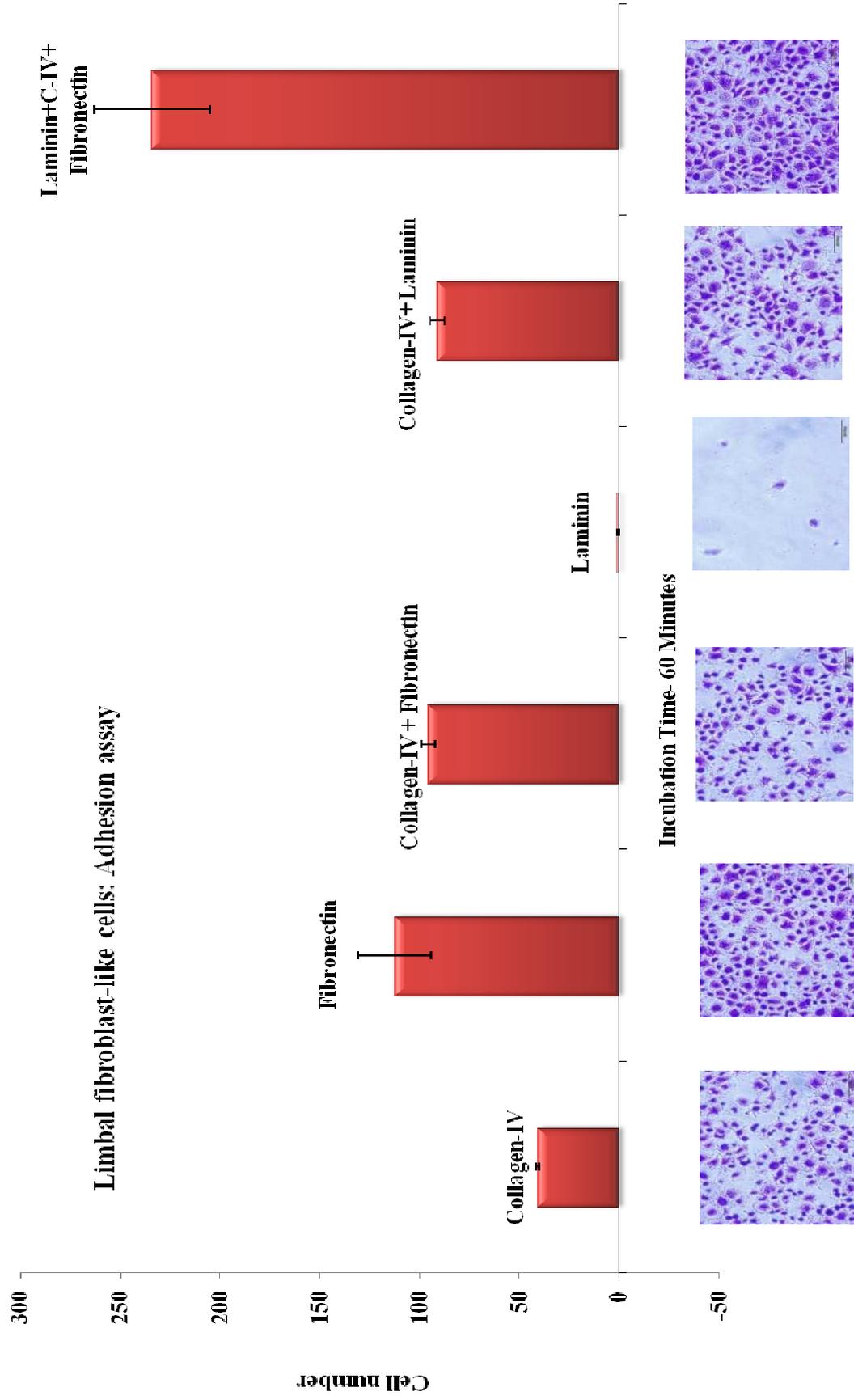


**Figure: 3.15** Limbal ‘fibroblast-like’ cells senescence-associated  $\beta$ -galactosidase. Light microscope images of passage 4, 14 and 20. Senescence in limbal ‘fibroblast-like’ cells increased as passage number increases. Mean  $\pm$  S.D and n=3 (sample number 20, 22, 25).

### **3.16 Extra cellular matrix adherence assay for limbal ‘fibroblast-like’ cells**

Cell attachment and adhesion properties of LFLc were tested for different extra cellular components. Adhesion assays were performed as discussed in materials and methods **section 12.5**. **Figure 3.16** illustrates the adhesive properties of LFLc to various extra cellular matrix, graph plotted incubation time vs. cell number with three biological replicates.

LFL cells attached to collagen IV, however greater adhesion was observed on fibronectin matrix. Interestingly the LFL cells showed a slight decrease in attachment to a matrix composed of collagen IV and fibronectin. Very poor attachment was observed with LFL cells plated on a laminin matrix. However, a matrix composed of collagen IV and laminin showed a significant increase in attachment compared to either matrix on its own. In the case of a matrix composed of laminin, collagen IV and fibronectin a significant increase in adhesion was observed compared to any other matrix either singly or in combination. The morphology of LFL cells appeared to be small and epithelial-like with all ECM components with 60 minutes adhesion time.



**Figure: 3.16** Limbal fibroblast-like cells adhesion assay with ECMs, respective graph plotted cell number Vs type of ECM, and light microscopy pictures of stained adhered limbal fibroblast –like cells. Mean = ± S.D (n=3 biological replicates)

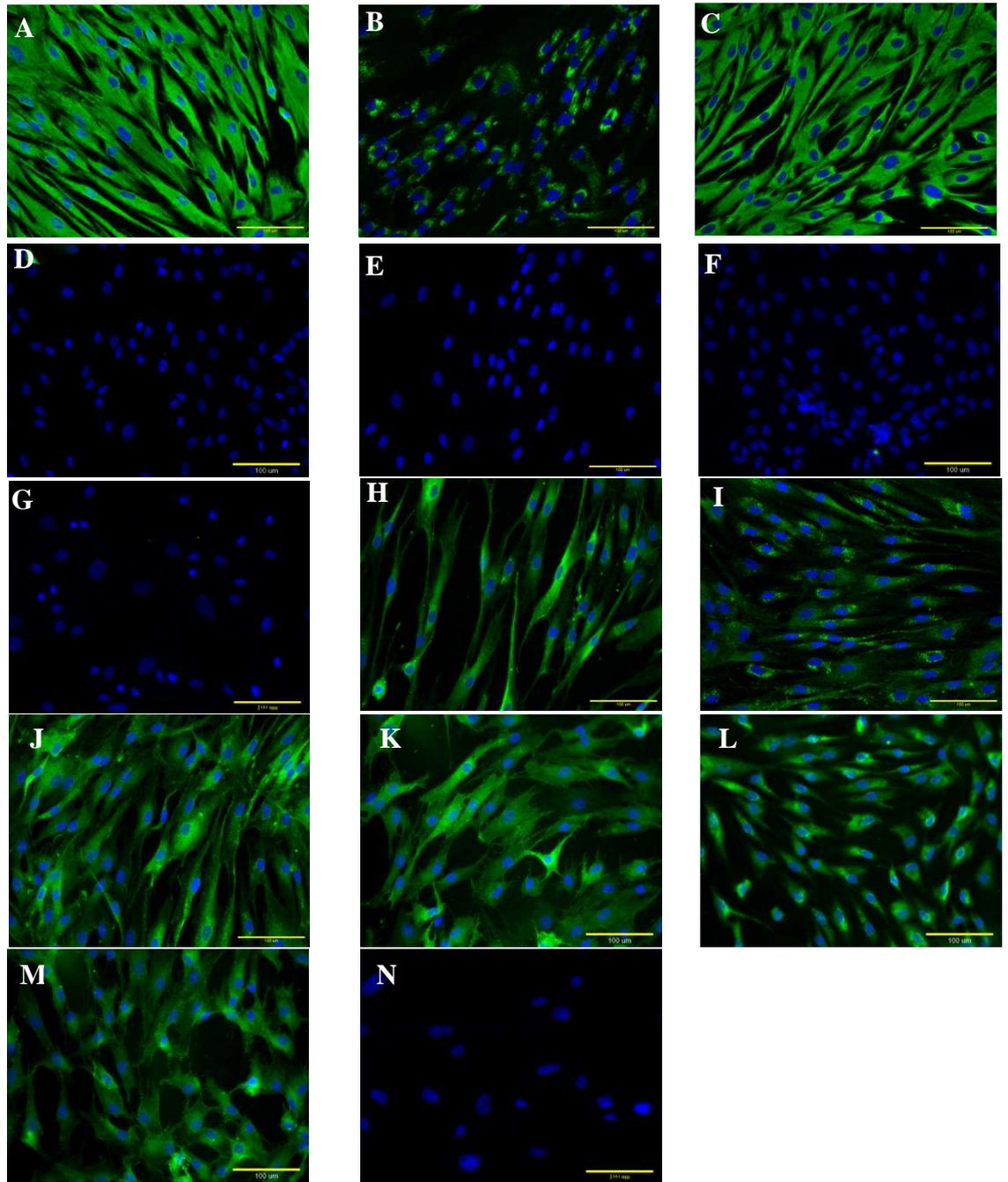
### 3.17 Cellular characterisation of limbal ‘fibroblast-like’ cells

Identification of LFL cells typically relies on use of cell surface markers cluster of differentiation (CD) antigens that denote the expression of particular proteins associated with genomic activity related to a particular differentiation state of the cell and cytokeratin markers to rule out epithelial contamination.

One recent study suggested that numerous bone marrow-derived cells existed in mouse corneal stroma. Funderburgh *et al.*, have shown that some cells in the adult corneal stroma express stem cell markers and have the ability to generate adult keratocytes (Funderburgh *et al.*, 2005). More recently Polisetty *et al.*, demonstrated the presence mesenchymal-like cells in limbal stroma (Polisetty *et al.*, 2008).

#### 3.17.1 Immunofluorescence of LFL cells for specific markers

Immunofluorescence staining for the cytoskeletal protein vimentin was positive in all LFL cells (**figure 3.17 a**), similarly, a high level of  $\alpha$ -SMA was observed LFL cells (**figure 3.14 c**). Whereas, fibroblast surface protein expression was low compared to vimentin and  $\alpha$ -SMA (**figure 3.14 b**). The panel of cytokeatins [(CK3, CK12, CK19, and Pan-cytokeratin (CK5, 6, 8)], showed negative expression in LFL cells (**figure 3.17 d to g**). The trans-membrane protein E-cadherin showed positive expression in all LFL cells. Low levels of neuron specific enolase was seen (**figure 3.17 h and i**), along with high levels of the CD panel (CD 29, CD31, CD49b, CD105) were seen in LFL cells. CD34 appeared negative in LFL cells (**figure 3.17 j to n**).



**Figure 3.17:** Morphological and phenotypic expression limbal ‘fibroblast-like’ cells. Mesenchymal markers (A) vimentin, (B) Fibroblast surface protein, (C)  $\alpha$  smooth muscle actin (SMA), (D) CK3, (E) CK12, (F) CK19, (G) Pan-cytokeratin (CK5, 6, 8), (H) E-cadherin, (I) Neuron Specific Enolase (NSE), (J) CD29, (K) CD31, (L) CD49b, (M) CD105, (N) CD34.

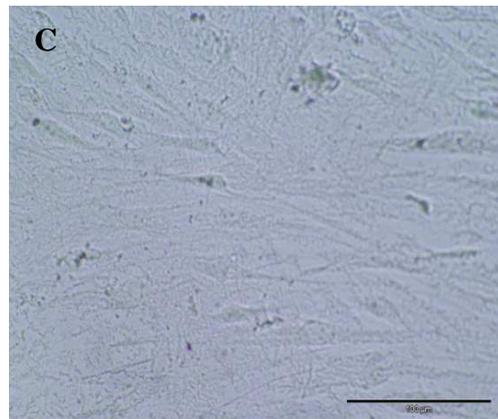
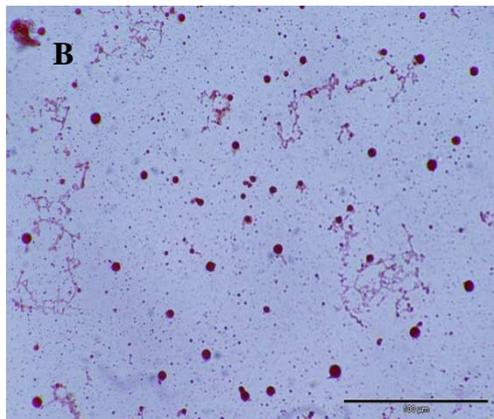
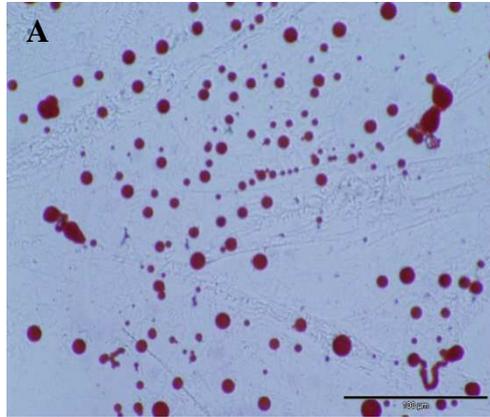
### **3.18      *In Vitro* differentiation potential of LFL cells**

There have been reports in the literature that LFL cells with bone marrow mesenchymal stem cell (BMSC) properties can be isolated (Lu *et al.*, 2010, Polisetty *et al.*, 2008). To investigate this, a study was conducted to determine the potential of LFL cells to differentiate into adipogenic and osteogenic lineages in comparison to bone marrow mesenchymal stem cells. Experiments were conducted as described in materials and methods **section 2.16.1** and **2.16.2**.

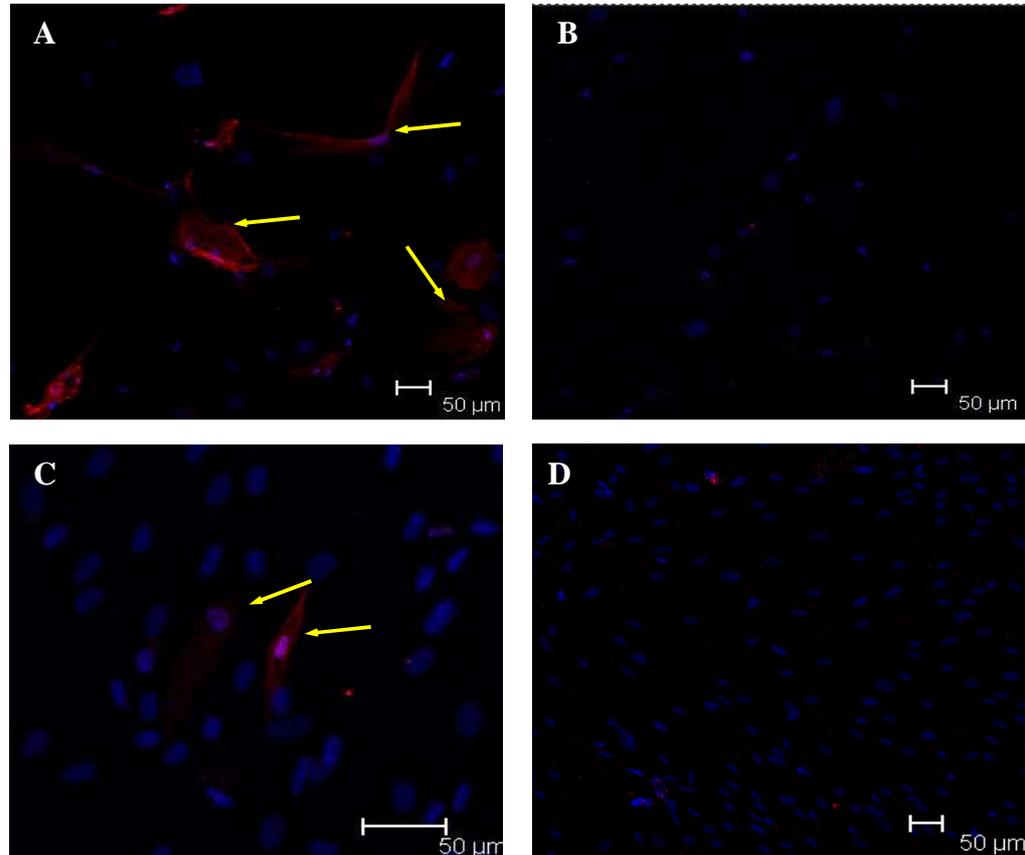
#### **3.18.1      Differentiation of LFL cells to adipocytes**

To generate adipocytes, confluent passage 3 LFLc and bone marrow mesenchymal stem cells (BMSc) as a positive control cells were induced by treating with adipogenic differentiation media (hydrocortisone, isobutylmethylxanthine, and indomethacin). Differentiation into adipose tissue was apparent by the accumulation of lipid-rich vacuoles. Oil Red O lipid staining was observed in treated LFL cells and control BMS cells (**figure 3.18 a, b**). The negative control using un-induced limbal fibroblast-like cells did not pick up any Oil Red staining, indicating that the cells were not spontaneously differentiating into adipocytes.

Human bone marrow mesenchymal cells (BMSc) were differentiated *in vitro* for 21 days using adipogenic differentiation medium and were stained with the adipogenic lineage-specific antibody to FABP-4, untreated BMS cells showed negative for FABP-4 antibody. Similarly, the LFLc differentiated to adipogenic lineage showed positive for FABP-4 antibody staining; undifferentiated LFLc were negative for FABP-4 staining (**figure 3.18.1**).



**Figure 3.18 :** Adipogenic differentiation; adipogenic differentiation of human bone marrow mesenchymal cells show the presence of Oil-Red O positive lipid (A), and differentiated limbal fibroblast-like cells positive for Oil Red O stain (B), undifferentiated LFLC were negative for Oil-red O stain (C).



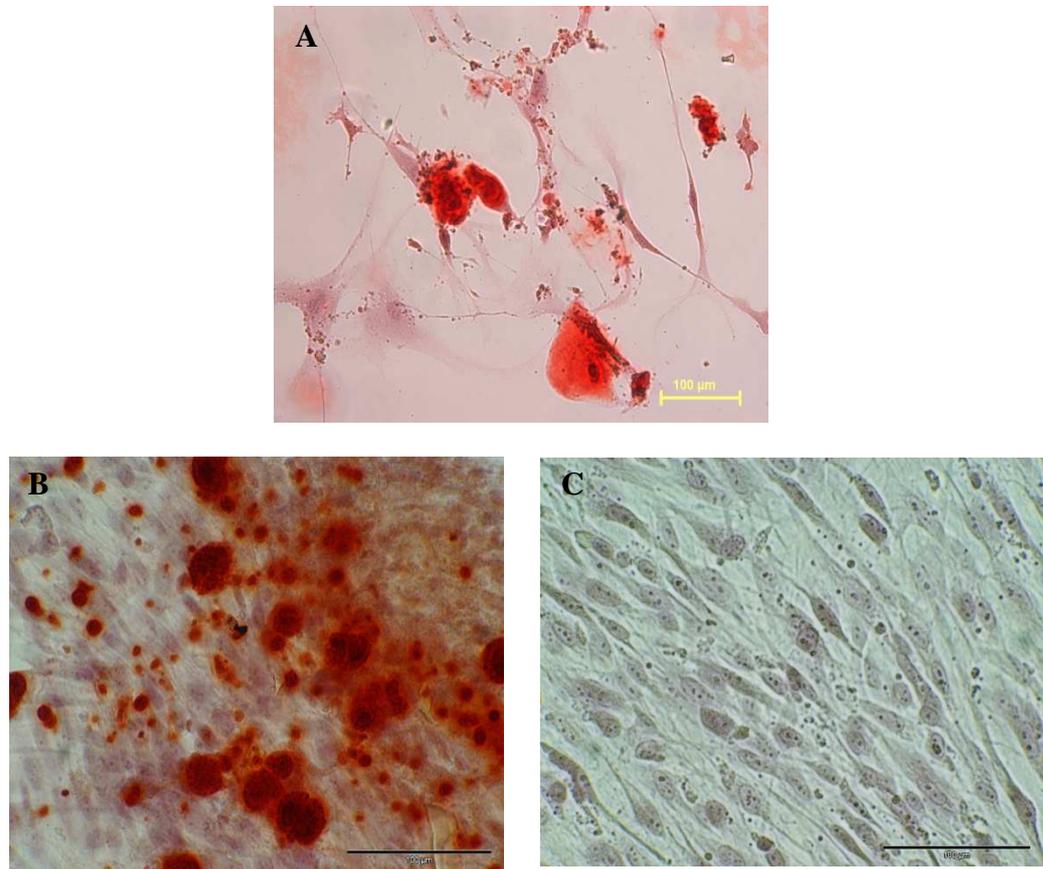
**Figure 3.18.1:** Adipocyte differentiation of limbal fibroblast-like cells (LFLc); (A) human bone marrow mesenchymal cells (BMSc) differentiated *in vitro* for 21 days using adipogenic differentiation medium were stained with the adipogenic lineage-specific antibody to FABP-4, (red) (arrows). (B) Untreated BMSc are negative for FABP-4. (C) LFLc differentiated to adipogenic lineage showed positive staining for FABP-4; (D) undifferentiated LFLc were negative for FABP-4 staining. Cells were counterstained with DAPI (nuclei, blue).

### 3.18.2 Differentiation of LFL cells to osteocytes

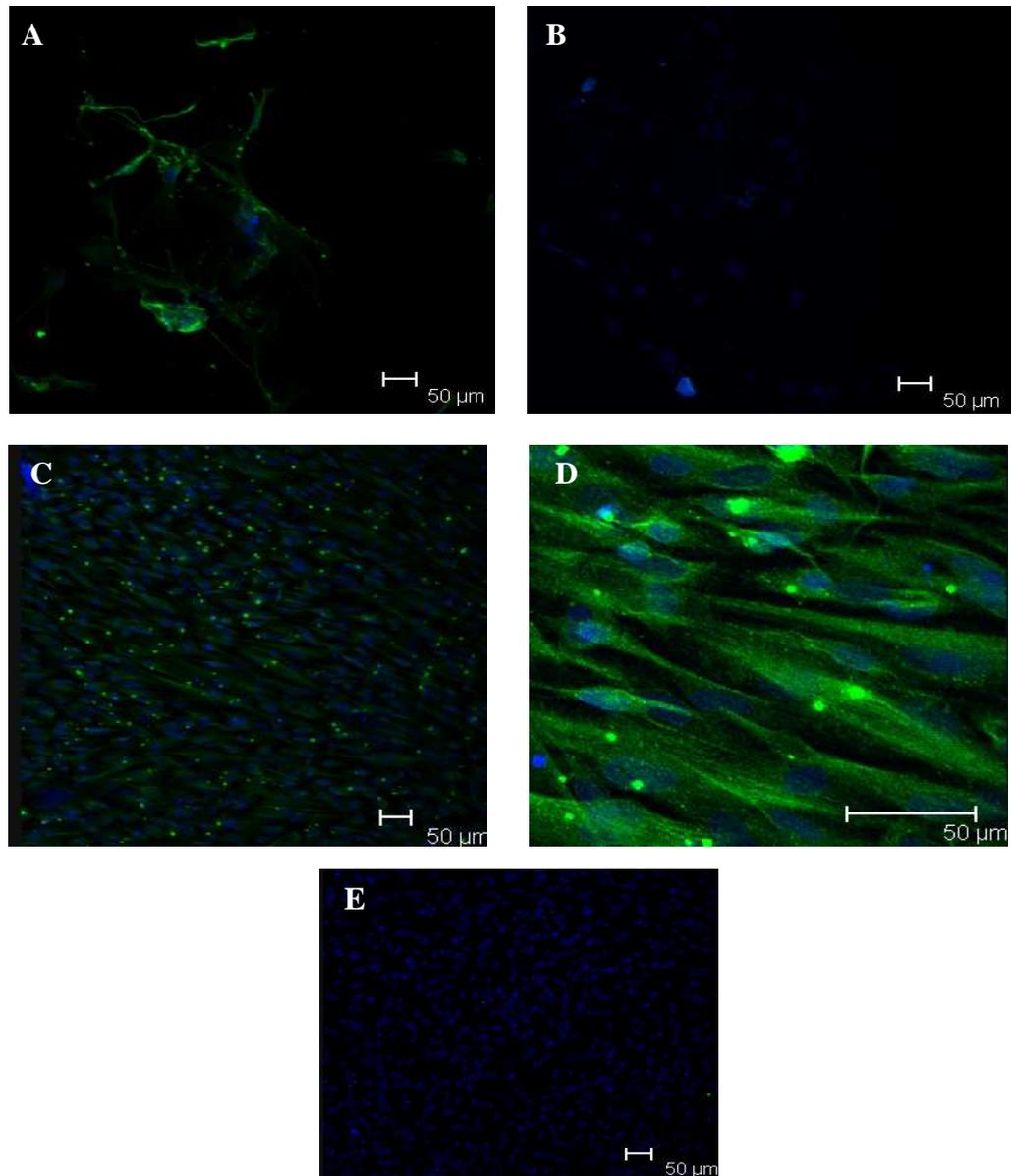
Adipogenic differentiation of LFL cells and bone marrow mesenchymal cells (BMSc) as a positive control was induced by treating with osteogenic differentiation media (dexamethasone, ascorbate-phosphate, and  $\beta$ -glycerolphosphate). After incubating with defined osteogenic differentiation media for 21 days, induced cells formed aggregates with calcium deposits.

The positive control human bone marrow mesenchymal cells differentiated to osteocyte showed presence of alizarin stained calcium deposits (**figure 3.18.2a**). The osteocyte differentiation of limbal fibroblast-like cells showed the alizarin stained calcium deposits as indicative of calcification upon induction. The undifferentiated LFLc were negative for calcification, indicating that the cells were not spontaneously differentiating into osteocytes (**figure 3.18.2 b and c**).

Osteogenic differentiated limbal fibroblast-like cells and bone marrow mesenchymal cells were positive for osteogenic lineage marker osteocalcin, and the undifferentiated cells were negative for osteocalcin, an indication that limbal fibroblast-like cells can be differentiated to similar lineages as bone marrow mesenchymal stem cells (**figure 3.18.2b**).



**Figure 3.18.2:** Osteocyte differentiation of limbal fibroblast-like cells; osteocyte differentiation of human bone marrow mesenchymal cells with presence of alizarin stained calcium deposits (20x) (A), and differentiated limbal fibroblast-like cells alizarin stained positive (40x) (B), undifferentiated LFLc were negative for calcification (40x) (C).



**Figure 3.18.2.1:** Osteocyte differentiation of limbal fibroblast-like cells (LFLc); (A) osteogenic induction of human bone marrow mesenchymal stemcells (BMSc) positive for osteogenic lineage marker osteocalcin (green). (B) Undifferentiated BMSc negative for osteocalcin. Differentiated LFLc positive for osteogenic (C), higher magnification (D). Undifferentiated LFLc negative for osteocalcin (E). Cells were counterstained with DAPI (nuclei, blue).

# 4.0 Section

---

# Discussion

#### 4.1 *In vitro* cultivation methods for limbal-corneal epithelial cells

As discussed in **section 1.2**, the limbus acts as a niche for the stem cell population of the corneal epithelium, and as such, is the tissue used to generate *in vitro* cultures of limbal-corneal epithelium. The first report of the *in vitro* cultivation of limbal-corneal epithelial cells was by Sun and Green in 1977(Sun and Green, 1977). Since this initial work, a large number of studies have been published on the *in vitro* cultivation of limbal-corneal epithelial cells. To date, there is no agreed common method for the cultivation of limbal-corneal epithelial cells with various studies using different culture methods and/or media and /or culture surface.

In Sun and Green's 1977 study, cells were isolated from the limbus and sub-cultured using the 3T3 fibroblast co-culture method. It was first shown in 1997 that limbal stem cell deficiency (LSCD) could be reversed successfully by transplanting expanded cultures of autologous human limbal epithelium (Pellegrini *et al.*, 1997) co-cultured with 3T3 mouse fibroblasts by an enzymatic method. In contrast, Tsai *et al.* used the epithelial outgrowth from the limbal explants (1 by 2 mm size) to initiate the primary culture of limbal-corneal cells on human amniotic membrane as culture surface (Tsai *et al.*, 2000b).

There are significant variations in the culturing of limbal epithelial cells on an amniotic membrane. Two notable examples are the removal, or not, of amniotic membrane epithelial cells and the use, or not, of 3T3 fibroblasts within the culture, in addition to the amniotic membrane (Sudha *et al.*, 2008, Grueterich *et al.*, 2003b, Koizumi *et al.*, 2007).

As a variety of methods are used in the culture of limbal-corneal cells, this thesis aimed to investigate the culture environment, e.g. the presence or absence of 3T3 feeder cells and/or denuded human amniotic membrane (dHAM) influences the stem cell niche of limbal-corneal cultures.

#### **4.2 Initial assessment of methods to generate limbal-corneal epithelial cell cultures**

Most primary cultures of limbal-corneal epithelial cells are generated by using enzymatic means or explants technique. These methods have different advantages and disadvantages associated with them. Enzymatic methods use different proteolytic enzymes to digest/degrade the extracellular matrix, releasing the cells. Enzymes frequently used include trypsin, collagenase, and dispase. Each enzyme will have certain advantages and disadvantages associated with it. For example, trypsin will break up tissue into its component cells but prolonged exposure can be detrimental to cell membranes (loss of cell surface receptors and adhesion molecules). In comparison dispase is considered less harmful to cells, but usually gives an incomplete digestion of the tissue.

Explants methods involve mechanical disruption of the tissue by finely cutting (2-3mm<sup>2</sup>) the tissue. The resulting tissue fragments are plated and outgrowth from the explants examined. This method is ideally suited to softer tissues, as tissues with cells tightly integrated into the ECM may not be able to grow out of the explants.

Initial experiments were conducted to compare enzymatic, i.e. trypsin, and explants methods for the generation of limbal-corneal epithelial cell cultures. The initial experiments also looked at how the culture environment might influence success in generating initial cell growth.

#### **4.2.1 Enzymatic cell suspension culture of limbal-corneal epithelial cells with a 3T3 feeder layer**

Enzymatic techniques for generation of primary cultures of limbal-corneal epithelial cells have usually used either trypsin (Pellegrini *et al.*, 1997) or dispase (Koizumi *et al.*, 2002). In this study 0.25% trypsin with 0.01% EDTA was used to dissociate the limbal tissue into single cells. These cells were seeded onto pre-plated irradiated 3T3 cell (**Section 2.4.1**). The cells that initially adhered to the culture surface had a round morphology (**figure 3.1 a**). The growth of these cells appeared to be slow initially, then becoming more rapid as the culture progressed. This initial round cell morphology and slow growth may have been due to the cells recovering from the trypsinisation process and adapting to the culture environment. As the primary culture cells grew, they formed cell sheets with cobblestone morphology (**figure 3.1 b**). This morphology is usually indicative of cells with an epithelial lineage. This cobblestone or cuboidal morphology has been reported by others as representing less differentiated cells (Koizumi *et al.*, 2002, Miyashita *et al.*, 2008, Notara *et al.*, 2007). Confluent cultures were obtained by approximately day 16 (**figure 3.1 c**). At this stage the cultures consisted of compact cells with cobblestone morphology. There also appeared to be areas where cells with a large, more flat morphology were growing on top of the cell sheet. These large flatter cells may represent a more differentiated cell type in the culture. Studies by others have shown that such stratification occurs in the primary culture of limbal-corneal epithelial cells, with cells in the different stratification layers showing differences in morphology and differentiation (Koizumi *et al.*, 2002, Miyashita *et al.*, 2008, Grueterich *et al.*, 2003b)

At day 16 the cultures were examined by immunofluorescence for two markers, cytokeratin 3 (CK3) and cytokeratin 19 (CK19). CK3 was used to investigate if the primary culture generated was of corneal epithelial lineage, as CK3 expression is reported to be a marker for corneal epithelium (Schermer *et al.*, 1986, Schlotzer-Schrehardt and Kruse, 2005). A positive staining for CK3 was observed in the primary culture (**figure 3.2 c**) thus indicating that the cells were most likely of a corneal epithelial lineage.

The intermediate filament cytokeratin 19 (CK19) has been reported to be expressed in the basal limbal epithelium and in all layers of the cornea epithelium (Chen *et al.*, 2004). However, it has been reported that CK19 expression is higher in the basal limbal epithelium (Chen *et al.*, 2004). This expression profile for CK19 has led to suggestions that it might act as a marker for limbal-corneal epithelial stem cells and hence cells with proliferative capacity *in vitro* (Chen *et al.*, 2004, Horenstein *et al.*, 2009). The limbal-corneal primary cultures generated using trypsin and cultured in the presence of a 3T3 feeder layer showed positive staining for CK19 (**figure 3.2 f**), thus suggesting that the culture may contain cells with a less differentiated phenotype. However, as no marker is definitive for “stem cell-ness” further analysis would be required to verify this.

While the marker analysis of CK3 and CK19 was considered promising and the cell morphology was deemed to be good, the success rate was poor. Only two out of six attempts yielded cultures (**table 3.1**). There are a number of possible reasons for this low success rate. Firstly, the limbal epithelial cells may be very sensitive to the effects of trypsin and may lose vital cell surface proteins. It is also possible that the cell culture surface is not ideally suited for the adhesion of limbal-corneal epithelial stem cells, thus remaining in suspension and perhaps undergoing cell death.

Other groups have reported success with the use of trypsin to generate limbal-corneal epithelial cultures (Sharifi *et al.*, , Pellegrini *et al.*, 1997, Kim *et al.*, 2006). This was only a short pilot study however, and it is possible that with more extensive study, refinements could have been introduced to the process to increase its success rate.

#### **4.2.2 Limbal explants on tissue culture plates**

A number of studies used the explants method to generate limbal-corneal epithelial cultures (Tsai *et al.*, 2000a, Tsai *et al.*, 2000b, Kolli *et al.*, 2008, Sangwan *et al.*, 2005). As stated previously, a benefit of this method is that less damage to cell surface proteins occurs, however the cells do have to grow/migrate out of the explants. The culture matrix and feeder cells will also influence the culture, thus explants cultures were investigated using a number of different culture conditions. The first condition investigated was explants method on tissue culture plastic.

In this study the corneo-scleral rim was separated from limbal tissue as described in **section 2.4.2**. The limbal ring was cut into small pieces of approximately 1-2mm<sup>2</sup> and plated on a 35-mm plastic culture dish. A cluster of cells were observed from explants by day 2 (**figure 3.3 a**) with compact cell morphology. These primary cultures developed a monolayer of cells coming from the explants with morphologically distinct flattened cells by day 8 (**figure 3.3 b**). These large flatter cells may represent the differentiation nature of epithelial cells as they migrate away from the limbal explants niche (Kolli *et al.*, 2008). These cells appeared to be more differentiated showing larger, flatter cell morphology by day 16 (**figure 3.3 c**). The possible reason for the differentiated cells could be lack of limbal stromal niche and feeder cell support

to maintain the stem cell niche, as observed by other groups (Kolli *et al.*, 2008, Arpitha *et al.*, 2005).

At day 16 the cultures were examined by immunofluorescence for two markers CK3 and CK19. CK3 was used to investigate if the primary culture generated was of corneal epithelial lineage as CK3 expression is reported to be a marker for corneal epithelium (Schermer *et al.*, 1986, Schlotzer-Schrehardt and Kruse, 2005). High expression of CK3 (**figure 3.4 c**) was observed in limbal-corneal epithelial cells grown on cell culture plastic, possibly indicating more differentiated cells in this culture condition. An intermediate filament protein CK19 was observed with low intensity in these cells (**figure 3.4 f**), suggesting that the culture may contain cells with differentiated phenotype. A similar observation was noted in a study by Kolli *et al.* 2008, where limbal epithelial cells grown without any feeder cell support become differentiated upon migration away from the limbal explants (Kolli *et al.*, 2008).

Limbal explants cultures on cell culture plastic show distinctive differentiated cell morphology and high expression of CK3, which suggests that this culture condition might have more differentiated cells and corneal epithelial lineage. The success rate with this method is good with four successful cultures out of five attempts (**table 3.1**). With good success rate of limbal-corneal epithelial cell culture with explants method, a further investigation was required to maintain stem cell niche in culture system.

Other groups have reported successful use of explants culture system in maintenance of limbal stem cell population by culturing on suitable substrate and feeder cell support (Balasubramanian *et al.*, 2008, Dravida *et al.*, 2008, Grueterich *et al.*, 2003a, Grueterich *et al.*,

2002a). The explants culture technique with suitable substrate and feeder cells might be an alternative method for maintenance of stem cell niche in culture system.

#### **4.2.3 Limbal explants culture on gelatine coated plates**

Explants cultures on cell culture plastic showed distinctive large size of epithelial cells with high expression of the differentiation marker CK3. In this section limbal explants were grown on gelatine (0.025%) coated plates to investigate the success rate, morphology and marker expression of limbal-corneal epithelial cells. Various substrates have been used to support growth and proliferation of limbal-corneal epithelial cells, such as surface coated contact lenses (Deshpande *et al.*, 2009, Di Girolamo *et al.*, 2009) and biopolymers (Dravida *et al.*, 2008), .

The initial growths of limbal-corneal epithelial cells on gelatine-coated plates was observed on day 2 (**figure 3.5 a**) with typical round cell morphology of outgrowth from the explants. In contrast to epithelial cells observed on plastic, these cells appeared to be of a squamous compact nature, a morphology that is usually indicative of cells with an epithelial lineage. Cells further expanded on gelatine-coated plates displayed distinctive cobblestone morphology by day 8 (**figure 3.5 b**), which is similar to the morphology of cells grown on polymers in a study by Dravida *et al.*,2008. A confluent culture was obtained by day 16, approximately (**figure 3.5 c**). At this stage, the culture consists of compact cells with cobblestone morphology with multi-layers and large cell morphology in some areas.

At day 16 the cultures were examined by immunofluorescence for two markers, CK3 and CK19 (**figure 3.6 c, f**). These cells expressed CK3 corneal epithelial marker similar to the cultures observed in **section 4.2.2** and low levels of CK19 positive cells, which suggests the presence of differentiated cells in culture system. While the marker analysis of CK3 and

CK19 was considered, this culture system appeared to be similar to explants cultures on plastic, with good success of growth rate i.e. four attempts out of five yielded cultures (**table 3.1**).

This was only a short study of the culture of limbal-corneal epithelial cells on gelatine coated plates; a pilot study with extensive marker analysis could have been applied to the process to increase the success rate and aid understanding of epithelial nature on coated plates.

#### **4.2.4 Limbal explants on tissue culture plastic with irradiated 3T3 feeder layer**

As discussed in previous **sections 4.2.2** and **4.2.3**, limbal explants cultures supported by 3T3 feeder cells might be an alternative method to maintain stem cell population in culture system. We investigated further limbal explants cultures on irradiated 3T3 feeder cell system.

In this method, limbal explants were prepared and plated as described in **section 2.4.3** on pre-plated irradiated 3T3 feeder cells. The cells that grew from limbal explants have typical epithelial morphology migrating away from limbal explants by day 2 (**figure 3.7 a**). Epithelial cell growth was initially slow, becoming more rapid as the culture progressed. The initial slow growth may have been due to the presence of 3T3 feeder cells, which are in contact with epithelial cells growing from the limbal explants. As the primary cultures grew, by day 8 they formed cell sheets with cobblestone morphology (**figure 3.7 b**). In contrast to the limbal-epithelial cells grown on cell culture plastic, a confluent culture was obtained by approximately day 16 in this culture (**figure 3.7 c**), containing stratified epithelial cells with smaller cells size at the apical layer, an indication of less differentiated cells (Koizumi *et al.*, 2001a, Kim *et al.*, 2004, Balasubramanian *et al.*, 2008). The morphological appearance of

cells leads to the suggestion that 3T3 feeder cells might have a considerable effect on maintenance of the limbal stem cell population in the culture system.

At day 16, the cultures were examined by immunofluorescence for two markers, CK3 and CK19. Limbal-corneal epithelial cells grown in the presence of 3T3 feeder cells were positive for CK3 (**figure 3.8 c**), which suggests cultures generated with this method were of corneal epithelial lineage (Schermer *et al.*, 1986, Schlotzer-Schrehardt and Kruse, 2005). Limbal-corneal epithelial cells in this condition were also positive for CK19 expression (**figure 3.8 f**), which was similar to limbal-corneal epithelial cells isolated with enzymatic digestion and cultured on 3T3 feeder cells, thus suggesting that the culture may contain cells with a less differentiated phenotype. However, as no marker is definitive for “stem cell-ness” further detailed analysis would be required to verify this.

The marker analysis of CK3 and CK19 was considered to be promising along with good cell morphology, and good success rate, five out of five attempts yielded cultures (**table 3.1**).

#### **4.2.5 Limbal-corneal epithelial cultures on cell culture inserts with irradiated 3T3 feeder layers**

As discussed in **section 4.2.4**, the generation of limbal-corneal epithelial primary cultures from explants with 3T3 feeder layers appeared to be the most favourable method. This was based on the tight cobblestone morphology of the cells with some stratification present, the expression of CK3 and CK19 by immunofluorescence, and the generation of five cultures from five attempts. Thus it was decided to use this as the main culture method in further studies. However, as a trans-well system was going to be used in future studies a validation run was performed. This was necessary as the culture surface and culture environment of the trans-well was likely to be slightly different from that of tissue culture plastic. Similar results were observed to those obtained in **section 4.2.4** explants culture with 3T3 feeder layers.

The morphology of the culture showed cell sheets being formed with tight cobblestone morphology (**figure 3.9**) that appeared to be stratified at confluency. Strong staining similar to that observed for the explants cultures with 3T3 feeder layers on tissue culture plastic was obtained for the corneal-epithelial marker CK3 (**figure 3.10 c**) and the limbal epithelial marker CK19 (**figure 3.10 f**).

Thus it was determined that the use of culture trans-wells would not impact detrimentally on the generation of limbal-corneal epithelial primary cultures.

### **4.3 Investigation of the role of 3T3 feeder cells in limbal-corneal epithelial cell proliferation and differentiation:**

#### **4.3.1 General discussion**

Stem cells, postulated to exist in all self-renewing tissues, serve as a reserve for proliferation where they maintain the balance between cell production and cell loss (Lajtha, 1979a, Lajtha, 1979b, Lavker *et al.*, 1993a, Miller *et al.*, 1993, Lavker *et al.*, 1993b). These cells are relatively undifferentiated, slow cycling and have a large capacity for proliferation. Thus, the unique properties of these special kinds of cells include:

- Capability of self-renewal
- Asymmetric DNA segregation
- Low mitotic activity
- Quiescence
- Error free proliferation
- Poor differentiation
- Special anatomical protection

The stem-ness of the stem cells governed by both intrinsic factors such as metabolically active enzymes, receptors and genes, as well as extrinsic factors, which essentially are the milieu or niche in which the stem cells exist. Stem cells are relevant not only in tissue homeostasis and regeneration, but in carcinogenesis because DNA mutations in critical genes can accumulate over time in these permanent residents of the tissue (Morris *et al.*, 1997). In addition, stem cells represent a key target for gene therapy, as alteration in stem cell DNA

can result in permanent functional changes of the cellular population (Greenhalgh *et al.*, 1994). It is essential to study stem cells in isolation by growing them *in-vitro*.

Despite decades of experience with different forms of limbal surgery, there is still no reliable therapy for severe forms of limbal stem cell deficiency (LSCD). In 1997, it was first shown that LSCD could be reversed successfully by transplanting expanded cultures of autologous human limbal epithelium (Pellegrini *et al.*, 1997) co-cultured with 3T3 mouse fibroblasts by enzymatic method. This has led to the recent developments of therapeutic strategies involving transplantation of *in vitro* expanded limbal epithelial cells (Tsai *et al.*, 2000a, Sangwan *et al.*, 2003b).

It is important to establish an effective culturing protocol for future clinical trials to ensure that *in vitro* expanded limbal-epithelial progenitor cells indeed retain stem cell characteristics. During preliminary experiments in establishing limbal-corneal epithelial cells, we were able to successfully grow cells with explants culture system in trans-well inserts, with/without 3T3 feeder layers for the growth and differentiation of limbal-corneal epithelial cells. Taking cues from the studies described above, in this thesis experiments were designed with/without 3T3 feeder cells (see below) to understand the role of 3T3 feeder cells in limbal-corneal epithelial cells (**figure 3.2.1.**)

- A) Explants on trans-well membrane with no 3T3 cells (No-3T3):** In this condition where limbal-epithelial cells were grown without any effect of 3T3 feeder cells.
- B) Explants on trans-well membrane co-culture with 3T3 cells (Co-3T3):** In this condition, the limbal-epithelial cells are in contact with 3T3 feeder cells, with supply of soluble factors secreted by feeder cells.

**C) Explants on trans-well membrane with 3T3 separated (Bot-3T3):** In this condition, there is no physical contact between limbal-epithelial cells and 3T3 feeder cells, and this provides only soluble factors secreted by feeder cells.

The above-mentioned culture systems are helpful to aid our understanding of limbal-stem cell biology in relation to role of 3T3 feeder layer. However, application of limbal-corneal epithelial cells for clinical use requires a suitable substrate or carrier for transplantation like petrolatum gauze (Pellegrini *et al.*, 1997) or contact lens (CL) (Di Girolamo *et al.*, 2009). More recently, human amniotic membrane (HAM) has been used as a culture substrate for *in vitro* cultured limbal epithelial stem cell (LECs) for transplantation (Sangwan *et al.*, 2007, Nakamura *et al.*, 2004b, Nakamura *et al.*, 2006a). The ability of HAM to maintain stem cell characteristics (stem cell marker retention and proliferative capacity) in *in vitro* culture has led to the suggestion that it acts as an effective stem cell niche (Koizumi *et al.*, 2000, Grueterich *et al.*, 2003a, Meller *et al.*, 2002, Koizumi *et al.*, 2007). Experiments were conducted to understand the role of 3T3 feeder layers in the maintenance of limbal-corneal epithelial cells on denuded human amniotic membrane (dHAM) as an extracellular matrix. Limbal epithelial cells are always cultured on the epithelial side of the amniotic membrane rather than the stromal side (**figure 3.2.1**).

**D) Explants on Human Amniotic Membrane (dHAM) with no 3T3 cells (dHAM No-3T3):** limbal-corneal epithelial cells were grown without 3T3 feeder cells.

**E) Explants on Human Amniotic Membrane co-culture with 3T3 cells (dHAM Co-3T3):** In this condition, the limbal-corneal epithelial cells are in contact with 3T3 feeder cells, with supply of soluble factors secreted by feeder cells.

**F) Explants on Human Amniotic Membrane with 3T3 separated (dHAM Bot-3T3):** in a condition there is no physical contact between limbal-epithelial cells and 3T3 feeder cells, this provides only a soluble factors secreted by feeder cells.

**Culture conditions were designated condition A to F for the remained for this thesis.**

Given Name
Condition A no-3T3
Condition B Co-3T3
Condition C Bot-3T3
Condition D dHAM no-3T3
Condition E dHAM Co-3T3
Condition F dHAM Bot-3T3

#### **4.3.2 Effects of organ culture, optisol-GS storage and tissue preservation time on limbal-corneal epithelial cells**

The process of preservation and storage of cadaveric limbal tissue/corneo-scleral rim in eye bank has potential effects on the outgrowth of limbal-corneal epithelial cells in comparison to live donor tissue, where studies have shown that fresh limbal tissue is superior to cadaveric limbal tissue (James *et al.*, 2001, Vemuganti *et al.*, 2004). **Table 3.2.1a** in **section 3** illustrates the number of samples used in culture of limbal-corneal epithelial cells on cell culture inserts with conditions A, B and C, details of donor age, time between death and enucleation (D-Enuc-), preservation to culture time (Pre-Cult-), growth start in three culture conditions, and termination day. The average age of donor tissue was 55.1 years, with maximum age of 69 years, and minimum age of 19 years. No obvious correlation was found between donor age and the day growth initiation was observed. Similarly, no correlation was observed between donor age and confluency/ termination day. However, it should be noted that 18 of the tissue samples for which donor age were available were over 50 in age. If tissue from younger donors was available, a difference may be observed in time for confluency.

The time spent by most of the tissue samples in optisol transport media prior to cultivation was around 7 days (average 12.55). No obvious correlation seems to exist between the time spent in optisol transport media and the time at which primary culture growth is observed. However, for the sample that was in optisol transport media the longest (20 days) growth was not observed until day 6 and confluent culture was not observed until day 20, both the maximum time points. Long-term storage of tissue in optisol may affect the growth of

cultures as observed by other groups (James *et al.*, 2001, Vemuganti *et al.*, 2004, Shanmuganathan *et al.*, 2006). However, as there is a lack of repeats it is not possible to say if there is a correlation or if the data is due to biological variation.

**Table 3.2.1b** in **section 3** illustrates the number of samples used in culture of limbal-corneal epithelial cells on dHAM with conditions D, E and F, details of donor age, time between death and enucleation (D-Enuc-), preservation to culture time (Pre-Cult-), growth start in three culture conditions, and termination day. The average age of donor tissue was 46.8 years with a maximum age of 66 years, and a minimum age of 30 years. No obvious difference was observed between age and growth/success rate on dHAM. Similarly, no correlation was observed with time of preservation and the utilization time on the growth of limbal-corneal epithelial cells. On dHAM cultures of all conditions the average day for growth start is 5.1 with a maximum of 8 days delay in growth, and the average termination day is 20 days with a maximum of 23 days in culture to attain epithelial sheet.

The data in **table 3.2.1a** and **3.2.1b** indicates that time spent in optisol transport media up to day 16 does not appear to impact on the ability to successfully generate primary cultures of limbal-corneal epithelium.

### 4.3.3 Cell outgrowth, and ability to generate cell sheets

The epithelial outgrowth from explants in all conditions was measured at days 2, 5, 8, 12, 14 and 17 (day 20 for dHAM cultures) in six biological replicates, and a graph was plotted with growth rate vs. number of days. Condition A no-3T3 was used as poor culture condition in order to set a base line to compare the growth rate of other culture conditions. Explants outgrowth in cell culture inserts regardless of feeder or feeder-free conditions was observed at day 2. In contrast, outgrowth was only observed in condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 at day 8, regardless of feeder or feeder-free conditions. A possible reason for this apparent difference was the technical difficulty in observing outgrowth on the HAM and the need of cells to possibly produce ECM remodelling enzymes which involves serial and complex processes. After attachment of the budding limbal epithelial cells to the underlying HAM, migration of the expanded cells is necessary for subsequent proliferation of the limbal epithelial cells.

Outgrowth was initiated in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 on same day. The epithelial outgrowth rate is slower in condition A no-3T3, possibly due to the lack of 3T3 feeder cell support for the proliferation of limbal-corneal epithelial cells (Balasubramanian *et al.*, 2008) and condition B Co-3T3 in initial stage (day 8) observed slow growth due to presence of high number of 3T3 cells in contact with the epithelial cells which might have effect on migration of cells. However, all culture conditions attain confluency by day  $15 \pm 2$  covering entire cell culture insert ( $4.2 \text{ cm}^2$ ). Due to early growth initiation in condition C Bot-3T3, the number of cells and multilayer condition is higher compared to

condition A no-3T3 and condition B Co-3T3, this could possibly be due to continuous supply of soluble factors to the limbal-corneal epithelial cells for proliferation and migration, due to absence of contact inhibition of feeder cells to epithelial cells in this condition (**figure 3.2.2**). These findings are in contrast to those of Miyashita *et al.*, where separate feeder layer system had shown less multilayer growth in comparison to contact feeder cell layer condition where this group used single cell suspension method for isolation of limbal-corneal epithelial cells (Miyashita *et al.*, 2008).

In dHAM cultures the growth rate is exponentially increased as the number of days increases, epithelial cells completely covered the membrane by day  $20 \pm 2$  (**figure 3.2.2**) with slight increase in condition F dHAM Bot-3T3 outgrowth compared to condition D dHAM no-3T3 and condition E dHAM Co-3T3.

This data demonstrated that the outgrowth of limbal-corneal epithelial cells was enhanced by 3T3 feeder cells not being in direct contact with limbal-corneal epithelial cells both in cell culture inserts and dHAM. This observation is in contrast to the findings of Miyashita *et al.*, and Ahmadiankia *et al.*, who used enzymatic digestion to isolate limbal-corneal epithelial cells (Ahmadiankia *et al.*, 2009, Miyashita *et al.*, 2008) and duplex culture system. The explants culture system with separate 3T3 feeder cells (Bot-3T3) yielded superior compact multilayered cell cultures. To confirm this, further studies of morphology and the stem cell nature of these cells is required, which is discussed in later sections.

#### 4.3.4 Explants culture system: cell sheet morphology

The morphology of limbal-corneal epithelial cells condition A no-3T3 showed large flat squamous cells in a loose cell-to-cell arrangement (**figure 3.2.3**). In contrast, the epithelial cells in condition B Co-3T3 and condition C Bot-3T3 showed morphologically distinct cobblestone-like cells similar to normal corneal epithelial cells in culture (Shortt *et al.*, 2007, Sharifi *et al.*, , Pellegrini *et al.*, 1999a), with robust (continuous or uniform) epithelial sheet formation of 2-3 layers (**figure 3.2.4**, and **3.2.5**). In the current study findings of multilayered epithelial sheets in condition B Co-3T3 and condition C Bot-3T3 are similar to the study by Miyashita *et al.*, 2008 where multilayer cultures of cuboidal basal cells and flattened superficial cells were observed. Condition A no-3T3 showed large flat cell morphology with loose cell-cell contact in comparison to condition D dHAM no-3T3, where cells are small, compact and uniform with round cell borders.

Apart from cell outgrowth differences in condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3, we have not observed a significant morphology change in cultures grown on dHAM. The morphology of limbal-corneal epithelial cells are small, compact and uniform with round cell borders, and continuous expansion area with semi-circular/regular margins. Cells are morphologically distinct two types (spindle-shaped and polygonal, example: **Figure 3.6.1 and 3.6.2**, represented different cell morphology) were observed which are similar to cultured limbal epithelial cells from explants observed by other group (Ghoubay-Benallaoua *et al.*, 2011), with variation in layers (2-3 layers). The morphology of cells varied within the culture condition due to sample-to-sample variation, multilayer/monolayer nature of culture system and type of stain (marker) performed to

characterise cells. For example, CK19 stain has shown small spindle in basal and large polygonal flat cell morphology at apical surface in all conditions (**Page 192 and 193**). Whereas, CK14 in condition C and F Bot-3T3 has shown round spindle –shaped cells (**Page 197 and 198**). This observation suggests that the variation in cell morphology could be due to multilayer nature of cells as well as type of marker used for analysis. Despite an absence of extracellular matrix/ basement membrane, condition B Co-3T3 and condition C Bot-3T3 have shown similar cell morphology to that observed in condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3, with 3T3 feeder cell support in explants culture system.

#### **4.3.5 Limbal-corneal epithelial cells motility/migration and invasion assay**

In corneal epithelial wound healing, the three basic distinct components are cell migration, cell proliferation, and cell adhesion. All the three components are part of a continuous process, but the contribution of each can vary depending on the size and depth of the wound and nature of injury. Cultured limbal-corneal epithelial cells on cell culture inserts with/without 3T3 feeder cells have shown high migration/motility and invasion rate compared to a positive control cell line BT-20 which is highly motile and invasive, and negative control cell line MCF-7 which is low motile and invasive (**figure 3.3 and 3.4**).

The motility/migration of corneal epithelial cells is a highly complex process in wound healing with matrix metalloproteinases (MMPs). Increasing evidence suggests that regulation of signalling molecules by MMPs contributes to the communication between cells and their microenvironment (Sivak and Fini, 2002). Moreover, MMP-9 transcripts can be up-regulated in cultures of limbal-corneal cells on intact HAM (Sun *et al.*, 2005) which suggests the role of

corneal epithelial cells *in vitro* wound healing. The high levels of migration/invasion of cultured corneal epithelial cells, suggests that MMP production and cell motility are important in the growth of limbal-corneal epithelial cultures. It is possible that these mechanisms are also considered to be important in clinical application of cultured limbal-corneal epithelial cells in their integration onto the patients eye (Sun *et al.*, 2005, Sivak and Fini, 2002, Li *et al.*, 2006).

## 4.4 Characterisation of limbal-corneal epithelial culture with specific markers

### 4.4.1 Introduction

Despite decades of experience in introducing limbal-corneal epithelial cells for therapy in limbal stem cell deficiency (LSCD), their biology is still poorly understood, due to lack of knowledge about the nature of limbal stem cells (LSC). Thus, a major challenge in limbal stem cell biology is the identification of stem cells (SC) *in-vitro* and *in-situ*. This directly relates to the issue of availability of reliable SC markers. A number of molecular markers for limbal stem cells have been proposed (Espana *et al.*, 2003a, Chen *et al.*, 2004, Schlotzer-Schrehardt and Kruse, 2005).

During the past years, progress has been made towards identification of phenotypic (molecular) markers that may distinguish SCs *in-situ*. In recent years, several research groups compared limbal and central cornea with the currently available high throughput nucleic acid profiling and proteomic techniques. The differences in protein expression in limbal and corneal epithelial cells found SOD2 and CK15 where these markers exclusively expressed in clusters of basal limbal epithelium (Lyngholm *et al.*, 2008). A proteomic analysis of limbal-corneal epithelial cells cultured on dHAM expressed more p63 and K19 (SC markers) and less CK3 and connexin43 (corneal differentiation markers) in comparison with other extracellular matrices (ECMs) (Baharvand *et al.*, 2007). Figueira *et al.*, have performed a focused microarray analysis on fetal cornea and cultured limbal explants epithelium and identified CK15, CK14, CDH3, and Wnt-4 in basal limbal epithelial cells (Figueira *et al.*, 2007).

The two principal defining characteristics of stem cells are their capacity to replenish themselves and their capability of providing the origin of differentiated cells. Fine tuning of techniques that make isolation and expansion of various adult tissues possible *in vitro* has opened enormous applicative potential for regenerative medicine. Diverse tissues, from which it is possible to isolate stem cells (SCs) in the laboratory, have now been identified. These are capable not only of generating cells of the original tissue but also of accomplishing regeneration of different tissues, if cultures are appropriately stimulated *in vitro* by selective growth factors, and they could be transplanted *in vivo* in compatible recipients.

A difficulty in the study of eye surface stem cells is that often a series of markers may be accurately selected according to the most recent data in the literature, yet these are insufficient to discriminate true stem-ness of the cells in an unequivocal manner, as there is often a considerable fraction of transit-amplifying cells (TAC) (even if very primitive), that still retain a phenotype very close to that of true stem cells (Vascotto and Griffith, 2006), the presence, absence or relative expression of stem cell markers in the corneal epithelium allows the description of putative stem cell phenotype.

Although a variety of putative limbal stem cell, markers have been proposed; their role for identification of limbal stem cells is controversial due to the specification of different markers. These markers could be present on the surface of the cells, in the cytosol, nuclear membrane, mitochondria or nucleus etc. Thus, broad knowledge regarding these markers would hold the key to providing an insight to the identification of limbal stem cells. The proposed criteria for corneal epithelial stem cells are-

1. While the markers do not have to be present exclusively on cells of the ocular surface, their presence or absence alone or in combination must provide a reliable means for the enrichment and/or isolation of the corneal epithelial stem cells.
2. The markers must also allow for the identification of the limbal-corneal epithelial stem cells within their native tissue in healthy individuals.

As no significant markers can be used to identify and characterise limbal-epithelial stem cells, a panel of markers was used. This panel of markers was divided into markers associated with differentiation (**table 4.4.1**) and markers associated with stem cells (**table 4.4.2**). The ideal expression pattern in cultured limbal-corneal epithelial cells could be a low differentiation marker expression and high stem cell marker expression.

#### 4.4.1: Limbal-corneal epithelial cell differentiation markers

<b>Differentiation Marker</b>	<b>Specification</b>	<b>Reference</b>
<b>Cytokeratin 3</b>	Major cytokeratin in corneal epithelium	(Schermer <i>et al.</i> , 1986, Liu <i>et al.</i> , 1993, Liu <i>et al.</i> , 1999)
<b>Cytokeratin 12</b>	Major cytokeratin in corneal epithelium	(Chaloin-Dufau <i>et al.</i> , 1990, Liu <i>et al.</i> , 1993, Liu <i>et al.</i> , 1999)
<b>E-cadherin</b>	Cell-cell contact	(Chen <i>et al.</i> , 2004, Scott <i>et al.</i> , 1997, Gumbiner, 2000)
<b>Connexin43</b>	Cell-cell contact (tight junction protein)	(Shortt <i>et al.</i> , 2007, Matic <i>et al.</i> , 1997, Chen <i>et al.</i> , 2004)
<b>ZO1</b>	Cell-cell contact	(Yoshida <i>et al.</i> , 2009, Sugrue and Zieske, 1997)
<b>Occludin</b>	Cell-cell contact	(Yoshida <i>et al.</i> , 2009)

**Table 4.4.2:** Limbal-corneal epithelial Stem cell markers

<b>Putative/Stem cell Markers</b>	<b>Specification</b>	<b>Reference</b>
<b>Cytokeratin 19</b>	Limbal corneal epithelial cells	(Cooper and Sun, 1986, Chen <i>et al.</i> , 2004, Lauweryns <i>et al.</i> , 1993)
<b>Cytokeratin 14</b>	Limbal basal cells	(Dua <i>et al.</i> , 2003, Moll <i>et al.</i> , 1982, Lavker <i>et al.</i> , 2004)
<b><math>\Delta</math>Np63<math>\alpha</math></b>	Nuclear	(Pellegrini <i>et al.</i> , 2001, Di Iorio <i>et al.</i> , 2005)
<b>ABCG2</b>	ATP-binding cassette transporter	(de Paiva <i>et al.</i> , 2005, Watanabe <i>et al.</i> , 2004, Budak <i>et al.</i> , 2005)
<b>p63</b>	Nuclear	(Chen <i>et al.</i> , 2004, Dua <i>et al.</i> , 2003)
<b><math>\alpha</math>-2 Integrin</b>	Membrane	(Schlotzer-Schrehardt <i>et al.</i> , 2007, Schlotzer-Schrehardt and Kruse, 2005)
<b><math>\beta</math>-1 Intergrin</b>	Membrane	(Chen <i>et al.</i> , 2004, Jones and Watt, 1993, Watt and Jones, 1993)

## **4.5 Expression of differentiation markers in cultured limbal-corneal epithelial cells**

### **4.5.1 Cytoskeletal proteins**

Cytokeratins or keratins are a group of proteins that form intermediate filaments in epithelial cells and are expressed in distinct patterns during epithelial development and differentiation. The family comprises of at least 20 different polypeptides, which are expressed in paired combinations of acidic and basic molecules according to the type of epithelium and its state of differentiation (Sun *et al.*, 1983). Epithelial cells contain different types of keratins, some of which indicate a high level of differentiation, whereas others are found mostly in less differentiated cells (Moll *et al.*, 1982, Cooper and Sun, 1986).

Cytokeratins CK3 and 12 are confined to corneal epithelial cells and are considered as markers for corneal epithelial cell differentiation. Epithelial cells in the basal layer of the limbus, the proposed niche for corneal epithelial stem cells are devoid of these two cytokeratins, indicative of their undifferentiated nature (Liu *et al.*, 1993, Liu *et al.*, 1999).

#### 4.5.1.1 Expression of CK3

The limbal basal epithelium is believed to contain the least differentiated cells of the corneal epithelium (Liu *et al.*, 1993, Liu *et al.*, 1999). It is believed that the expression of the cytokeratins are linked to the differentiation status of various epithelia (O'Sullivan and Clynes, 2007). In humans, expression of CK3 *in vivo* is selective for the most differentiated cells of the central part of the cornea, in the suprabasal and epibasal/apical layers (Sun *et al.*, 1983). *In vitro* cultured limbal-epithelial cells express CK3 in prolonged cultures and differentiation stage cells due to lack of stem cell niche support (Miyashita *et al.*, 2008, Koizumi *et al.*, 2000, Koizumi *et al.*, 2002, Grueterich *et al.*, 2003a, Cristovam *et al.*, 2008).

In this study, staining patterns showed positive CK3 in all six conditions with variation in basal and apical cells. The immunofluorescence staining of CK3 in condition A no-3T3 appeared to be uniform between basal and apical cells i.e. positive expression in large differentiated and small basal cells, suggesting the more differentiated cells in absence of 3T3 feeder cells (Balasubramanian *et al.*, 2008, Parnigotto *et al.*, 1996). In contrast, the basal cells of condition B Co-3T3 showed lower expression of CK3 than the apical cells. This staining pattern for CK3 appeared in condition B Co-3T3, condition C Bot-3T3, condition D no-3T3dHAM, condition E Co-3T3 dHAM and condition F Bot-3T3 dHAM (**figure 3.5.1** and **3.5.2**). Where CK3 specifically expressed in corneal epithelial cells and are regarded as markers of corneal epithelial differentiation (Kasper *et al.*, 1988, Liu *et al.*, 1993), the strong positive staining of CK3 in apical cells of all six conditions suggests that in culture systems

apical cells are differentiating to corneal epithelial cells regardless of 3T3 feeder cells support and dHAM .

Similarly, immunofluorescence of culture conditions, condition D dHAM no-3T3, condition E dHAM Co-3T3, and condition F dHAM Bot-3T3 showed lower expression of CK3 in basal cells, the presence of HAM provides a suitable extracellular matrix and maintains cells in a progenitor state. Where dHAM with 3T3 feeder cells known to maintain the high stem cells population and less differentiation markers (Balasubramanian *et al.*, 2008, Grueterich *et al.*, 2002a) as similar pattern observed in this study.

Expression of cytokeratins is consistent with observations reported in other studies. Where *in vitro* fetal bovine serum (FBS) induces cytokeratins (Schramek *et al.*, 1997) in addition to the presence of epidermal growth factor (EGF), insulin and other supplements in media which help in the maintenance of cytokeratin expression (Chen *et al.*, 2007), as in this study limbal-corneal epithelial cells were grown with EGF and insulin for all six conditions.

In contrast to immunofluorescence expression data, western blot analysis (**figure 3.5.3**) did not show altered expression of CK3 in the six culture conditions. In the literature differential expression of CK3 was only found when the epithelial cells were subjected to air-lifting to differentiate further as terminal differentiation, Chen *et al.*, demonstrated low level of CK3 protein expression in epithelial cells on intact human amniotic membrane (iHAM) before airlifting compare to iHAM after airlifting (Chen *et al.*, 2010). All culture systems designed in this study were in a submerged condition, where the cells were maintained in an undifferentiated state (Li *et al.*, 2008, Papini *et al.*, 2005). In another study Minami *et al.*, used the airlifting method for bovine corneal cell culture in three-dimensional collagen gel

culture condition, which promoted corneal epithelial cell proliferation and differentiation (Minami *et al.*, 1993).

Interestingly no altered CK3 mRNA (**figure 3.5.4**) and protein expression was seen, the possible reason for the unaltered expression of total protein in limbal-corneal epithelial could be due to submerged culture. In addition, conditions where type II cytokeratin protein (CK3) might not be well differentiated that can be detect differential expression by western blot analysis where posttranslational modification or processing of cytokeratins does not appear to be involved in these culture systems (Quinlan *et al.*, 1985).

#### 4.5.1.2 Expression of CK12

The formation of cytokeratin filaments requires type II keratin paired with a type I keratin (Quinlan *et al.*, 1985), specific keratin pairs predominate both the epithelial cell type and the differentiation state. As discussed previously, the type II keratin CK3 is a major cytokeratin of differentiated corneal epithelium and type I collaborate is CK12 in the cornea.

Thus, as expected CK12 expression pattern *in vivo* matches that of CK3, which was expressed abundantly at both the protein and mRNA levels in the corneal epithelium. In contrast only very weak or no expression is detected in the limbal epithelia (Kurpakus *et al.*, 1994, Liu *et al.*, 1993, Chen *et al.*, 2004, Schlotzer-Schrehardt *et al.*, 2007). It is this lack of expression by the basal cells of the limbus (proposed location of the corneal epithelial cells) compared to the cornea that results in CK12 being a marker of differentiation and inversely proportional to ‘stem cell-ness’.

In the present six-culture conditions study, low level, sporadic staining was observed in cells of the basal layer. This low, sporadic staining may indicate cells that are un-differentiated or partially differentiated. However, the staining in condition C Bot-3T3 was stronger than in the other five culture conditions, suggesting that these cells might more differentiated. However, other analysis such as flow cytometry (**figure 3.5.7**) and western blot analysis (**figure 3.5.8**) did not appear to support this finding obtained from immunofluorescence for condition C Bot-3T3. It is unclear why such positive staining is seen in the basal layer of condition C Bot-3T3. It could be speculated that since type I keratins depend on interaction

with polysaccharide type II keratins for proteolytic stability and filaments formation (Fuchs *et al.*, 1981), it is possible that CK12 in condition C Bot-3T3 is indeed being expressed at a low level but it is being stabilised by CK3 in a manner that allows its visualisation by immunofluorescence.

In condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 immunofluorescence staining of CK12 was observed on the apical cell layers suggesting that these cells may be more differentiated than those on the basal layer, and as noted before, these cells had a large flat morphology. In contrast, the cells in the apical layer of growth condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 showed similar immunofluorescence staining i.e. low and sporadic compared to that observed in the basal layer. This suggests that these cultures may have less differentiated cells. The low and sporadic nature of the CK12 immunofluorescent staining suggests that basal cell layers of all six conditions have a heterogeneous population with precursors and transient amplifying cells (TAC). In comparison, a similar observation and conclusion was reached by Du *et al.*, who found primary cultures of human fetal and adult limbus expressed CK12 (Du *et al.*, 2003).

In this study, the western blot analysis shown a decrease in CK12 protein in conditions E, C and F compared to condition A no-3T3, whereas conditions D dHAM no-3T3 and condition B Co-3T3 have shown no difference compared to condition A no-3T3 (**figure 3.5.8**). A decrease in CK12 protein was observed in dHAM with 3T3 cells when compared to condition D dHAM no-3T3, an indication of low differentiation of limbal-corneal epithelial cells in conditions with dHAM + 3T3 cells. This observation was similar to other group findings where 3T3 cells with HAM express low differentiated marker (CK3/12) (Balasubramanian *et al.*, 2008, Ban *et al.*, 2003, Grueterich *et al.*, 2003b). A semi-

quantitative RT-PCR analysis showed an inconsistent expression pattern among the biological samples across the culture conditions (**figure 3.5.9**). In this study, no correlation was observed between protein expression level and mRNA level of CK12. CK12 mRNA is the main regulatory element of type I keratins in proteolysis. It is possible that a 3T3 feeder cell interaction with the primary cultures activates this pathway in some manner (Rothnagel *et al.*, 1993). It should also be noted that the synthesis and control of keratin types can occur at different levels (Knapp *et al.*, 1989). Other possible mechanisms affecting keratin expression are mRNA degradation (Blouin *et al.*, 1992) and a block on mRNA translation (Schweizer and Winter, 1983, Winter and Schweizer, 1983, Winter *et al.*, 1983). The inconsistent expression of CK12 in biological samples across culture conditions could be due to difference in age, culture conditions, relative stem cell quiescent environment (Kasper *et al.*, 1988, Tseng *et al.*, 1996).

The feeder cells would be exerting an effect on CK12 expression by physical cell-cell interactions or by soluble factors. In case of cell-cell factors, Higa *et al.*, showed the importance of N-cadherin interactions in CK12 expression using a duplex culture system (Higa *et al.*, 2009), the interactions in 3T3 feeder cells controls the expression of CK12 where N-cad plays a vital role in the maintenance of the progenitor phenotype in cultured limbal epithelial cells. These cell-cell interactions are important in culture system to control differentiated state. Similarly, in this study CK12 expression was lower in condition E dHAM Co-3T3 compared to condition B Co-3T3, which speculates that a cell-cell interaction and dHAM controls the differentiation of limbal-corneal epithelial cells in culture system.

However, in this study it was observed that flow cytometry analysis of condition C Bot-3T3, and western blot analysis of condition F dHAM Bot-3T3 had low CK12 protein expression. It is likely that soluble factors may also be important along with any cell-cell interaction.

#### **4.5.2 Introduction to cell-cell and cell-matrix interaction molecules**

Gap junctions are communication cell-cell junctions consisting of six trans-membrane proteins called connexins enabling diffusion of ions, low molecular weight metabolites, and second messengers. Connexin43 (Cx43), and connexin50 (Cx50) are abundantly expressed throughout all layers with Cx43 being mainly confined to the basal cell layer of cornea (Dong *et al.*, 1994) *in vivo*. In contrast, both connexins have been reported to be absent in the basal layer of the human, mouse, chicken and neonatal rabbit limbal epithelium. Limbal basal cells that are completely devoid of Cx43 are thought to be stem cells, whereas those that stain weakly may represent early progenitor cells *in vitro* (Grueterich *et al.*, 2002a, Balasubramanian *et al.*, 2008)

In culture systems cell–cell interactions play crucial roles in maintaining normal physiology. The cell–cell interactions may be mediated in three ways: soluble factors including cytokines; extracellular matrices (ECMs); and cell–cell adhesions. Cell–cell adhesion mediated by various molecules is an important factor in regulating differentiation and proliferation of cells. E-cadherin is a member of the classic cadherin family and is expressed mainly in epithelial cells (Takeichi, 1991). The cadherins are a family of  $\text{Ca}^{2+}$  dependent trans-membrane receptors that mediate cell-cell adhesion. E-cadherin mediated cell-cell contact results in cell activation and an increase in key signalling molecules that are involved in cell proliferation and survival. In humans, E-cadherin expression resembled that of Cx43 (Chen *et al.*, 2004)

i.e. in suprabasal cells of limbal epithelial cells, whereas in rats the expression was seen in corneal epithelium. The lack of intracellular communication has been suggested to help maintain stem cells and their niche (Matic *et al.*, 1997) by protecting the cells from damage affecting adjacent neighbours (Chee *et al.*, 2006).

The molecular components of the tight junction include, occludins which are the recently identified trans-membrane protein and a set of proteins associated to the cytoplasmic face of the junction (Tsukita and Furuse, 1999). Tight junctions are present at the apical side of epithelia, and play an important role in the establishment and maintenance of the barrier function and cell polarity. The immunolocalisation of ZO1 in the corneal epithelium indicates that ZO1 is associated to two distinct junctional components, the tight junction of the most superficial cells and the adherence junction of the basal wing cells (Sugrue and Zieske, 1997).

#### 4.5.2.1 Expression of connexin43

Absence of Cx43 (gap junctions) was considered to be an attribute of stem cells, as a protection from any noxious influence from adjacent cells. Moreover, Cx43 is also known to have non-gap junction functions such as suppression of cell and tumour growth and in cell differentiation and migration (Menichella *et al.*, 2003). Yeung *et al.*, have demonstrated Cx43 expression in infant limbus, basal and suprabasal cells of cornea *in vivo* (Yeung *et al.*, 2009).

Limbal basal cells that are completely devoid of Cx43 are thought to be SC, whereas those that stain weakly may represent early progenitor cells. It has been suggested that the expression of Cx43 denotes the differentiation of corneal transient amplifying cells (TAC). And the absence of gap junction-mediated intercellular communication may reflect a mechanism in corneal SC, which retains their stem-ness in a distinct micro-environmental niche (Quinlan *et al.*, 1985, Matic *et al.*, 1997). The expression of gap junction proteins is rarely detectable in the limbus and corneal domain that is supposed to be found in cells that from the first layer above the basal row of the epithelium *in vivo* (Lavker *et al.*, 2004, Wolosin *et al.*, 2000, Wolosin *et al.*, 2004).

In this study, connexin 43 protein expression was observed in all six-culture conditions. **Figure 3.5.10** representative picture with two biological replicates for conditions A, B, C and conditions D, E, F respectively. The expression of connexin 43 with 3T3 feeder cells is low and consistent across biological samples. Expression was higher in condition A no-3T3 and

condition D dHAM no-3T3 compared to the other conditions where 3T3 feeder cells were present. This observation supports the previous similar observations made by different groups which have shown low expression of Cx43 in presence of 3T3 feeder cells (Grueterich *et al.*, 2003b, Koizumi *et al.*, 2007).

The study by Grueterich *et al.*, shown that removal of the amniotic epithelium by EDTA, mechanical removal exposed the amniotic basement membrane and promoted the human limbal epithelial cells to adopt a phenotype that showed significantly higher Cx43 expression in dHAM with 3T3 compared to intact HAM and dHAM alone (Grueterich *et al.*, 2002a). This study observed higher expression of Cx43 in condition D dHAM no-3T3 compared to dHAM with 3T3, indicating the possible importance of feeder cell support on dHAM which is in contrast to observation made by Grueterich *et al.* This variation in expression of Cx43 in this study and Grueterich *et al.* could be due to different culture method adopted in the study. This study observed expression of Cx43 in explants culture method, whereas the above-mentioned study used cell suspension culture.

#### 4.5.2.3 Expression of E-cadherin

The extracellular domain of E-cadherin interacts with E-cadherin molecules on neighbouring cells in a homotypic calcium-dependent manner, thereby facilitating cell–cell contact such as epithelial islands formed by epithelial cells. In mouse eye development E-cadherin is expressed during terminal differentiation of corneal epithelial cells (Xu *et al.*, 2002). A study by Chen *et al.*, has shown that human corneas express E-cadherin in corneal and limbal epithelia, resembled expression of Cx43, with positive membrane staining of suprabasal layers and negative staining of basal layers at the limbus (Chen *et al.*, 2004).

This study demonstrated the higher expression of E-cadherin protein in five culture conditions (**figure 3.6.11**). The immunofluorescence of E-cadherin shown bright and high level expression in basal cells of all six conditions, confined to the membrane bound stain, with decreased intensity of staining in apical cells, staining appeared to be cytoplasmic. E-cadherin is considered as major mediator of the intercellular interactions of basal limbal epithelial cells (Higa *et al.*, 2009). In contrast to immunofluorescence of E-cadherin condition E dHAM Co-3T3, protein expression was lower compared to other five culture conditions. In corneal epithelial cells the higher expression of basement membrane and junction proteins, indicate key role in wound healing (Suzuki *et al.*, 2000). A decrease in E-cadherin protein expression in condition E dHAM Co-3T3 could be due to contact of feeder cells with dHAM, whereas immunofluorescence analysis has shown similar expression pattern observed in condition F dHAM Bot-3T3.

#### 4.5.2.4 Expression of ZO-1 and occludin

Tight junctions have two important functions in the establishment of epithelial barriers: firstly, they regulate barrier formation by modulating cell proliferation, differentiation and polarisation. Secondly, they control barrier function by restricting paracellular diffusion. Some reports observe that in corneal epithelium tight junctions are present only between the most superficial cells (McLaughlin *et al.*, 1985, Tanaka *et al.*, 1983).

Barrier characteristics of tight junctions vary considerably among different types of epithelium and endothelium, depending on physiological requirements (Powell, 1981). Occludin and ZO1 are considered differentiation markers, which are expressed on apical cells of cornea, appearance of these tight junction proteins on cultured limbal-corneal epithelial cells is not fully understood (Sugrue and Zieske, 1997).

The occludin and ZO1 proteins are more highly expressed in condition A no-3T3 than in other culture conditions, indicating the presence of a more differentiated cell phenotype in condition A no-3T3 due to absence of feeder cell support (**figure 3.5.12** and **3.5.13**). Similarly, higher expression of occludin in condition D no-3T3 dHAM represents cells under differentiated state compared to condition E dHAM Co-3T3 and condition F dHAM Bot-3T3, further evidence of the importance of 3T3 feeder cells in limbal-corneal epithelial cells *in vitro* (Balasubramanian *et al.*, 2008, Grueterich *et al.*, 2003a, Grueterich *et al.*, 2002a, Sudha *et al.*, 2008).

## 4.6 Expression of stem cell markers in cultured limbal-corneal epithelial cells

### 4.6.1 Expression of CK19

Cytokeratin 19 (CK19) is a known marker for proliferating keratinocytes in the skin, CK19 has been used to localize epidermal SC in hair follicles (Michel *et al.*, 1996). CK19 along with another intermediate filament protein- vimentin has been found to locate in the basal cells of human and murine limbal epithelium (Kasper, 1992). *In vivo* co-localization of CK19 and vimentin was particularly pronounced in basal cells of the transition zone between cornea and limbus (Barnard *et al.*, 2001). Chee *et al.*, has classified CK19 as a marker for basal layer of limbal epithelial cells (Chee *et al.*, 2006). Other groups have described that CK19 expression becomes sporadic towards the centre of the cornea and finally disappears in the centre (Pitz and Moll, 2002, Kasper *et al.*, 1988, Kivela and Uusitalo, 1998). In contrast to the above observations by different groups, some groups categorise CK19 as a conjunctival marker *in vitro* (Colabelli Gisoldi *et al.*, 2010, Ahmad *et al.*, 2007, Kolli *et al.*, 2010).

In this study, CK19 expression was observed in limbal-corneal epithelial cells in six culture conditions, where apical layer of cultured limbal-corneal epithelial cells had high intense fluorescence staining and lower expression in basal cells. CK19 expression was lower in basal cells of condition A no3T3, condition D dHAM no-3T3, condition F dHAM Bot-3T3, compared to condition B Co-3T3 and condition E dHAM Co-3T3, which indicates the limbal-corneal epithelial cells in contact with feeder layer have higher/increased expression of CK19 in basal cells (**figure 3.6.1** and **3.6.2**). This observation is in contrast to the other

group observation where cells expressed CK19 without feeder cell support in epidermal skin cells (Yang *et al.*, 2007).

As discussed above, in human ocular surface the expression pattern of CK19 is controversial, where CK19 is expressed strongly in basal limbal cells (Barnard *et al.*, 2001, Chen *et al.*, 2004) and all layers of the conjunctival epithelium (Diebold *et al.*, 2001, Pitz and Moll, 2002, Schlotzer-Schrehardt and Kruse, 2005, Yoshida *et al.*, 2006). *In vivo* the appearance of CK19 was observed after injury or in limbal stem cells deficiency (Donisi *et al.*, 2003) this might be the possible explanation for the expression of CK19 in cultured limbal-corneal epithelial cells. Where the cells under stress conditions due to culture environment may induce possible changes in cytokeratins (Endres *et al.*, 2005). Current study demonstrated flat, polygonal large cells, with high expression of CK19, which would be possible due to high differentiation of cells at apical layers; this would vary across the biological samples that were observed in conditions.

CK19 expression was confirmed by western blot analysis in the six culture conditions, (**figure 3.6.3**). No significant change in the level of protein expression was observed across the conditions. The differential localisation of CK19 in the six conditions could be due to the sensitivity of immunofluorescence staining technique, whereas the total protein isolated from all layers of cultured cells showed equal amount of protein.

#### 4.6.2 Expression of CK14

CK14 has been proposed as a marker for proliferating keratinocytes in skin and has been used to isolate epidermal stem cells (Bickenbach, 2005). Like most of the undifferentiated cells in stratified epithelia, basal cells of both corneal and limbal epithelia have been known to express the keratin pair K5/K14. Indeed, CK14 expression in the basal layer of corneal epithelium has been reported in humans, mice, and rats (Moll *et al.*, 1982, Kurpakus *et al.*, 1994, Kasper *et al.*, 1992, Hsueh *et al.*, 2004). Published data indicates that CK14 is possibly an indicative marker for progenitor cells in the limbus rather than in terminally differentiated cells (TDCs) (Dua *et al.*, 2003, Moll *et al.*, 1982, Lavker *et al.*, 2004). However, one group showed expression of CK14 in transient amplifying cells (Figueira *et al.*, 2007).

In this study, CK14 expression was observed in limbal-corneal epithelial cultures grown on cell culture insert conditions, with and without 3T3 cells. CK14 displayed low intensity staining in basal cells, with slight increase in expression in the apical layers of condition A no-3T3. In condition B Co-3T3, CK14 expression was higher in apical cells; with low/no stain in basal cells. In contrast to conditions A no-3T3 and B Co-3T3, condition C Bot-3T3 (**figure 3.6.4**) showed strong intense staining of CK14 in basal cells. The dHAM cultures showed sporadic strong staining of apical cells for CK14 in condition D dHAM no-3T3, condition E dHAM Co-3T3. Whereas expression of CK14 in apical cells of condition F dHAM Bot-3T3 was not available. CK14 intensity was increased in apical cells of condition D dHAM no-3T3 and condition E dHAM Co-3T3 (**figure 3.6.5**). Thus, suggesting that culture condition A no-3T3 is more differentiated and has less proliferative / transient

amplifying cells (TAC) cells compared to the other growth conditions. A similar observations were made in vitro and in vivo by other groups where, CK14 was at basal cells of limbal epithelial cells *in vivo* and TAC cells *in vitro* (Dua *et al.*, 2003, Moll *et al.*, 1982, Lavker *et al.*, 2004).

In the current study, western blot analysis **figure 3.6.6** represents expression of CK14 in all six-culture conditions, with low expression in condition A no-3T3, the variation in expression of CK14 was similar across biological samples, and conditions. Overall, CK14 protein expression was increased in conditions with 3T3 feeder cells and/ or dHAM when compared with condition A no-3T3. CK14 might be stem cell marker under specific circumstances, i.e. within the limbus of a quiescent cornea (Yoshida *et al.*, 2006).

A study by, Chen *et al.* 2010 recently found increased expression of CK14 in airlift culture of bovine corneal epithelial cells (Chen *et al.*, 2010). They suggest that CK14 is an unreliable marker for undifferentiated cells *in vitro*. Thus, CK14 positive cells are likely to include cells in various stages of differentiation i.e. from stem cells to transient amplifying cells (TAC) and finally to terminally differentiated cells. Moreover, in one study demonstrated that CK14 continues to be expressed in limbal derived explant epithelial cultures through five trypsinized passages, suggests that CK14 is expressed by both limbal and transient amplifying cells (Figueira *et al.*, 2007). This study observed the appearance of strong positive CK14 staining in apical cells of cultured limbal-corneal epithelial cells (**figures 3.6.4 and 3.6.5**) which would suggest that this marker is not selective for limbal stem cells but rather may also detect the transient amplifying cell population and those cells undergoing differentiation.

### 4.6.3 p63 and $\Delta$ Np63 $\alpha$ (Nuclear proteins)

#### 4.6.3.1 p63

The transcription factor p63 has been suggested to be a limbal stem cell marker (Pellegrini *et al.*, 2001). This protein is known to be involved in tumour suppression and morphogenesis. p63 is a p53 homologue consistently expressed in basal cells of the stratified epithelia which is essential for epithelial development and differentiation (Yang *et al.*, 1999). Subsequent studies confirmed the presence of p63 in limbal basal layer (Chen *et al.*, 2004, Kim *et al.*, 2004, Lavker *et al.*, 2004). However, in another study by Dua *et al.*, shown positive staining of p63 not only in the limbal region but also among most of the basal cells of the peripheral and central corneal epithelium in both frozen and formalin-fixed specimens (Dua *et al.*, 2003). In this study western blot analysis showed no obvious difference in p63 protein expression in all six conditions, which could be a possible detection of transactivation (TA) domain of p63 (Krishnan *et al.*, 2010) (**figure 3.6.17**).

Studies on murine corneas have shown that p63 expression throughout the corneal epithelium with maximal expression levels in paracentral areas of increased proliferative potential as demonstrated by BrdUrd-labelling (Moore *et al.*, 2002). Damaged conjunctivalized corneas displayed an abnormal p63 expression pattern when compared to normal corneas. In the conjunctival epithelium, a regular pattern of p63 expressing cell clusters was noted in basal epithelial cells. The authors concluded from the observations that p63 expression was not

confined to SC alone, but was maximally increased in areas of increased proliferative potential (Moore *et al.*, 2002).

Two groups Wang *et al.* 2003 and Hsueh *et al.* 2004 found p63-positive signals specifically in basal and suprabasal cells of the limbal epithelium in rabbit eyes, whereas the corneal epithelium was negative. However, most of the basal and suprabasal limbal epithelial cells were positive for both p63 and Ki67, a proliferating cell nuclear marker, and only few suprabasal cells were Ki67-positive but p63-negative (Wang *et al.*, 2003, Hsueh *et al.*, 2004). Together, different studies reflect large discrepancies between p63 expression patterns, which might be due to differences concerning technical procedures, interindividual variations, age-related changes, and cross-species variations. As mentioned earlier, this study identified no differential protein expression of p63 in all six-culture conditions. It is suggested that a used primer and antibody did not differentiate p63 isoform, it has been proposed that only the  $\alpha$  isoform of p63 is maintenance of limbal stem cell characteristics (Di Iorio *et al.*, 2005).

#### **4.6.3.2 $\Delta$ Np63 $\alpha$**

There are six isoforms of p63 protein. These are generated by the existence of two different promoter sites, which give rise to different N-termini. An upstream promoter, which generates the transactivating forms and a downstream intronic promoter that generates the  $\Delta$ Np63 $\alpha$  isoforms. The  $\Delta$ Np63 $\alpha$  isoform has been shown to be important in sustaining the proliferative potential of keratinocyte stem cells (Parsa *et al.*, 1999). Alternate splicing of the  $\Delta$ Np63 $\alpha$  mRNA transcript gives rise to three C-termini proteins, which are designated,  $\alpha$ ,  $\beta$ , and  $\gamma$ . In 2005 Di Iorio and colleagues using in situ hybridization showed that the expression of these isoforms different in expression and location within the cornea and the limbus in

vitro. In particular that 8% of the basal cells of the limbus, which is the suspected location of corneal limbal stem cell population, were positive for the isoform  $\Delta\text{Np63}\alpha$  (Di Iorio *et al.*, 2005). This strong localisation of  $\Delta\text{Np63}\alpha$  in basal and intermediate layers of the limbus in vitro was validated by a similar study by Kawasaki et al (Kawasaki *et al.*, 2006). Both studies showed either minimal or no expression of  $\Delta\text{Np63}\alpha$  elsewhere in the cornea. The study by Di Iorio showed that the level of  $\Delta\text{Np63}\alpha$  expression and number of cells positive increased following injury (Di Iorio *et al.*, 2005). In addition, this response to injury also induced the expression of the  $\Delta\text{Np63}\beta$  and  $\Delta\text{Np63}\gamma$  isoforms in limbal and corneal epithelium. As p63 has been proposed as being important in the maintaining the proliferative capacity of epithelial stem cells (Yang *et al.*, 1999) and its expression was reported in the transamplifying and stem cells of epidermal and corneal epithelia it is suspected in being important in potential marker in stem cells. The expression of the  $\Delta\text{Np63}\alpha$  isoform is believed to give greater specificity as to the potential stem cell population. With this in mind we investigated  $\Delta\text{Np63}\alpha$  expression in the different culture conditions using immunofluorescence, western blotting and RT-PCR (Pellegrini *et al.*, 2001).

The immunofluorescence study showed all six culture conditions were positive for  $\Delta\text{Np63}\alpha$  (**figure 3.6.7 to 3.6.14**). In particular, bright nuclear staining was observed in all cells of the basal cell layer in each of the culture conditions. These cells of the basal layer had a small cuboidal morphology compared to the large squamous morphology of the negative cells of the apical layer. In addition, occasionally sporadic positive staining was observed in the nuclei of apical cells of Condition E dHAM Co-3T3 (**Figure 3.6.12**). While only 8% of cells in the limbus *in vivo* were estimated by Di Iorio and colleagues to be positive for  $\Delta\text{Np63}\alpha$ , the same study also noted its elevation following cornea injury and in limbal-cornea epithelial cell culture (Di Iorio *et al.*, 2005). While no specific analysis was conducted in this study to

quantify the number of cells positive  $\Delta\text{Np63}\alpha$ , it would appear that there was no obvious difference in the number of positive cells in basal layers of each of the six conditions. The lack of difference in the number of cells positive for  $\Delta\text{Np63}\alpha$  among different conditions would appear to contradict a study by Galindo et al 2009, which looked at the staining pattern of the  $\Delta\text{Np63}\alpha$  in different culture conditions and treatments (Galindo *et al.*, 2009). Galindo and colleagues showed a higher  $\Delta\text{Np63}\alpha$  staining in cells cultured on human amniotic membrane (81.4%  $\pm$ 12.1% positive) compared to those cells cultured on tissue culture plastic (66.6% $\pm$ 16.5% positive). Interestingly they noted that while the  $\Delta\text{Np63}\alpha$  expression was homogenous in cells cultured on human amniotic membrane the staining was more heterogeneous in cells cultured on tissue culture plastic. In particular, Galindo and colleagues generated their cultures from a single explant and the  $\Delta\text{Np63}\alpha$  expression reduced in cells from the centre of the culture towards the periphery. This finding was further studied by Kolli et al 2008, who noted three zones (A,B, and C) around a single explant culture on human amniotic membrane, Zone A nearest the explant which highly expressed  $\Delta\text{Np63}\alpha$  while zone C, the most peripheral zone, showed lower expression as shown by qRT-PCR. They also noted that this different expression in  $\Delta\text{Np63}\alpha$  in each of the three zones was also reflected in the CFE potential of each zone with zone A showing the highest %CFE. Kolli and colleagues hypothesised that the use of an explant allowed maintenance of the stem cell niche and that as the cells migrated far away from the explant they did not receive the specific signals from this niche to prevent differentiation (Kolli *et al.*, 2008). They also speculated that it was the presence of such a niche in the culture and not contact with amniotic membrane that kept the limbal cells from differentiating. Although it was not looked for or shown in this, study, no obvious regional difference in expression in the basal layers of the different cultures was observed. This might be due to the fact that these cultures contained multiple explants which meant that there would be a greater exposure to niche signals thorough out the culture.

However positive staining for  $\Delta\text{Np63}\alpha$  is not the sole indicator of the stem cell potential, consideration of the level of expression within cells and cultures is also required (Di Iorio *et al.*, 2005, Rama *et al.*, 2001). A preliminary flow cytometry analysis conducted on conditions A No 3T3, B Co-3T3, and C Bot-3T3 appeared to show all cells positive within the culture. However, a difference did exist between the cultures with their mean fluorescence intensity (**figure 3.6.15**). In particular condition C Bot-3T3 had the highest mean fluorescence intensity for  $\Delta\text{Np63}\alpha$  compared to condition B and A respectively. Which would potentially suggest that the condition C bot-3T3 culture might possess a greater stem cell potential. However, this result by flow cytometry analysis is only preliminary and requires further development and validation prior to any confirmation of these results.

Western blot analysis was performed and it confirmed that  $\Delta\text{Np63}\alpha$  was present in each of the six culture conditions. There were some indications that the expression of  $\Delta\text{Np63}\alpha$  might vary between culture conditions. In particular the addition of 3T3 feeder cells in condition B Co-3T3 and condition C 3T3-Bot appeared to increase the expression of  $\Delta\text{Np63}\alpha$  compared to condition A No 3T3 (**figure 3.6.16 A**). Similarly the level of  $\Delta\text{Np63}\alpha$  protein expression appeared to increase slightly when 3T3 feeder cells were present as in condition E dHAM Co-3T3 and Condition F dHAM Bot-3T3 compared to Condition D dHAM no-3T3 (figure 3.6.16 A). However, this effect was not always as pronounced among the different biological samples as seen in **figure 3.6.16 B**. In this set of biological repeats there is no obvious difference between Condition A No 3T3 and Condition B Co-3T3, There is a very slight increase in  $\Delta\text{Np63}\alpha$  protein expression in Condition C Bot-3T3 compared to Condition A No 3T3. This lack of pronounced difference is also seen with condition E dHAM Co-3T3 and Condition F dHAM Bot 3T3 showing very slight increase in  $\Delta\text{Np63}\alpha$  protein expression

compared to Condition D dHAM no-3T3. This lack of clear, strong, reproducible differential expression between the different culture conditions may reflect the differences in stem cell quality from the donors used to generate the cultures.

RT-PCR analysis did not show any differential expression of  $\Delta$ Np63 $\alpha$  mRNA levels in any of the six conditions (**figure 3.6.18**). This appeared to be a consistent finding across a number of biological repeats. This would suggest that  $\Delta$ Np63 $\alpha$  mRNA level is not altered by culture condition and that if  $\Delta$ Np63 $\alpha$  protein expression is altered it is due to post-transcriptional control. Huang and colleagues proposed in 2008, one possible post-transcriptional control mechanism for  $\Delta$ Np63 $\alpha$  protein levels. They proposed that phosphorylation of  $\Delta$ Np63 $\alpha$  by three kinases ATM, CDK2 and p70s6K resulted in the targeting of  $\Delta$ Np63 $\alpha$  to a proteasome-dependent degradation pathway. Thus by controlling this phosphorylation of  $\Delta$ Np63 $\alpha$  a cell could regulate the level of  $\Delta$ Np63 $\alpha$  protein (Huang *et al.*, 2008).

The fact that was a potential  $\Delta$ Np63 $\alpha$  protein increase Condition C Bot-3T3, condition E dHAM Co-3T3 and Condition F dHAM Bot 3T3 compared to controls would suggest that the feeder cells might potentially regulate  $\Delta$ Np63 $\alpha$  protein levels. A possible candidate is keratinocytes growth factor (KGF) also known as FGF7, and is known to be secreted by the stromal cells of the cornea (Wilson *et al.*, 1993). This growth factor was shown by Cheng *et al.* to increase  $\Delta$ Np63 $\alpha$  protein expression in limbal explant cultures on amniotic membrane (Cheng *et al.*, 2009). They propose that KGF signals via the MAPK P38 pathway to phosphorylate Sp1, which has a putative binding site in the  $\Delta$ Np63 promoter region (Romano *et al.*, 2006). However, it is unclear if this is the reason for the observed slight increases in  $\Delta$ Np63 $\alpha$  protein expression, as the RT-PCR data would indicate the mRNA levels remain unchanged throughout the different culture conditions. It should be noted that for both the

western blot analysis and the RT-PCR analysis sample were taken only at about day 14 when a confluent culture was obtained. Thus, clear differences in expression at an mRNA and protein level might have occurred at earlier time point within the culture.

Interestingly the Cheng and colleagues suggested that while  $\Delta Np63\alpha$  alone was important for proliferation of cells in culture, its over expression alone did not promote outgrowth from explants and that KGF may play additional roles in promoting outgrowth and proliferation of limbal-cornea epithelial cells.

#### 4.6.4 Expression of transporter molecule ABCG2

Stem cells from bone marrow, skeletal muscle, and other tissues can be isolated based on their ability to exclude the vital dye Hoechst 33342 (bisbenzimidazole), which defines a 'side-population' (SP) phenotype. ABCG2 is a member of the ATP binding cassette (ABC) transporters, which localizes predominantly to the plasma membrane and is actively involved in Hoechst efflux. ABCG2 has been proposed as a universally conserved marker for SC from wide variety of tissues (Kim *et al.*, 2002). A study by Chen *et al.*, demonstrated for the first time that the ABCG2 transporter was exclusively expressed by the limbal epithelium on the ocular surface (Chen *et al.*, 2004). Recent studies have provided convincing evidence that a subset of limbal epithelial cells belongs to the SP phenotype expressing ABCG2 protein (Watanabe *et al.*, 2004). About 0.3-0.5% of cells from total limbal population exhibited the SP phenotype, whereas no SP cells were seen in corneal epithelium. SP cells isolated from limbal epithelium expressed ABCG2 and possessed colony forming efficiency *in-vitro* suggesting ABCG2 as a limbal stem cell marker (de Paiva *et al.*, 2005). Cells from limbal explants expressing ABCG2 showed high clonogenic potential and expressed high levels of  $\Delta Np63\alpha$ , similar to side population cells (de Paiva *et al.*, 2005, Meyer-Blazejewska *et al.*, 2010). Recently two studies have demonstrated that ABCG2 has been found in cell clusters of basal limbal epithelium and but not in central cornea by Immunofluorescence analysis (Schlotzer-Schrehardt and Kruse, 2005, Watanabe *et al.*, 2004).

As mentioned above, in a number of studies ABCG2 has been shown to indicate the location of limbal epithelial stem cells in basal epithelial layers of the limbus where clusters of positive cells are observed. However, in this study basal and apical layer cells of all six

conditions expressed ABCG2 (**figure 3.6.19 to 3.6.26**). The basal layer of cells in all six conditions which are tightly packed epithelial cells were more brightly stained than enlarged differentiated cells. The presence of a brightly stained cells was increased in condition B Co-3T3 and condition C Bot-3T3 compared to condition A no-3T3 in cell culture inserts. Similarly, brightly stained cells were observed in condition F dHAM Bot-3T3 and E dHAM Co-3T3, compared to condition D no-3T3 dHAM. As such, the localisation of ABCG2 marker was not convincing for limbal stem cells *in vitro*, as this study identified the localisation in basal and apical cells. This observation is in contrary to the findings by other groups where ABCG2 expression was only at basal cells of limbus in *in vivo* and *in vitro* (de Paiva *et al.*, 2005, Watanabe *et al.*, 2004, Budak *et al.*, 2005).

A flow cytometry analysis of limbal-corneal epithelial cells grown in cell culture inserts with/without 3T3 feeder cells agreed with immunofluorescence analysis, the mean fluorescence intensity (MFI) of ABCG2 was different in culture conditions. Condition B & C (Co-3T3 & Bot-3T3) showed similar MFI, with decreased MFI in condition A no-3T3 (**figure 3.6.27**). These flow cytometry analyses is in contrast to the observations where only 2.5%–3.0% ABCG2 positive cells were identified by flow cytometry in limbal corneal epithelial cell cultures (de Paiva *et al.*, 2005). This might be due to differences concerning technical procedures, inter individual variations, and age-related changes.

A further study was performed for differential expression of ABCG2 by total protein analysis. Total protein analysis for ABCG2 has shown slight increased expression in cultures with 3T3 feeder layers in cell culture inserts and dHAM, across biological samples, compared to limbal-corneal epithelial cells alone (condition A no-3T3) (**figure 3.6.28**). The higher expression of ABC-G2 in level for condition D dHAM no-3T3 than condition A no-3T3

suggests that dHAM is suitable substrate for stem cells niche *in vitro*. There were some indications that the expression of ABCG2 might vary between culture conditions. A dHAM with irradiated-3T3 (Ir-3T3) feeder cells away or in contact acts as suitable condition for maintenance of more stem cell/progenitor population compared to limbal epithelial cells grown on plastic with Ir-3T3 feeder cells, where ABCG2 protein expression was slightly increased in dHAM cultures with 3T3 feeder cells. However, this effect was not always as pronounced among the different biological samples as seen in figure 3.6.28 B. In this set of biological repeats, there is slight increased expression in Condition B Co-3T3 compared to Condition F dHAM Bot-3T3. There is a very slight increase in ABCG2 protein expression in Condition F dHAM Bot-3T3 compared to Condition B Co-3T3. This lack of pronounced difference is also seen with condition E dHAM Co-3T3 and Condition F dHAM Bot-3T3 showing very slight increase in **figure 3.6.28**. This lack of clear, strong, reproducible differential expression between the different culture conditions and biological samples may reflect the differences in stem cell quality from the donors used to generate the cultures in different conditions.

In three biological samples ABCG2 mRNA expression was higher in condition D no-3T3 dHAM compared to condition A no-3T3, which is the evidence of higher stem cell population in limbal-corneal epithelial cells grown on dHAM compared to epithelial cells on plastic. We have not observed major mRNA expression level across all other conditions for ABCG2; this could be due to a small change that was undetectable by end point PCR across biological variations (**figure 3.6.29**).

As described previously, in this study ABCG2 was expressed in basal and apical cells of cultured limbal-corneal epithelial cells. This would not be expected if this was a true limbal

epithelial stem cell (LESC) marker *in vitro*. This suggests that *in vitro* expression of ‘stem cell’ markers on cultured cells alone should be interpreted only as an indicator of the presence of early transient amplifying cells. Further characterisation would still be required to identify LESCs. Moreover, it does not provide evidence that all of the positive cells are stem cells. The CFE assay is used to enhance and supplement the results from the putative stem cell markers ABCG2 and P63 $\alpha$ , as a more specific stem cell marker remains elusive.

#### 4.6.5 Integrins in corneal epithelial cells

Integrins function as heterodimers consisting of individual  $\alpha$  and  $\beta$  chains. They can be classified into three major groups:  $\beta 1$ ,  $\beta 2$ , and  $\alpha$ .  $\beta 1$  family consists of the first integrin to be discovered,  $\beta 1$  can form heterodimers with at least 12 distinct  $\alpha$  chains. In the corneal epithelium, several integrins are expressed in a pattern that is indicative of their function. In human and mouse central cornea, there is  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha v\beta 5$ , and  $\alpha 6\beta 4$ , all of which have a polarized localization within the epithelium (Kurpakus *et al.*, 1991, Stepp *et al.*, 1993, Grushkin-Lerner and Trinkaus-Randall, 1991, Murakami *et al.*, 1992, Paallysaho *et al.*, 1992, Latvala *et al.*, 1996)

The  $\alpha 2$  and  $\beta 1$  Integrins are known to express most intensely in the basal cells with expression progressively lost in the more apical layers. In human cornea the most intense staining for integrins is found where the basal aspect of the basal cells comes into contact with the basement membrane (Filenius *et al.*, 2001, Filenius *et al.*, 2003, Li *et al.*, 2005b, Li and Lu, 2005). *in vivo*  $\alpha 2$  integrin is present in limbus and basal cells of the cornea, due to difference in the composition between basal membranes of the cornea and limbus which could, at least in part, explain the differences in phenotype and in behaviour of the basal cells (Dietlein *et al.*, 1997, Fukuda *et al.*, 1999, Ljubimov *et al.*, 1995)

#### 4.6.5.1 Expression of $\alpha$ -2 integrin

In the present culture system (all six conditions), a uniform strong staining of  $\alpha$ -2 integrin was observed in basal layer cells (**figure 3.6.30 and 3.6.31**) except for condition D dHAM no-3T3. In contrast to the basal layer of cells, the apical cells with large flatter morphology showed weaker staining. Other studies have shown up-regulation of integrins in the presence of specific extracellular matrix proteins or glycoproteins, such as laminin or collagen type IV (Grushkin-Lerner *et al.*, 1997). This supports high expression of  $\alpha$ -2 integrin in basal cells of corneal epithelial cell cultures where epithelial cells interacts with basement membrane of dHAM rich in type IV collagen (Hopkinson *et al.*, 2006) and 3T3 feeder cell interactions with compact limbal-corneal epithelial cells (Li *et al.*, 2005a, Li *et al.*, 2005b, Frisch and Francis, 1994).

Western blot analysis for  $\alpha$ -2 integrin showed no significant difference between culture conditions, and observed similar trend in level of expression across the biological samples (**figure 3.6.32**). Integrins (alpha and beta family) mediate cell matrix adhesion; the abundance of  $\alpha$ -2 integrin in the cultured limbal-corneal cells explains the strong adhesion of the extra cellular matrix. Moreover,  $\alpha$ -2 integrin is important in the formation of ocular phenotype, wound healing process (Chen *et al.*, 2002), and express in limbal-corneal epithelial cells. The expression of  $\alpha$ -2 integrin might be due to high proliferation rate of the cells in all culture conditions where protein synthesis in the cells increases as they migrate in wound healing process (Stepp *et al.*, 1993). Where, in the current cultures, cells migrate on

dHAM and cell culture inserts from the limbal explants to form thick cells sheet, which express  $\alpha$ -2 integrin.

In summary, the immunofluorescence study found  $\alpha$ -2 integrin in the limbal-corneal epithelium at sites of cell-cell interaction (tight compact cell basal cells) and at sites of cell-substrate interaction, (dHAM + 3T3 cells) and this distribution changed little on apical cells where cell-cell interaction appeared to be less due to differentiation of the cells. At this stage it is difficult to differentiate between the stem cells and the TAC in the corneal epithelium with  $\alpha$ -2 integrin expression. The relationship between migration (from explants) and appearance of  $\alpha$ -2 integrin can be studied with time lapse experiments to understand the role of integrin in cultured limbal-corneal epithelial cells and its importance in maintenance of stem cells/progenitors in cultures.

#### 4.6.5.2 Expression of $\beta 1$ integrin

Integrin  $\beta 1$  has been proposed as a keratinocyte stem cell marker (Jones and Watt, 1993, Watt and Jones, 1993). In cultures of human corneal epithelial cells expanded *in vitro*, more small cells (presumed to be stem cells) have been found to express integrin  $\beta 1$  as compared with larger cells (presumed to be more differentiated cells) (Li *et al.*, 1998). In human cornea, integrin  $\beta 1$  is uniformly expressed by basal cells of both limbus and cornea, and lacks any real specificity in distinguishing human limbal from corneal basal cells (Chen *et al.*, 2004). Experimental studies suggest expression of  $\beta 1$  integrin in corneal epithelial cultures, predominately in undifferentiated cells.

In this study, immunofluorescence staining of  $\beta 1$  integrin showed strong expression in basal layer of cells in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3 with tight compact cell-cell membrane stain, with decrease in membrane bound localisation in apical cells of condition B Co-3T3 and condition C Bot-3T3. Whereas condition A no-3T3 appeared to have no/very low expression level in apical cells (**figure 3.6.33**). A high intense stain of  $\beta 1$  integrin in basal cells of condition B Co-3T3 and condition C Bot-3T3 could be the effect of 3T3 feeder cells in contact with limbal-corneal epithelia cells or soluble factors.  $\beta 1$  integrin expression in dHAM cultures with/without 3T3 feeder cells showed lower intensity stain in basal cells for three conditions in contrast to the insert cultures, with decreased intensity in apical cells for condition F dHAM Bot-3T3 (**figure 3.6.33**).

These findings suggest that localisation of  $\beta 1$  integrin is not very specific and may not be a very specific marker for stem cells as it is originally suggested to be a keratinocyte marker

(Jones and Watt, 1993). High level of expression of  $\beta_1$ -integrin in apical cells (large cell size), non-stem cells of limbal-corneal epithelial cultures caused concern over how effective an enrichment strategy might be based on the expression of  $\beta_1$ -integrin.

The staining appears to be relatively non uniform and appears to contradict the findings of Chen *et al.*, that  $\beta_1$  integrin is not specific enough to distinguish differences in limbal-corneal cell population (Chen *et al.*, 2007). Whereas this study, demonstrated differential expression in basal and apical layers for all six conditions as discussed above.

Western blot analysis of  $\beta_1$ -Integrin showed an increase in the level of expression in all conditions compared to condition A no-3T3, with variation in expression level across biological samples (**figure 3.6.36 A and B**), whereas expression pattern was similar among samples i.e. high protein expression compared to condition A no-3T3. No substantial increase in protein expression was observed between 3T3 feeder cell addition and / or 3T3 dHAM cultures (**figure 3.6.35**). Integrins impact cell adhesion, matrix assembly, and cell survival; they are implicated as stem cell markers (Stepp *et al.*, 1993). The abundance of  $\beta$ -1 integrin in the cultured limbal- corneal cells may help to explain the strong adhesion of the limbal corneal cells to the extra cellular matrix. At this stage, it is difficult to differentiate between the stem cells and the TAC in the cultured limbal-corneal epithelial cells with this marker. One may speculate that the expanded limbal epithelium might interact with its underlying extracellular matrix and plays an important role in maintaining stem cell characteristics and preventing epithelial differentiation, a view that has been proposed for keratinocytes (Adams and Watt, 1989). Jones and colleagues first showed that cultured human keratinocytes expressing high levels of  $\beta_1$ -integrin had higher proliferation potentials than those expressing low levels of the integrin (Jones *et al.*, 1995). Additional studies continued to implicate the

$\beta$ 1-integrin family of epithelial integrins as potential markers of epidermal stem cells (Jones and Watt, 1993, Jensen *et al.*, 1999). Nevertheless, the high level of expression of  $\beta$ 1-integrin in surrounding nonstem cells in the basal layers of epithelial tissues caused concern over how effective an enrichment strategy might be, based on the expression of  $\beta$ 1-integrin (Pajoohesh-Ganji *et al.*, 2006).

#### 4.7 qRT-PCR analysis of limbal-corneal epithelial cells

Quantitative RT-PCR is the most accurate way of both detecting and quantifying a given DNA sequence and represents the most sensitive way of detecting differences in mRNA expression irrespective of exact cell number. The standard reference gene used for the studies below was beta actin (ACTB). The putative limbal stem cell (LSC) markers used to quantify stem cell numbers in the limbal outgrowths were p63,  $\beta 4$  and  $\alpha 6$  integrins. The limbal-corneal differentiation markers used are CK3 and CK12.

In this study, the expression analysis of stem cell and differentiation marker of limbal-corneal epithelial cells by qRT-PCR has given differential levels of expression across culture conditions for target genes. As described in **section 2.20.6**, qRT-PCR was analysed by considering condition A no-3T3 as calibrator sample (considered as the worst condition to grow limbal-corneal stem cells) for comparison against other culture conditions.

Differentiation marker CK3 has shown non-significant up regulation in condition B Co-3T3 and condition C Bot-3T3. Whereas, CK12 has shown down regulation in conditions B, D, E, F with significant down regulation in condition E dHAM Co-3T3, similarly down regulation of CK12 protein was observed in this condition (**figures 3.7**). Integrin  $\beta 4$ ,  $\alpha 6$  have shown no significant effect in culture conditions.

The SD values are higher due to large variation in biological and culture conditions in this study. The limitation in this study is the lack of a control (variation within the calibrator sample) in calibrator sample in terms of age of the donor, storage of the tissue before process are the factors that are not under control in this study. Comparing samples requires normalization to compensate for differences in the amount of biological material in the tested samples. In most tissues under reasonably well defined conditions, only a fraction of the genome is active, and a limited number of genes have their transcriptional levels that might alter by external stimuli or moderate environmental changes.

qRT-PCR may not be the best technique to identify the differential gene expression level in differentiation experiment studies with high variation in biological calibrator sample. This approach is highly unsuitable for differentiation studies, because no gene seems to be expressed at a constant level during differentiation and, with primary tissue culture samples where the tissue-to-tissue samples vary in donor age, preservation, and culture conditions. Supplementary data (**Appendices**) illustrates the raw values and calculations of different genes in cultured limbal-corneal epithelial cells for three biological replicates, and variation within the calibrator  $C_T$  values in sample (three biological replicates) for each gene. A further study with large amount of samples might give a better understanding of these markers at transcriptional level.

#### 4.8 Colony forming efficacy assay of limbal-corneal epithelial cells

A Colony forming efficacy (CFE) assay was performed to determine the proliferative potential of cells in the cultured limbal-corneal cell sheets by prolonged culturing time and a lower seeding density, which could maintain SCs. The interaction between epithelial cells and 3T3 feeder cells via diffusible factors promotes the proliferation and differentiation of epithelial cells (Smola *et al.*, 1993, Szabowski *et al.*, 2000). A clonal analysis of squamous human epithelia, including the limbus and cornea, has indeed identified three types of keratinocytes, known as holoclones, meroclones and paraclones (Pellegrini *et al.*, 1999a, Barrandon and Green, 1987).

Holoclonal cells give rise to large colonies (10-30 mm<sup>2</sup>) with a smooth circular perimeter and appeared to be in the limbus but not in the cornea (Pellegrini *et al.*, 1999a) and have the hallmarks of a stem cell, including self-renewing capacity (Rochat *et al.*, 1994, Barbaro *et al.*, 2007, Claudinot *et al.*, 2005). A single holoclone can generate the entire epidermis of a human being (Rochat *et al.*, 1994). Expression of high holoclone cells in culture systems suggests the maintenance of stem cell population and this requires the presence of a proper feeder-layer of irradiated 3T3 cells, an appropriately selected substrate and fetal calf serum (De Luca *et al.*, 2006).

Meroclones give rise to intermediate sized colonies (5-10 mm<sup>2</sup>) with a slightly irregular or wrinkled perimeter (Pellegrini *et al.*, 1999a). When sub-cultured, the colonies formed by meroclones give rise to 5-100% terminal looking colonies (Pellegrini *et al.*, 1999b). Because meroclones have limited growth potential but are by no means terminal, they have been

compared with the epithelial transient amplifying cells. Whereas paraclones give rise to small colonies ( $\leq 5 \text{ mm}^2$ ) with a highly irregular perimeter, these colonies are referred to as terminal colonies (Rochat *et al.*, 1994). Recently, CFE in limbal –corneal epithelial cells were demonstrate by selection of colonies greater than  $2 \text{ mm}^2$  with compact circular edges as holoclones, and non consideration of colonies with irregular morphology for count (Notara *et al.*, 2007). In this study, the selection criterion for CFE of limbal-corneal epithelial cells was based on the colonies greater than  $2\text{-}10 \text{ mm}^2$  (**figure 3.8.1 and 3.8.1b**). Selection of colonies based on binary image and original images together for best selection of colonies, refer appendences 8 (page number 449-450) for binary and original images.

In this study, colony-forming efficacy (CFE) was assessed in all six-culture conditions (**figure 3.8.1a and 3.8.1c**). In the first set of experiments with cell culture inserts, the ability of CFE is higher in condition C Bot-3T3 ( $2.32 \pm 0.30 \%$  CFE) than condition B Co-3T3 ( $1.17 \pm 0.24 \%$  CFE). A very low CFE percentage at condition A no-3T3 ( $0.42\%$  CFE) suggest a low stem cell/progenitor population. In support to this, condition A no-3T3 showed low expression of stem cell/putative markers  $\Delta\text{Np}63\alpha$  and ABCG2 (**Figure 3.3.16 and 3.6.28**). In contrast, condition B (Co 3T3) and condition C (3T3-Bot) showed a higher CFE count then condition A (No-3T3). This study speculates that a soluble factor or factors secreted by 3T3 feeder cells into the media promotes a higher CFE and hence a higher number of stem/progenitor cells. The High CFE in condition C Bot-3T3 ( $2.32 \pm 0.30 \%$ CFE) compared to condition B Co-3T3 ( $1.17 \pm 0.24 \%$  CFE) observation was in contrast to Miyashita *et al.*, study which demonstrated that CFE formation was higher in contact feeder cells than away feeder cells in limbal-corneal epithelial cells (Miyashita *et al.*, 2008). However, in this study explants were used to generate the cultures in contrast to the single cell method used by Miyashita and colleagues. It is possible that the presence of the explant during the duration of

the culture provided a stem cell niche and provided additional factors that encouraged growth and maintenance of the stem/progenitor cell phenotype. Support for this hypothesis comes from Kolli et al 2008, who noted that cells closer to the explant had a higher CFE than those at a distance from it. Thus, the combination of this stem cell niche in combination with additional soluble factor(s) from the 3T3 feeder layers promoted stem/progenitor cell growth and maintenance. A possible factor produced by the 3T3 feeder cell is considered to be a major regulator of keratinocyte growth and differentiation factor in cell culture studies (Szabowski *et al.*, 2000, Maas-Szabowski *et al.*, 1999).

The second set of cultures in this study on dHAM with/without Ir-3T3 feeder cell system has shown high percentage of CFE than the first three culture conditions (condition A, B and C). This could be due to the extracellular matrix (HAM) and/or 3T3 cells supporting the stemness of limbal epithelial cells, a similar observation was made by other groups (Grueterich *et al.*, 2002b, Levis and Daniels, 2009, Shortt *et al.*, 2008). In detail the CFE of limbal-corneal epithelial cells grown in condition D no-3T3 dHAM is  $5.25 \pm 0.19\%$ , which shows higher percentage than condition A no-3T3 of 0.42% CFE, even in the absence of Ir-3T3 feeder cells. This increase in percentage of CFE with dHAM (condition D dHAM no-3T3) alone indicates the possible importance of a suitable substrate for limbal-corneal epithelial cells *in vitro*. Studies have shown that human amniotic membrane is the best suitable substrate for culture of limbal-corneal epithelial cells due to presence of rich extracellular matrix proteins (Koizumi *et al.*, 2000, Grueterich *et al.*, 2003b, Grueterich *et al.*, 2003a). Recently Balasubramanian *et al.*, studied the importance of 3T3 feeder cells in maintenance of stem cell niche in limbal-corneal cells, where the presence of 3T3 feeder cells with dHAM have shown high stem cell markers (Balasubramanian *et al.*, 2008). Similar observation was made in this study in conditions E and F with 3T3 feeder cells and dHAM.

Moreover, the percentage of CFE was higher in condition E dHAM Co-3T3 with  $8.95 \pm 2.18\%$  CFE than condition F dHAM Bot-3T3 with  $7.2 \pm 1.96\%$  CFE, which suggests the importance of Ir-3T3 feeder cell location in maintenance of stem cell population *in vitro*. The decrease in percentage of CFE in condition F dHAM Bot-3T3 is possibly due to limited supply of growth factor exchange between 3T3 feeder cells and limbal-epithelial cells due to thickness of the membrane. A short study was conducted to understand importance of soluble factors in limbal-corneal epithelial cultures and discussed in the next section.

#### **4.9 Explants culture system in cell culture inserts with 3T3 conditioned medium**

The effect of 3T3 feeder cells on limbal explants growth showed a positive impact on growth potential (**Section 3.9**), either in contact or away from limbal-epithelial cells. Feeder cells provide a suitable environment in co-culture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interaction (Ehmann *et al.*, 1998), production of soluble growth factors and removal of toxicants from the culture medium.

The initial outgrowth of limbal-corneal epithelial cells with 3T3 conditioned media was greater than condition A no-3T3 ( $2.1\text{mm}^2$  at day 5), in conditioned medium with average outgrowth was  $3.6\text{mm}^2$  at day 5. Whereas the outgrowth rate was shown to be slower compared to condition C Bot-3T3, with average outgrowth rate of  $5.8\text{mm}^2$  at day 5 (**figure 3.9.1**). Culture medium conditioned with 3T3 feeder cells stimulates epithelial cell growth (Barreca *et al.*, 1992) better than condition A no-3T3.

Morphological assessment of limbal-corneal epithelial cells grown with 3T3 feeder cell conditioned medium was carried out during culture period. A compact round epithelial cell morphology was observed at day 5, which is similar to the condition C Bot-3T3, with monolayer formation by day 8, the end cultures morphology was similar to that of condition C Bot-3T3 with compact tight cell-cell contact morphology with 1-2 cell thick sheet (**figure 3.9.2**). The colony forming efficacy (CFE) of limbal-corneal epithelial cells grown in conditioned medium was superior to condition A no-3T3 (**figure 3.9.3**), whereas condition C Bot-3T3 has shown the highest number of CFE compare to conditioned media, suggesting the importance of a continuous supply of 3T3 feeder cell. These findings are in contrast to the observations made by Miyashita *et al.*, that soluble factors are not sufficient to propagate colony forming progenitor cells, where his observations were made in relation to cell suspension culture system, where limbal-corneal epithelial cells lack their own niche from stroma of limbal tissue. In the current study use of limbal explants cultures maintain the stem cell population in presence of 3T3 feeder cells or conditioned medium and limbal tissue niche (high number of CFE in conditioned medium compared to condition A no-3T3).

These preliminary results suggest the importance of feeder cell conditioned media or separate feeder cell system to limbal-corneal epithelial cell proliferation and migration, where conditioned medium from 3T3 feeder cells can be used in place of 3T3 feeder cells in contact with limbal-corneal epithelial cells to maintain the stem cell niche (High CFE).

This was only a short pilot study and it is possible that with more extensive study refinements could identify key growth factors in the conditioned media important for maintenance of limbal-corneal epithelial stem-ness.

#### 4.10 Summary of immunofluorescence for differentiation markers

Marker	Cell culture inserts						Denuded Human Amniotic Membrane (dHAM)					
	Condition A		Condition B		Condition C		Condition D		Condition E		Condition F	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
<b>CK3</b>	+++	++ +	+++	++	+++	++	+++	+	+++	++	+++	+
<b>CK12</b>	++	+	++	+	+++	++	++	++	++	++	+	+
<b>E-cadherin</b>	+++	++ +	+++	++ +	+++	+++	+++	++ +	+++	++ +	+++	++ +

-, not present; +, mild; ++, moderate; + + +, severe.

#### 4.10.1 Summary of immunofluorescence for Stem cell markers

Marker	Cell culture inserts						Denuded Human Amniotic Membrane (dHAM)					
	Condition A		Condition B		Condition C		Condition D		Condition E		Condition F	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
<b>CK19</b>	++	+	+++	++ +	+++	++	+++	+	+++	++	+++	+
<b>CK14</b>	++	+	++	+	ND	++	+++	++ +	+++	++ +	ND	++
<b><math>\Delta</math>Np63a</b>	-	++	+	++ +	++	+++	+	++ +	++	++ +	+	++ +
<b>ABCG2</b>	+	+	+	++	++	+++	+	+	++	++	++	++ +
<b><math>\alpha</math>-2 Integrin</b>	+	++	+	++ +	+	+++	+	++	++	++ +	++	++ +
<b><math>\beta</math>-1 Integrin</b>	+	++	++	++ +	++	+++	++	+	++	++	+	++

-, not present; +, mild; ++, moderate; + + +, severe, ND; no data

#### 4.10.2 Summary of western blot analysis for differentiation markers

Marker	Cell culture inserts			Denuded Human Amniotic Membrane (dHAM)		
	Cond.A	Cond.B	Cond.C	Cond.D	Cond.E	Cond.F
CK3	+++	+++	+++	+++	+++	+++
CK12	++	++	+	++	+	+
Connexin 43	+++	+	+	++	+	+
E-cadherin	+++	+++	++	+++	++	+++
Occludin	+++	+	+	++	+	+
ZO1	+++	+	+	++	+	+

-, not present; +, mild; ++, moderate; + + +, severe

#### 4.10.3 Summary of western blot analysis for stem cell markers

Marker	Cell culture inserts			Denuded Human Amniotic Membrane (dHAM)		
	Cond.A	Cond.B	Cond.C	Cond.D	Cond.E	Cond.F
CK19	++	++	++	++	++	++
CK14	+	++	++	++	++	++
$\Delta$ Np63 $\alpha$	+	++	+++	+++	+++	+++
p63	++	++	++	++	++	++
ABCG2	+	++	+++	++	+++	+++
$\alpha$ -2 Integrin	+++	+++	+++	+++	+++	+++
$\beta$ -1 Integrin	+	++	++	++	++	++

-, not present; +, mild; ++, moderate; + + +, severe

#### 4.10.4 Summary of CFE assay for limbal-corneal epithelial cells

Culture Type	Cell Culture Inserts			Denuded Human Amniotic membrane (dHAM)		
	Condition A	Condition B	Condition C	Condition D	Condition E	Condition F
% OF CFE	0.42	1.17	2.32	5.25	8.92	7.2
$\pm$ SD	$\pm$ 0.00	$\pm$ 0.24	$\pm$ 0.3	$\pm$ 0.19	$\pm$ 2.18	$\pm$ 1.96

Values are mean  $\pm$ SD, n=6.

#### 4.10.5 Concluding remarks

In this study, the preliminary experiments were conducted to establish limbal-corneal epithelial cell culture with cell suspension and explants culture systems with various substrates and feeder cell support (**table 3.1**). In this short pilot study, the explants culture system with 3T3 feeder cell support was the best condition in terms of success rate (growth rate), cell morphology and marker expression.

In 1977 Sun and Green were the first to demonstrate that corneal epithelial cells could also be successfully cultured and sub-cultured using 3T3 feeder cells (Sun and Green, 1977). The exact role of 3T3 fibroblasts in the culture is not completely understood. It is possible that 3T3 feeder cells either lay down an extracellular matrix that promotes epithelial culture, or that they release factors into the medium that promote cell growth and expansion or through direct cell-to-cell contact between the 3T3 fibroblasts and the epithelial cells (Miyashita *et al.*, 2008, Sudha *et al.*, 2008).

However, not much success has been achieved in terms of understanding the basic biology of these stem cells. The question of whether the limbal stem cells maintain their undifferentiated state even in simulated *in vitro* conditions remains unanswered. With the tremendous potential of these stem cells, it becomes all the more important to characterize *in vitro* expanded limbal epithelial cells. The cells could be characterized based on morphology and antigenic expression.

The technique of limbal epithelial expansion investigated in this study is the limbal explants method that was established during preliminary experiments, which involves the use of intact small limbal biopsies plated on cell culture inserts, and denuded amniotic membrane (dHAM) with/without 3T3 feeder cells.

Recently Kolli *et al.*, demonstrated the importance of limbal tissue niche in maintenance of stem cell population in culture system (Kolli *et al.*, 2008) where the loss of stem cell characteristics appeared when cells migrate away from the explants. Taking cues from studies of explants culture systems and 3T3 feeder layer systems, the purpose of this study was to examine the effect of 3T3 feeder cells in different culture setups, and to analyze limbal stem cells and their properties to provide further knowledge of limbal stem cells. This study used cell culture inserts; this culture-insert method enables the additional use of 3T3 feeder cells within the culture, which can be placed on the cell culture inserts (Co-3T3) and below the culture insert and amniotic membrane (Bot-3T3).

In conclusion, out of six culture systems, condition A no-3T3 system showed highest level of differentiation cells with large size, hexagonal/polygonal morphology, and 1-2 cells thick, showed high expression of limbal- corneal differentiation marker CK12, Cx43, ZO1, and low expression of putative stem cell markers with low percentage of CFE (0.42%). Whereas the morphology of limbal-corneal epithelial cells on dHAM without 3T3 feeder cells is small compact with clear cell-cell gap, 2-3 cell thick cell sheet has formed with high expression of stem cell makers like ABCG2,  $\Delta$ Np63 $\alpha$ , low expression of differentiation marker CK12 at protein and mRNA level, and high percentage of CFE (5.25 $\pm$ 0.19%). The increase in stem

cell population/CFE in limbal-corneal epithelial cells even in absence of Ir-3T3 feeder cells is possibly due to the presence of extracellular matrix HAM, which is a suitable substrate containing collagen IV, laminin and fibronectin (Cooper *et al.*, 2004, Grueterich *et al.*, 2003b) for culture of limbal-corneal epithelial cells.

Higher percentage of stem cells in explants culture system in condition C Bot-3T3 compared to condition B Co-3T3 was observed, this suggests that feeder cells in contact with limbal-epithelial cells have some effect on maintenance of stem cell niche. Condition C Bot-3T3 cells show a high percentage of CFE ( $2.32 \pm 0.3\%$ ), we believe that growth factors provide a driving force for many cellular processes, it is important to identify the specific factor required for the requisite approach. The increased percentage of CFE and high expression level of stem/progenitor markers ABCG2,  $\Delta Np63\alpha$  in condition C Bot-3T3 in cell culture inserts could be a paracrine effect of 3T3 cells, along with diffusion factors, between limbal-corneal epithelial cells and 3T3 feeder cells. Gradients of growth factors play a key role in culture system, as they provide a mechanism by which cells can obtain spatial and directional cues. To form a gradient, a growth factor is released from a localised source (e.g., specific cells), and as it diffuses it is degraded or bound to the matrix, typically resulting in steep, local gradients. A population of nearby and distant cells that are capable of responding to the signalling molecule in a concentration dependent way can sense the concentration gradient.

The possible hypothesis for the expression of low level of stem cell marker in condition B Co-3T3 compared to condition C Bot-3T3 is due to cell-cell contact of limbal-corneal epithelia cells with 3T3 feeder cells. The plasma membrane of 3T3 cells or glutaraldehyde-fixed 3T3 cells has been reported to support the growth of epithelial cells seeded at a high density (Yaeger *et al.*, 1991), although epithelial cells seeded at low clonal densities cannot

form colonies when in contact with non-viable 3T3 cells (Tseng *et al.*, 1996). Therefore, direct cell-to-cell contact and soluble factors secreted by viable feeder cells both seem to be involved in maintaining epithelial cells in an undifferentiated state, which was observed in this study in condition B Co-3T3, but with lower colony forming efficacy compared to condition C Bot-3T3. Thus, the possible reasons for the expression of higher progenitors could be multi factorial such as stem cell niche from limbal explants and spatial gradient sensing mechanisms, their sensitivity to the relative steepness of the gradient. Which typically express as the percentage difference in chemoattractant, concentration across a cell, as well as their dependence on the absolute (midpoint) chemoattractant concentration (Schneider and Haugh, 2005, Schneider and Haugh, 2006). In conclusion, these experiments define the critical role of 3T3 feeder cells in culture system in relation to location (in contact and away from limbal-corneal cells) of growth factor secretion gradient. The preliminary short study demonstrated the importance of 3T3 feeder cell within the culture system (Bot-3T3) compared to conditioned media that supplies growth factors in controlled manner due to constant communication between limbal-corneal epithelial cells and 3T3 feeder cells.

This study demonstrated that denuded human amniotic membrane (dHAM) alone with explants culture is superior to limbal-epithelial cells grown on plastic with Ir-3T3 feeder cells, for maintenance of sufficient stem cell population (CFE  $5.25 \pm 0.19\%$ ). As well as low expression of differentiation markers CK3 and CK12, compared to other published data with cell suspension culture system on human amniotic membrane (Koizumi *et al.*, 2002).

In contrast to condition B Co-3T3 with low stem cell/progenitors compared to condition C Bot-3T3, limbal-corneal epithelial cells grown with condition E dHAM Co-3T3 showed the highest number of stem/progenitors cells (CFE  $8.95 \pm 2.18\%$ ) with 3T3 in contact with limbal-

corneal epithelial cells compared to condition F dHAM Bot-3T3. This difference could be related to substrate and 3T3 feeder cell influence on limbal-corneal epithelial cells in condition E dHAM Co-3T3, and separation of 3T3 feeder cells in dHAM Bot-3T3, where a possible effect of exchange of growth factors between limbal-corneal epithelial cells and 3T3 feeder cells due to thick extra cellular matrix (HAM). In contrast to the direct contact with 3T3 fibroblast feeder layers without a direct contact, co-cultured 3T3 fibroblasts are still effective in delaying epithelial differentiation by limbal epithelial cells seeded on denuded AM (Grueterich *et al.*, 2003b). A duplex of 3T3 fibroblast feeder layers has been used to promote CK15-expressing corneal epithelial cells (Miyashita *et al.*, 2008). These results suggest that soluble factors derived from 3T3 fibroblasts might be involved in promoting niche regulation of limbal SCs.

Thus, it is believed that the explants culture system with dHAM in presence of Ir-3T3 feeder cells, condition E dHAM Co-3T3 is the best culture system for the maintenance of the stem cell niche and the best system to study stem cell biology *in vitro*. However, when it comes to clinical applications dHAM with explants culture system is the best suitable condition as it maintains its stem cell niche in presence of explants (own niche) *in vitro*, as described by other groups (Kolli *et al.*, 2008, Grueterich *et al.*, 2003a, Sangwan *et al.*, 2005, Sangwan *et al.*, 2003a, Sudha *et al.*, 2008). Moreover, these findings have important practical implications in terms of improving/maximising the limbal-corneal epithelial culture system for high stem cell populations, by paracrine effect and maintenance of original niche from tissue.

*In vitro* expansion of limbal explants on HAM in the treatment of LSCD represents a successful stem cell therapy in the field of regenerative medicine. Although limbal stem cell

(LSC) numbers can be successfully expanded using such techniques, this study demonstrated the stem cell properties of outgrowths in six different culture systems in a single study.

The study of six different culture systems enabled us to understand the importance of 3T3 feeder cells and dHAM, and showed an improved level of stem cell population in dHAM and dHAM with 3T3 feeder cells, where these conditions easily replicate *in vitro* and thus make the process of culturing limbal stem cell more efficient.

These findings have important practical implications in terms of improving the surgical technique to maximize limbal stem cell (LSC) delivery and location during transplantation in addition to improving current techniques of LSC expansion, which will lead to higher rates of stem cell engraftment and therefore improved clinical outcomes. Thus, these findings are forecasting the possibility of generating corneal epithelial sheets for transplantation under better defined, standardised conditions without the use of animal material (3T3 feeder cells).

#### **4.11 Limbal-corneal epithelial cultures translation to clinic**

Human amniotic membrane (HAM) has been used successfully in ocular surface reconstruction as a biological bandage and as a substrate for stem cell expansion (Kim and Tseng, 1995, Shimazaki *et al.*, 1997, Tsubota *et al.*, 1996, Tseng *et al.*, 1998). The combination of anti-inflammatory, antimicrobial, antiviral, antifibrotic, and antiangiogenic properties (Solomon *et al.*, 2001, Tseng *et al.*, 1999, Talmi *et al.*, 1991, Hao *et al.*, 2000, Akle *et al.*, 1981) of HAM provides a favourable environment for cellular attachment and expansion in both *in vivo* and *in vitro* settings.

Culturing cells on the human amniotic membrane (HAM) does not prove to be easy especially since the membrane is thin, needs correct orientation and has a tendency to curl and wrinkle prompting differentiation of the cultured cells. Allogeneic tissue has an implicit risk of infectious disease transmission. In general, amniotic membrane is procured from potential donors undergoing caesarean section that have been screened for communicable disease, such as HIV, hepatitis and syphilis. The placenta is cleaned with a mixture of balanced salt solution, penicillin, streptomycin, neomycin and amphotericin B.

The general procedure for HAM preparation is that the amnion separated from chorion by blunt dissection under sterile conditions, and attached to the nitrocellulose paper strips and stored in glycerol solution. The tissue is either stored in that solution for fresh use or cryopreserved at a temperature of  $-80^{\circ}\text{C}$  (Sangwan *et al.*, 2007). However, this is not without

complications of potential contamination during processing due to the large amount of buffers and materials.

Thus, proper and reliable sterilization of HAM is vital to render it completely safe for clinical applications (Nakamura *et al.*, 2006a). In conclusion, although AMT is a safe procedure, some serious biological and logistical problems remain, such as the deficiency of appropriate sterilization and difficulties in transport and storage. Thus, many important assays have been suggested for environmental monitoring as well as to standardise microbiological controls in stem cell banks to prevent contamination (Pessina *et al.*, 2008).

Recently commercially available HAM products, such as AmnioGraft and ProKera (Bio-Tissue, Inc, Miami, Florida) have been used, which are cryopreservation at  $-80^{\circ}\text{C}$  following harvesting to render amniotic epithelial cells nonviable and the tissue nonimmunogenic (Fernandes *et al.*, 2005). This approach has two main drawbacks, namely incomplete sterilisation with the possibility of disease transmission and the need for an expensive and cumbersome deep freeze. In the literature, few studies have shown the use of freeze-dried AM for ocular surface reconstruction (Nakamura *et al.*, 2004c, Jang *et al.*, 2006, Lim *et al.*, 2010).

To overcome the risk of infections in cultured limbal-corneal epithelial cells in the clinic, in this study we checked the feasibility of using some of commercially available human amniotic membranes for culture of limbal-corneal epithelial cells.

Commercially available HAM used in this study

Trade Name	Properties
<b>Acelagraft™</b>	Amniotic membrane with decellularized, leaving a product composed of a complex collagen-based matrix, with 25 micron thick.
<b>AmbioDry2™</b>	Human amniotic membrane consists of collagen layers including basement membrane, and stromal matrix. With 40µm thick.
<b>Ambio5™</b>	Processed for full thickness by RETAINING additional collagen matrices from the placental interface. Cells are devitalized and Nominal thickness: 110+ microns.
<b>EpiFix™</b>	The multi-layer structure of EpiFix™ is comprised of an epithelial layer and two fibrous connective tissue layers.

#### 4.11.1 Acelagraft™

Acelagraft™ (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey) is a commercially available de-cellularised and dehydrated HAM (DDHAM) product. The tissue is sterilized by  $\gamma$ -irradiation and is acellular and freeze dried, with a thickness of 25 microns. Initial culture runs were performed using the Acelagraft™ human amniotic membrane.

The culture protocol used did not use trypsin on the membrane as the membrane appeared to be denuded of epithelial cells. In all other aspects the culture protocol followed was the same as for fresh frozen amniotic membrane (explants method). The appearance of cells and the growth rate appeared to be similar to that obtained with denuded human amniotic membrane (**figure 3.10**). Marker expression of CK3 and  $\Delta$ NP63 $\alpha$  appeared to be similar to that obtained from cultures on dHAM (**figure 3.10.1**).

However, the culture of limbal-corneal epithelial cells on the Acelagraft™ does not prove to be easy, especially since the membrane is thin, and observation of limbal-corneal epithelial cell growth on membrane was technically difficult due to undulations (**figure 3.10**) of membrane created during freeze dried process. This could lead to false positives in detection/identification of growth rate during culture period. Currently this membrane is no longer available on the market.

#### 4.11.2      **AmbioDry2™**

As the Acelagraft™ product is no longer commercially available, AmbioDry2™, a dried amniotic membrane 40µm thick was tested. The product specification noted that this was processed and bioburden and potential virulency were removed, though the mechanism of how this was achieved is not specified. The resulting membrane still contains intact but devitalised epithelial and fibroblast cells (**figure 3.10.2**).

The initial test run was with an intact AmbioDry2 that was rehydrated in growth media. No obvious growth was obtained. There was a complete epithelial layer present on AmbioDry2, this epithelial layer could be removed using trypsin (**figure 3.10.2**). Immunofluorescence analysis for E-cadherin (**figure 3.10.2.1**), demonstrated considerable staining from the devitalised amniotic epithelial cells.

A second experiment was performed in which AmbioDry2™ was rehydrated in 0.9% saline or rehydrated in growth media. Both rehydrated conditions were further subdivided to yield four culture conditions:

- 1)      Saline rehydrated
- 2)      Saline rehydrated de-epithelialised with trypsin-EDTA
- 3)      Growth media rehydrated
- 4)      Growth media rehydrated de-epithelialised with trypsin-EDTA

No growth was observed in condition 1- saline rehydrated and condition 3- growth media rehydrated. Limbal-corneal epithelial growth was observed in condition 2- saline rehydrated de-epithelialised with trypsin-EDTA and condition 4 growth media rehydrated de-epithelialised with trypsin-EDTA (**figure 3.10.2.3**).

Thus, the presence of the epithelial cells on the AmbioDry2™ product was deemed to be inhibitory to limbal-corneal epithelial outgrowth from the explants, and AmbioDry2™ product was a very thin membrane and difficult to manipulate during the de-epithelialisation process. The use of commercially available membranes in this process is to reduce process protocol to avoid steps like trypsinisation, washing steps, whereas AmbioDry2™ requires the process of trypsin treatment, and this membrane seems to be unsuitable for good epithelial growth as observed this study.

A thicker version of AmbioDry5™ membrane was tested for culture of limbal-corneal epithelial cells.

#### **4.11.3 AmbioDry5™**

AmbioDry5™ is approximately 110µm thick. It is similar to the AmbioDry2™ product as it contains devitalised epithelial cells on the membrane surface, an intact dense basement membrane, the presence of a loose collagen layer with fibroblasts but also contains an additional thick layer of retained collagen from the placental interface (**figure 3.10.3**). Due to the thickness of the membrane and presence of fibroblast layer, this membrane was treated with trypsin EDTA.

The AmbioDry5™ was divided to yield two different culture conditions:

- 1) Rehydrated in 0.9% saline and de-epithelialised with trypsin-EDTA.
- 2) Rehydrated in growth media and de-epithelialised with trypsin-EDTA.

Growth was observed in the AmbioDry5™ culture conditions and positive staining for the CK3 and 12, and stem cell marker  $\Delta$ Np63 $\alpha$  was obtained (**figure 3.10.3.2 to figure 3.10.3.3**).

The limbal–corneal epithelial cells grown on AmbioDry5™ are similar in morphology that appeared on fresh frozen HAM, with compact epithelial cells on basal layer, and large stretched cells on apical layer. Marker profile was similar as appeared on fresh frozen HAM with expression of CK3, CK12 and stem cell marker  $\Delta$ Np63 $\alpha$ .

However, both versions of AmbioDry required de-epithelisation with trypsin for growth of limbal-corneal epithelial cells, considered as an additional step under Good Manufacture Practices (GMP) process. For use of commercially available membrane, our aim is to minimise culture manipulations or procedure steps in culture of limbal-corneal epithelial cells at GMP level.

#### 4.11.4 EpiFix™

To avoid the use of trypsin in the GMP process, the human amniotic membrane product, EpiFix™ was investigated. This membrane is already de-epithelialised. However, the product specification on the company website states that it contains a single layer of epithelial cells, basement layer, compact layer and a fibroblast layer.

In the initial experiment using Epifix the membrane was divided in two to yield two culture conditions

- 1) Rehydrated with Saline and treated with trypsin EDTA
- 2) Rehydrated with growth media and treated with trypsin EDTA.

Growth was observed in both conditions (**figure 3.10.4**). The morphology of limbal-corneal epithelial cells grown on this membrane was not clearly visible under phase contrast microscope due to the hazy nature of membrane. These cultures stained positive for the cornea markers CK3 and CK12; epithelial morphology of these cultures was not satisfactory, as cells did not appear to be in thick compact epithelial sheet formation (**figure 3.10.4.1**). These cells were positive for stem cell marker  $\Delta Np63\alpha$  with sporadic appearance, and counter stain of E-cadherin showed large epithelial cell morphology with no cell-cell boundary stain (**figure 3.10.4.5 and 6**).

According to the manufacturer's instructions, EpiFix membrane is de-epithelialised, by considering manufacture instructions, a repeat experiment was performed without de-epithelialisation, with just rehydration of the membrane with 0.9% saline.

Explants showed growth of epithelial cells by day 3, whereas limbal-corneal epithelial cells were not growing further after day 8 to form good epithelial sheet (**figure 3.10.4.7**). Technically, it was difficult to observe cell growth on the membranes and close examination ultimately showed slow and non-uniform growth on membrane. It is possible that these membranes may not be the best substrate to culture epithelial cells, and freeze dried processing of membranes might lead to the loss of growth factors or key ECM that appear on native human amniotic membrane, which act as key factors for growth of epithelial cells.

In conclusion, the commercially available freeze-dried human amniotic membranes are not greatly suitable for the culture of limbal-corneal epithelial cells compared to fresh frozen human amniotic membrane, due to the thickness of membrane, presence of intact freeze-dried epithelial/stromal layer on membrane. Commercially available membranes are applicable to ocular surface disorders without culture of any epithelial cells. Due to the technical difficulty in handling commercially available membranes and inconsistency in growth on epithelial cells/limbal stem cells on these membranes, we adopted the fresh frozen amniotic membrane to culture limbal-corneal epithelial cells for clinical applications.

Szurman *et al.*, have illustrated the difficulties of membranes thickness, their propensity to tear during suturing and the time consuming manual realignment of the amniotic membrane after each individual stitch (Szurman *et al.*, 2006). Furthermore, it has been noted that some degree of graft shrinkage does occur, leading to puckered margins with indentations at suture

points. The high translucency of the membrane also poses difficulty and poor visualisation of the membrane edges in the surgical field has lead a number of groups to investigate staining properties of the membrane (Kandavel and Chuck, 2005), but it was not ideal and questions arose about the toxicity of the dyes used.

In order to address all of these issues with the membrane that culturing and transplantation, we developed a protocol for the culture of limbal-corneal epithelial cells on commercially available fresh frozen human amniotic membrane, without 3T3 feeder cell system. The protocol that was taken forward for development of a product under good manufacture practices (GMP) conditions, for clinical trial was fresh frozen HAM with de-epithelialisation.

The GLP culture process used shows categorically that a system of *in vitro* expansion of limbal tissue can be used to produce large sheets of cells, which can successfully regenerate the corneal epithelium. Analysis of the expanded sheets at the termination day showed the epithelium with a basal layer of cells have limbal stem cell like morphologic appearance and expressed a signature of putative limbal stem cell markers. These findings are in agreement with other laboratory (Grueterich *et al.*, 2002a, Grueterich *et al.*, 2003a, Koizumi *et al.*, 2001b, Sangwan *et al.*, 2003b, Shortt *et al.*, 2008) analysis of *in vitro* expanded cultures using explants cultures on dHAM.

In conclusion, the commercially available freeze dried HAMs do not reliably support the growth of limbal-corneal epithelial cells compared to fresh frozen human amniotic membrane.

#### 4.12 Introduction to limbal 'fibroblast-like' cells (LFLc)

The limbus of the eye, a tissue at the junction of the cornea and conjunctiva of the ocular surface, is now extensively used for ocular surface resurfacing in patients with limbal stem cell deficiency (LSCD) (Pellegrini *et al.*, 1997, Sangwan *et al.*, 2003b, Sangwan *et al.*, 2003a, Sangwan *et al.*, 2005, Schwab *et al.*, 2000b). The extensive work on limbal stem cells has led to some interesting observations, like the presence of spindle shaped cells. A number of studies with limbal stem cells cultures reported the presence of spindle shaped cells, which are believed to be similar to mesenchymal cells (Choong *et al.*, 2007, Uchida *et al.*, 2005, Yamagami *et al.*, 2007, Amano *et al.*, 2006, Dravida *et al.*, 2005, Polisetty *et al.*, 2008).

Corneal keratocytes are easily obtained and grown by any standard culture methods (Masur *et al.*, 1993, Beales *et al.*, 1999). Enzymatic action will release stromal cells as well as desired epithelial cells and both cell types will grow from the explanted full-thickness corneal piece (He and McCulley, 1991, Eggl *et al.*, 1989). Many groups successfully isolated multipotent, fibroblast-like cells from limbal and corneal stroma. (Dravida *et al.*, 2005, Polisetty *et al.*, 2008, Perrella *et al.*, 2007, Yamagami *et al.*, 2007, Du *et al.*, 2007, Du *et al.*, 2005).

Multipotent fibroblasts isolated from human cornea/limbal tissue that have been induced to differentiate *in vitro* into epithelial cells of the cornea. These cells were shown to possess stem cell like self-renewal property with plasticity *in vitro* (Dravida *et al.*, 2005, Choong *et al.*, 2007, Lu *et al.*). Similarly, Yoshida *et al.*, isolated a subset of cells termed neural crest

derived corneal precursors (COPs) from stromal cells of adult mice. These cells showed side population characteristics, were multipotent, clonogenic (sphere forming), and expressed various adult stem cell markers (nestin, Notch-1, Musashi-1, ABCG2) (Yoshida *et al.*, 2005). However there are no further reports which would throw light onto the origin of these cells and their lineage and extensive study in trans-differentiation of limbal fibroblast-like cells to corneal epithelial-like cells.

Taking cues from the above studies, a study was conducted to investigate if the limbal 'fibroblast-like' cells (LFLC) were of mesenchymal origin and if they behave similarly to mesenchymal stem cells in terms of differentiation. Thus, the present study aimed at extensive characterization of the limbal 'fibroblast-like' cells (LFLC) for a battery of mesenchymal and epithelial markers, cell behaviour for proliferation, migration, invasion, cell attachment/adhesion, and trans-differentiation potential of limbal 'fibroblast-like' cells to adipocytes and osteocytes.

In system *in vivo* the healing of stromal wounds is mediated by the activation of quiescent fibroblasts, migration of the activated fibroblasts to the site of injury, synthesis of new extracellular matrix (ECM), wound contraction, and ECM remodelling (Garana *et al.*, 1992). So far, limited *in vitro* studies are available on corneal or limbal fibroblast-like cells. In this thesis, LFLC were examined for migration, invasion and cell attachment/adhesion assay.

#### **4.12.1 Isolation and characterisation of limbal fibroblast-like cells**

Limbal ‘fibroblast-like’ cells were isolated from limbal explants, (section 2.11), with mechanical dissociation of limbal tissue after scraping the epithelial layer away from the stromal part. Initial growth of fibroblast cells started at day 8, with confluent cultures by day 20, cells had typical fibroblast morphology with stretched ends (figure 3.11), and parallel alignment in confluent culture (a typical fibroblast character). Adherent cells were sub-cultured once they attained confluence of 80%. Even after eight passages, cells were observed to maintain the spindle-shaped, fibroblast morphology seen using Wright-Giemsa staining. Fibroblast-like cells lost the spindle-shaped morphology at passage 15 and appeared larger cell body. The culture method used in this study is different from other groups in terms of subpopulation or group of cells with cell sort analysis, as described by another group (Dravida *et al.*, 2005). OR by enzymatic isolation of limbal fibroblast cells described by other groups (Du *et al.*, 2005, Majo *et al.*, 2008, Masur *et al.*, 1993, Polisetty *et al.*, 2008).

#### 4.12.2 Proliferation of limbal ‘fibroblast-like’ cells (LFLc)

Limbal fibroblast-like cells had high proliferation rates reaching confluence in 3, 5 and 7 days at low passage number and decreased as passage number increased (**figure 3.12**), the observation of a decrease in proliferative capacity of cells with increasing passages with rigorous growth at passages 2 and 4, proliferation rate decreased at passage 8 and 9 with changes in morphology from small spindle-shaped with stretched end to broad cell bodies and long thin cytoplasmic extensions, and no/poor proliferation at passages 13 and 15. These finding suggest that long-term cell growth (high passage number) *in vitro* is a more sensitive method to detect subtle changes in the kinetics of proliferation of the cell population compared with short-term cultures (early passage number).

#### 4.12.3 Limbal fibroblast-like cells (LFLc) migration/motility assay

*In vivo* stromal fibroblasts promote corneal neovascularisation (NV) through their proangiogenic matrix metalloproteinase (MMP) activity by three possible mechanisms: (1) breakdown of the extracellular matrix (ECM); (2) degradation of corneal antiangiogenic factors; and (3) transcriptional up-regulation of vascular endothelial growth factor (VEGF) (Onguchi *et al.*, 2009). Whereas motility or migration of limbal fibroblast-like cells are not studied extensively in literature, these studies showed high motility rate of LFLc with slight decrease in higher passage number (**figure 3.13**).

It is widely accepted that the major events of cell migration are extension of a leading edge, establishment of new adhesion sites at the front, cell body contraction, and detachment of adhesions at the cell rear (Lauffenburger and Horwitz, 1996) and it is well known that the small GTPases Rho and Rac are prime candidates for regulating the cytoskeletal and mechanical phenotype of fibroblasts during cell migration (Grinnell, 2000, Chrzanowska-Wodnicka and Burridge, 1994). Taken together, the short study of LFL cell migration suggests that the impact of these cells could have on ECM architecture during migration possibly depend on the balance of Rho and Rac activation induced by the cytokines to which they exposed or induced *in vitro*. Thus this mechanical plasticity of motility may ultimately make it possible to modulate this important aspect of cell behaviour during *in vivo* corneal wound healing. However, a detailed study on the role of GTPases Rho and Rac factors of fibroblasts requires understanding of wound healing mechanism in LFL cells.

#### 4.12.4 Limbal fibroblast-like cells (LFLc) invasion assay

An important step in human corneal wound healing is the invasion of activated keratinocytes into cell-free areas, initiated by apoptosis of keratinocytes in the stroma surrounding the lesion (Szerenyi *et al.*, 1994, Wilson *et al.*, 2001). Studies have shown that growth factors (PDGF, EGF and bFGF) increase the migration and chemotaxis of corneal fibroblasts (Kim *et al.*, 1999, Andresen *et al.*, 1997) *in vivo*. An intra-stromal invasion with limbal epithelial cells was studied by one group who concluded that epithelial-mesenchymal transition via the Wnt/ $\beta$ -catenin pathway influences the fate of limbal epithelial cells, likely to be progenitor cells, between regeneration and fibrosis when the stromal niche is activated (Kawakita *et al.*, 2005).

While the *in vitro* invasive property of limbal ‘fibroblast-like’ cells (LFLc) were not studied to date (*in vitro* and *in vivo*), we have studied invasiveness of LFLc. This experiment demonstrated high invasiveness of LFLc at initial passage and decrease invasiveness at higher passage number compared to a positive invasive cell line BT20 (**figure 3.14**). In this study, proliferation experiments (**section 4.12.2**) showed that LFLc have decreased proliferative capacity with higher passage number, this could be the reason for the decrease in invasiveness of LFLc in higher passage cells. This interesting finding could be the involvement of complex process in invading cells through extra cellular matrix (ECM), that affect the invasion of cells with higher passage number, whereas an *in vitro* setup for migration assay involved the movement of cells through particular pore size (8 $\mu$ ) under gradient conditions.

Studies have shown the importance of corneal epithelial cells in wound healing and invasion *in vitro* (Wang *et al.*, , Buss *et al.*, , Yoshioka *et al.*, , Hu *et al.*, 2009) and *in vivo* (Morishige *et al.*, , Chen *et al.*, 2009, Pal-Ghosh *et al.*, 2008, Mazzotta *et al.*, 2008). It could be argued that the primary culture of LFL cells could stimulate the wound healing response for injury.

#### 4.12.5 Senescence-associated $\beta$ -galactosidase (SA- $\beta$ -gal) assay

The senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) assay is based on a senescence-induced increase in levels of lysosomal  $\beta$ -galactosidase (Dimri and Campisi, 1994, Dimri *et al.*, 1995). In non-senescent cells, the lysosomal hydrolase  $\beta$ -galactosidase cleaves galactose from glycoproteins at an optimum pH of 4.0 to 4.5. Lysosomal  $\beta$ -galactosidase activity can be detected in most mammalian cells by performing a cytochemical assay at pH 4.0 in which cleavage of Xgal by  $\beta$ -galactosidase leads to the formation of a blue precipitate (Kurz *et al.*, 2000).

Senescence *in vitro* involves multiple changes due to inducers like DNA damage (Di Leonardo *et al.*, 1994, Robles and Adami, 1998), expression of supraphysiological mitogenic signal and genetic or pharmacological manipulations that open or de-condense chromatin (Serrano *et al.*, 1997, Zhu *et al.*, 1998). One change is often not a sufficient criterion for judging senescence in culture, senescence of many differentiated cells attributes to a decrease in functionality.

To investigate the mechanism of decreased maximal proliferative potential and invasion of LFL cells, these cells were examined for the presence of senescence markers. A senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) assay showed that, the number of SA  $\beta$ -gal<sup>+</sup> LFL cells increased from early- to late-passage cells, with increased rate of accumulation of SA  $\beta$ -gal in cultures (**figure 3.15**).

In summary limbal 'fibroblast-like' cells are highly motile, compared to negative control in motility experiments, suggesting that these cells might have an active role in the wound healing process, whereas the invasiveness of the cells decreased as passage number increased *in vitro*, and an increase in senescent cells in high passage cultures might suggest the importance of early passage cells for *in vitro* experiments. Understanding limbal 'fibroblast-like' cell (LFLc) interactions with corneal epithelial cells, as well as elucidating the behaviour of these cell types under physiological and pathological conditions might greatly increase our knowledge on corneal wound healing and regeneration.

#### 4.12.6 Extra cellular matrix (ECM) adherence assay

Limbal fibroblast-like cells were tested for attachment assay with various extracellular matrix (ECM) molecules at a time of 60 minutes with 10µg/ml, (collagen IV, Fibronectin (FN), Laminin, and combinations of Collagen IV+ FN, Collagen IV+Laminin and Collagen IV+Laminin+ FN). Collagen IV and Laminin are major basement membrane components of the limbal and corneal epithelia (Ljubimov *et al.*, 1995, Marshall *et al.*, 1993, Ihanamaki *et al.*, 2004). Fibronectin is an ECM glycoprotein that is deposited in the ECM in response to corneal epithelial wound healing and promotes epithelial adhesion and migration (Fujikawa *et al.*, 1984, Nishida *et al.*, 1992, Murakami *et al.*, 1992). Thus, in this study we investigated the single ECM and combinations of collagen IV, laminin and FN, for their suitability in attachment assay.

The limbal ‘fibroblast-like’ cells (LFLc) showed high affinity for FN compared to collagen IV, laminin (**figure 3.16**). This result is consistent with other studies which demonstrated that fibroblast cells have high affinity to FN and vitronectin (Wayner *et al.*, 1991, Charo *et al.*, 1990). The possible reason might be that limbal ‘fibroblast-like’ cells (LFLc) were removed from their collagen-rich environment and cultured *in vitro* with fetal bovine serum, which is rich in FN, vitronectin and growth factors (Zieske *et al.*, 1987, Masur *et al.*, 1993).

The cellular attachment/adhesion assay of limbal fibroblast-like cells showed the importance of different ECMs in adhesion of fibroblast like cells. An important observation is that laminin was found to be a poor substrate for the attachment of LFLc. In contrast, LFL cell

attachment was found to be high in fibronectin (FN). Fibronectin is known to induce cell spreading, the formation of stress fibres', and the establishment of focal adhesions containing paxillin in corneal fibroblast cells cultured in collagen gels (Liu *et al.*, 2006b). Type VI collagen was found to be a poor substrate for attachment of the limbal fibroblasts, despite the fact that type VI collagen is known to contain as many as 11 Arg-Gly- Asp sequences, which are high affinity ligands for the  $\alpha 5\beta 1$  integrin (Tervo *et al.*, 1991).

LFLc has shown highest affinity to the combination of collagen IV, laminin and FN, the combination of ECMs might be better application in proliferation, migration, differentiation of LFLc *in vitro*. Demonstration of cells with the capability of generating human limbal fibroblast-like cells *in vitro* is novel and has significant implications for corneal cell-based therapy and tissue engineering.

#### 4.12.7 Cellular characterisation of limbal fibroblast-like cells (LFLc)

Many groups have isolated corneal /limbal stromal cells and characterised them with a variety of markers to identify the origin of these cells in comparison with bone marrow mesenchymal cells (Polisetty *et al.*, 2008, Dravida *et al.*, 2005, Yoshida *et al.*, 2005, Lu *et al.*, , Choong *et al.*, 2007). Dravida *et al.*, isolated a distinctive population of fibroblast-like cells from de-epithelised stroma of limbal explants using positive binding of stage specific embryonic antigen 4 (SSEA-4) positive cells. These isolated SSEA-4 positive fibroblast-like cells, showed a unique marker profile, different from that of bone marrow mesenchymal or other adult stem cells but similar to that of embryonic stem cells (Dravida *et al.*, 2005), whereas other groups have propagated the fibroblast-like outgrowth from limbal explants, which were multipotent and exhibited a mesenchymal stem cell-like surface marker phenotype (Du *et al.*, 2005, Du *et al.*, 2007, Perrella *et al.*, 2007, Polisetty *et al.*, 2008, Yamagami *et al.*, 2007).

Thus, characterisation of limbal fibroblast-like (LFL) cells requires a panel of CD markers, the variation in degree of expression from one study to other indicates that source and isolation of fibroblast-like cells (LFLc) can vary in marker expression profile as listed **table 4.12.**

**Table 4.12:** Stromal cells found in Cornea/limbus and characterisation profile, in literature.

Stem cell (SC) type	Isolation/culture	Markers		Reference
		Positive	Negative	
Stromal mesenchymal cells	Spontaneous spindle cell outgrowth of limbal explants	<b>FACS:</b> CD105, CD106,CD54, CD166, CD90, CD29, CD71,Pax6	<b>FACS :</b> SSEA-1,Tra1-81,Tra1-61, CD31,CD45, CD11a, CD11c, CD14, CD138, Flk1-Flt1, VE cadherin	(Polisetty <i>et al.</i> , 2008)
Neural crest derived corneal stem cells (COPs)	Sphere forming assay from mouse central cornea	<b>FACS:</b> Sca-1, CD34, , c-kit, <b>IF:</b> ABCG2, Musashi-1, nestin, wnt-1	<b>FACS:</b> CD45- <b>IF:</b> NA	(Funderburgh <i>et al.</i> , 2005, Yoshida <i>et al.</i> , 2005)
Murine corneal stroma-derived cells (CSDCs)	Stromal cells isolations by digestion method	<b>FACS-</b> positive for CD29, CD90, CD105, and CD71;	<b>FACS</b> negative for CD34 and CD45.	(Ye <i>et al.</i> , 2008)
Mesenchymal stromal cell-like cells	Small bits of Corneal stroma in culture	<b>FACS</b> positive for- CD13, CD29, CD44, CD56, CD73, CD90, CD105 and CD133,	<b>FACS</b> Negative for- HLA-DR, CD34, CD117 and CD45	(Benchaouir <i>et al.</i> , 2007)
Stromal fibroblast-like cells	SSEA-4+ cells isolated by MACS	<b>FACS:</b> CD31, SSEA-4, CD73, CD105, <b>IF:</b> Oct-4, Sox-2, Tra1-60, Tra1-81	<b>FACS</b> Negative for CD34, CD45, CD123, CD133, CD14, CD106, HLA-DR	(Dravida <i>et al.</i> , 2005)

**IF** – Immunofluorescence; **FACS**- Fluorescence-activated cell sorting; **NA**-Not available

In this study, limbal fibroblast-like cells (LFLc) isolated from de-epithelised limbal explants from explants culture system were characterised with a panel of markers that were selected based on most commonly used CDs and cytokeratins (specific for corneal epithelial cells) by immunohistochemical staining to rule out contamination of epithelial cells. Second- to fifth-passage cells were used for all the experiments. List of markers use and results were summarised in **table 4.12.1**.

**Table 4.12.1** List of markers used for LFL cells characterisation by Immunofluorescence

S.No.	Marker	Result
1	Vimentin	Positive
2	Fibroblast surface protein	Positive
3	$\alpha$ smooth muscle actin (SMA)	Positive
4	CK3	Negative
5	CK12	Negative
6	CK19	Negative
7	Pan-cytokeratin (CK5,6,8)	Negative
8	E-cadherin	Positive
9	Neuron Specific Enolase (NSE)	Positive
10	CD29	Positive
11	CD31	Positive
12	CD49b	Positive
13	CD105	Positive
14	CD34	Negative

Cellular characterisation using immunofluorescence has demonstrated that isolated limbal fibroblast-like cells (LFLc) are positive for several mesenchymal related markers including vimentin, CD29 and CD105. Many investigators have explored CD105 as an important

antigenic determinant in the identification of mesenchymal stromal cells (MSC) (In 't Anker *et al.*, 2003, Zvaifler *et al.*, 2000, Romanov *et al.*, 2003). Pittenger has reported that CD29 is important cellular marker for MSC (Pittenger *et al.*, 1999).

In addition, LFLc are positive for  $\alpha$  smooth muscle actin, fibroblast surface protein, neuron specific enolase (NSE), CD31, CD49b, E-cadherin, and negative for CD34. These results are consistent with the findings by Sosnova *et al.*, (Sosnova *et al.*, 2005), which indicated that the corneal keratocytes are CD34 negative. However, these results are in contrast to the observation of another group, where human corneal fibroblast-like cells were shown to be positive for CD34 (Perrella *et al.*, 2007). This discrepancy could be attributed to the difference in the source of cells such as sorted and unsorted cells (**figure 3.17**). A positive expression of E-cadherin in LFL cells is an indication of epithelial mesenchymal transition (EMT), where the E-cadherin is a hallmark of EMT and the loss of E-cadherin expression in mesenchymal cells is indicative of mesenchymal transition. E-cadherin is a central component of cell-cell adhesion junctions and is required for the formation of epithelia (Thiery, 2002, Kang and Massague, 2004).

A positive result of E-cadherin and mesenchymal markers (CD105, CD49) in LFLc is a possible indication that these cells could be in the mid transition to mesenchymal cells or mesenchymal to epithelial transition, it could be speculated that these cells are probably either epithelial derived, or it may point toward mesenchymal differentiation. The present data on the expression of these markers is not sufficient to derive any further conclusions, due to the lack of a specific marker.

#### **4.13      *In Vitro* differentiation potential of limbal fibroblast-like cells (LFLc)**

Under defined culture conditions, corneal keratocytes could be induced to differentiate into cells of various mesenchymal lineages, such as adipocytes and osteogenic cells. In the presence of adipogenic, osteogenic medium, isolated LFLc were shown to develop into adipocytes and osteocytes respectively. The appearance and properties of adipocytes were similar to those differentiated from BM-MSc, as reported previously (Campagnoli *et al.*, 2001, Tondreau *et al.*, 2004, Polisetty *et al.*, 2008).

The finding of limbal fibroblast-like cells has always been a subject of debate about differentiation potential. Thus, in this study we demonstrated the potential of LFLc to differentiate into adipogenic and osteogenic lineages in comparison to bone marrow mesenchymal cells by using a human mesenchymal stem cell functional identification kit (Catalog Number SC006)

#### **4.13.1 Differentiation of LFLc to adipocytes**

Under defined culture conditions, LFLc are differentiated to adipocytes similar to human bone marrow mesenchymal stem cells, in accumulation of intracellular droplets, completely filled with lipid-rich vacuoles, which stained positively with Oil Red O (**figure 3.18**), an established lipid dye. Differentiation was demonstrated further by adipogenic lineage-specific antibody to FABP-4 (**figure 3.18.1**). The isolated LFLc were shown to have the potential to differentiate to adipocytes similar to bone marrow mesenchymal stem cells, similar to other studies, (Campagnoli *et al.*, 2001, Tondreau *et al.*, 2004, Polisetty *et al.*, 2008). This indicates that isolated LFLc from de-epithelialised limbal tissue has similar differentiation potential to corneal fibroblast cells.

#### **4.13.2 Differentiation of LFLc to osteocytes**

Isolated LFLc were also found to differentiate into cells of the osteogenic lineage. When exposed to osteogenic induction for 21 days, the centre of the colony increased gradually and finally formed mineralization nodules, which were stained with Alizarin Red for calcium deposits (a functional property of differentiated osteogenic cells). Osteogenic lineage marker osteocalcin was used for further demonstration of osteogenic differentiation; this differentiation potential is similar to bone marrow mesenchymal cells, (positive control cells). Thus, LFLc have differentiation potential similar to bone marrow mesenchymal cells as shown by other groups (Noort *et al.*, 2002, Roelen and Dijke, 2003, Choong *et al.*, 2007, Polisetty *et al.*, 2008). (**Figure 3.18.2 and 3.18.2.1**)

In summary, this study demonstrated that LFL cells could be isolated from de-epithelialised limbal tissue with mechanical disruption. The difference in cultured corneal epithelial cells and fibroblast-like cells are morphological, corneal epithelial cells were relatively small and cuboidal approximately 10.1  $\mu\text{m}$  in diameter (Romano *et al.*, 2003), structurally and biochemically primitive with low cellular granularity, and pigmented. On the other hand, limbal fibroblast-like cells were characteristically elongated and spindle shaped.

Limbal epithelial stem cells are shown to possess label-retaining property and to express CK3, CK12, CK14, whereas limbal fibroblast-like cells do not express epithelial markers such as CK3, 12, and pancytokeratins. These cells showed good proliferative capacity in early passage stage, and decrease in proliferation with higher passage number. LFLc expressed homogenously CD markers, which express are expressed by MSC-related surface antigens; they differentiate along the adipogenic and osteogenic cell lineages.

The isolated limbal fibroblast-like cells were highly invasive and motile in comparison to positive control cells, where these cells can be used to understand the role of invasive function in wound healing with cell culture models and tissue engineering by creating epithelial and stromal interactions within the culture system.

The ability to expand stromal stem cells *in vitro* may provide material for cell based therapy for stromal scarring. Accessibility to an essentially unlimited supply of differentiated keratinocytes also opens the possibility of producing stromal tissue for use in bioprosthetic corneas.

The practical applicability of stem cell based therapies and bioengineered tissues may be less complicated in the corneal stroma than in many of the other applications for which stem cells are being developed. The cornea enjoys an immune privileged status that results in a rejection rate of only 10% of unmatched allografts (Streilein, 2003).

# 5.0 Section

---

## Summary and conclusions

## 5.1 Limbal-corneal epithelial cells

1. Preliminary experiments in the establishment of limbal-corneal epithelial cell cultures were more successful with an explants culture system than with single cell suspension cultures in our laboratory.
2. To find the optimum culture condition for limbal-corneal epithelial cells in culture system, six culture systems were established in this study as follows:
  - I. Condition A no-3T3 (no 3T3 cell support)
  - II. Condition B Co-3T3 (3T3 cells in contact with limbal-epithelial cells)
  - III. Condition C Bot-3T3 (3T3 cells away from limbal-epithelial cells)
  - IV. Condition D dHAM no-3T3 (Limbal-epithelial cells on denuded human amniotic membrane without 3T3 cells)
  - V. Condition E dHAM Co-3T3 (Limbal-epithelial cells on denuded human amniotic membrane in contact with 3T3 cells)
  - VI. Condition F dHAM Bot-3T3 (Limbal-epithelial cells on denuded human amniotic membrane away from 3T3 cells)
3. The total number of samples used in this study for first three conditions was 39, and no obvious differences in relation to donor age (range 19 to 69 years) and growth rate in three conditions and in relation to time of death, preservation time, or process day were observed.

4. The average days for growth start from explants in all three culture conditions is  $2.6 \pm 0.96$  days, with a maximum delay in growth of 6 days, with early growth start as early as day 2. The average number of days for termination day is 15.5 mean  $SD \pm 1.57$  days, with a minimum of 12 days and a maximum of 20 days to attain thick cell sheet of limbal-epithelial cell from explants in cell culture inserts, where  $n=39$  biological replicates.
5. The number of samples used in denuded Human Amniotic Membrane (dHAM) cultures was 21. The average age of donors was 47 years, (range 28-66 years).
6. Cell outgrowth in dHAM cultures first appeared at  $5 \pm 1.29$  days, with expanded cell sheet formation by 20 mean  $SD \pm 2.7$  days, irrespective of culture conditions, where  $n=21$  biological replicates.
7. Limbal-corneal epithelial cells grown in condition A (no-3T3) had large size, hexagonal/polygonal morphology, and 1-2 cells thick, in other culture conditions with 3T3 cell support and dHAM substrate yielded small compact cells with clear cell-cell gap, in sheets 2-3 cell thick cells.
8. Limbal-corneal epithelial cells grown on cell culture inserts with three different culture conditions (Condition A, B and C) have shown high motility/migration and invasive capacity (compared to a positive control cell line BT-20) *in vitro*, possibly associated with the role of corneal epithelial cells in wound healing in corneal injury.

#### **Characterisation of limbal-corneal cell sheets:**

*In vitro* expanded limbal-corneal epithelial cells grown under different culture conditions were examined for differentiation and stem cell marker analysis, to understand the role of 3T3 cells in maintenance of stem cell population in different culture conditions.

9. Limbal-corneal epithelial cells grown in condition A no-3T3 appeared to be most differentiated cells *in vitro*, with high expression of corneal differentiation markers (CK3, CK12) at apical and basal layers, and low expression of stem cell markers (ABCG2,  $\Delta$ Np63 $\alpha$ ), with poor or low colony forming efficacy (CFE) 0.42%.
10. Limbal-corneal epithelial cells grown with 3T3 feeder cell support (either in contact with or away from epithelial cells) have shown high intense levels of stem cell markers ABCG2,  $\Delta$ Np63 $\alpha$  at basal and apical layer of cells by immunofluorescence, compared to the no-3T3 condition. Limbal-corneal epithelial cells grown away from 3T3 cells have shown high number of stem cells suggested by of CFE 2.32 mean SD  $\pm$  0.3% compared to condition B Co-3T3 1.17 mean SD  $\pm$ 0.24% of CFE, where n=6 biological replicates.
11. The number of colony forming units is higher in condition D dHAM no-3T3 (CFE 5.25 $\pm$ 0.19 %) compared to condition A no-3T3 with low CFE 0.42%. (n=6 biological replicates)
12. This study, demonstrated the highest number of colony forming units in limbal-corneal epithelial cells in condition E dHAM Co-3T3 with CFE of 8.92 mean SD  $\pm$ 2.18 %, and increased the expression of stem cell marker (ABCG2,  $\Delta$ Np63 $\alpha$ ) protein by western blot analysis. This suggests that limbal-corneal epithelial cells grown on dHAM with 3T3 feeder cells act as suitable condition to retain stem cell population *in vitro*.

In conclusion, this study established the culture of limbal-corneal epithelial cell culture from human cadaveric limbal tissue with explants culture system; and no obvious difference in growth of epithelial cells in relation to the donor age, time between tissue preservation and the process. During the expansion phase, limbal epithelial outgrowth exhibited a compact

and uniform cell layer in culture systems with 3T3 cell support. Limbal-corneal epithelial cells were characterised by a panel of markers. This data indicates the importance of a feeder cell system in the maintenance of limbal stem cell population *in vitro*. This study demonstrated a suitable culture system for limbal-corneal epithelial cells that has highest number of stem cells population (high CFE). These findings suggest that a culture system, which might mimic the original niche environment (explants, human amniotic membrane and 3T3 feeder cells), is important in maintaining the stem cells in an undifferentiated state. *In vitro* expansion of limbal explants on HAM in the treatment of limbal stem cells deficiency (LSCD) represents a successful stem cell therapy in the field of regenerative medicine.

## **5.2 Limbal-corneal epithelial cells for clinical application**

Human amniotic membrane (HAM) has been used successfully in ocular surface reconstruction as a biological bandage and as a substrate for stem cell expansion. In this section we studied the possible application of four types of commercially available HAM for culture of limbal corneal epithelial cells that could be used under Good Manufacture Practice (GMP) conditions and for clinical application.

1. Culturing cells on the Acelagraft<sup>TM</sup> does not prove to be easy, especially as the membrane is thin and observation of limbal-corneal epithelial cell growth on membrane was technically difficult due to undulations of the membrane created during freeze-drying process. This could lead to false positives in observation of growth rate during culture period, due to epithelial morphology appearance on membrane alone.
2. AmbioDry2<sup>TM</sup> and AmbioDry5<sup>TM</sup> products have amniotic epithelial cells, and are inhibitory to limbal-corneal epithelial outgrowth from the explants; also this

membrane was very thin and difficult to manipulate during the de-epithelialisation process, which is therefore unsuitable for GMP process due to lot more manipulation in the procedure.

3. According to the manufacturer's instructions, EpiFix™ membrane is de-epithelialised, but microscope observation revealed that this membrane is processed with stromal/epithelial cells intact. Further, EpiFix membrane needs additional process of trypsinisation to culture limbal-epithelial cells.
4. None of the commercially available Freeze-Dried membranes are suitable for culture of limbal-corneal epithelial cells. We standardised use of fresh frozen Human Amniotic membrane for culture of limbal-corneal epithelial cells in GLP conditions, which can be translated to clinical applications by processing at GMP conditions.

In conclusion, this study has demonstrated the feasibility of a sterile, dehydrated HAM for the culture of limbal-corneal epithelial cells. None of the four commercially available HAMs are entirely suitable for the culture of limbal-corneal epithelial cells due to technical difficulties of handling during the process, and in some cases this may be due to presence of amniotic epithelial cells on processed membrane. Fresh frozen dHAM with an explants culture system appears to be the best method. Altogether, we developed a simple, quick, and manipulation free technique to ensure transplantation of viable, proliferating limbal epithelial stem cells.

### 5.3 Limbal fibroblast-like cells (LFLc)

Human limbal mesenchymal stem cells are easy to isolate and multipotent with the capacity to differentiate into other lineages. Recent studies (Dravida *et al.*, 2005, Polisetty *et al.*, 2008) in isolation and differentiation of limbal fibroblast-like cells shown that these cells also express typical limbal stem cell markers such as ABCG2 and p63 $\alpha$ , which makes them strong candidates for use in corneal resurfacing, and understanding basic biology of mesenchymal like cells in cornea. In this study, we isolated limbal ‘fibroblast-like’ cells (LFLc) from de-epithelialised limbal tissue.

1. Limbal ‘fibroblast-like’ cells (LFLc) were isolated from de-epithelialised limbal tissue, that are of stromal origin and fibroblastic in nature. The phenotypic marker expression profile showed negative for corneal epithelial markers CK3, CK12, CK8 and CK19, and positive for mesenchymal markers vimentin, Fibroblast surface protein,  $\alpha$  smooth muscle actin ( $\alpha$  SMA), CD29, CD31, and CD49b.
2. Limbal fibroblast-like cells have spindle shape morphology and were confirmed by Wright-Giemsa staining, where cells appeared as fibroblastic. The morphology of LFLc at higher passage numbers appeared large cell size; from small spindle-shaped with stretched end to broad cell bodies and long thin cytoplasmic extensions and cells undergone senescence stage with higher passage number.
3. The LFLc has shown a decrease in proliferation rate as the passage number increases *in vitro*. Similarly, LFLc have shown increased senescence with higher passage number.

4. Limbal fibroblast-like cells were highly motile and invasive with no significant change with higher passage number.
5. Limbal fibroblast-like cells were differentiated *in vitro* using adipogenic and osteogenic induction media, where cells have shown positive upon induction for Oil red O (Adipocytes) and calcification when stained with alizarin red for calcium deposits (Osteocytes), similar to positive control bone marrow mesenchymal cells.

In conclusion, limbal ‘fibroblast-like’ cells (LFLC) have stem like properties where these cells can be differentiated into adipo and osteogenic lineages.

# 6.0 Section

---

# Future work

## 6.1 Limbal-corneal epithelial cultures

Culture models used in this study for the *in vitro* expansion of limbal-corneal epithelial cells leads to potential new experimental approaches that will be revolutionary in identification of molecules and growth factors, which enhance maintenance of stem cell population by applying proteomics and molecular biology techniques.

➤ Different culture setups used in this study can be extrapolated for gene and protein analysis, for identification of cytokines, growth factors and hormones, which are important for limbal-corneal epithelial cells, and maintenance of stem cell phenotype.

➤ In this study, we demonstrated the possible high stem cell niche maintenance in condition C Bot-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3 compared to condition A no-3T3. 2-D electrophoresis of conditioned media from above samples would allow for identification of proteins present at different levels in condition A no-3T3 vs. condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Such studies will enable the development of complete animal-cell and animal product-free culture conditions for limbal epithelial cultures.

➤ miRNA and microarray analysis of different culture systems could lead to the identification of new definitive limbal stem-cell markers, which can be used in the analysis of various culture systems. This can be achieved by using a number of different batches of limbal-corneal epithelial cultures, limbal fibroblast-like cells, and conjunctival cells to identify markers only associated with limbal epithelial cells.

- Identification of epigenetic changes in condition A no-3T3 vs. condition E dHAM Co-3T3, and condition F dHAM Bot-3T3 could reveal what epigenetic changes are leading to enhance stem cell enrichment in particular condition.

## **6.2 Limbal-corneal epithelial cultures translation to clinic**

- Simple, reliable, efficient limbal-corneal epithelial cell culture (Condition A dHAM no-3T3) protocol developed in this thesis was taken to Good Laboratory practises (GLP). This could be further modified according to Good manufacture practises (GMP) with controlled regulatory in-house processes, for the treatment of ocular surface disorders.

## **6.3 Limbal fibroblast-like cells:**

- Murine 3T3 feeder cells are commonly used for stem cell expansion, as demonstrated in this thesis; the investigation of limbal fibroblast-like cells for its potential use as a feeder layer could be an alternative source/ replacement for mouse feeder cells (3T3 feeder cells).
- The preliminary results of limbal fibroblast-like cells (LFLc) have shown that these cells are highly motile and invasive *in vitro*, where these results demonstrate plausibility of limbal fibroblast-like cells in corneal wound healing, which can be investigated further with wound healing experiments *in vitro*.
- The trans-differentiation potential of Limbal fibroblast-like cells (adipo and osteocytes) demonstrated in this thesis laid down to further investigation of LFL cells for the

potential trans-differentiation towards corneal epithelial-like cells, with suitable environment (ECM substrate) and by inducing them with corneal epithelial growth media.

# 7.0 Section

---

# References

- Adams, J. C. and Watt, F. M. (1989) Fibronectin inhibits the terminal differentiation of human keratinocytes, *Nature*, 340, 307-9.
- Adler's (1992) *Physiology of the Eye*, chapters 1-3.
- Ahmad, S., Kolli, S., Lako, M., et al. (2010) Stem cell therapies for ocular surface disease, *Drug Discov Today*, 15, 306-13.
- Ahmad, S., Stewart, R., Yung, S., et al. (2007) Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche, *Stem Cells*, 25, 1145-55.
- Ahmadiankia, N., Ebrahimi, M., Hosseini, A. and Baharvand, H. (2009) Effects of different extracellular matrices and co-cultures on human limbal stem cell expansion in vitro, *Cell Biol Int*, 33, 978-87.
- Akle, C. A., Adinolfi, M., Welsh, K. I., et al. (1981) Immunogenicity of human amniotic epithelial cells after transplantation into volunteers, *Lancet*, 2, 1003-5.
- Allen-Hoffmann, B. L. and Rheinwald, J. G. (1984) Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture, *Proc Natl Acad Sci U S A*, 81, 7802-6.
- Amano, S., Yamagami, S., Mimura, T., et al. (2006) Corneal stromal and endothelial cell precursors, *Cornea*, 25, S73-7.
- Andresen, J. L., Ledet, T. and Ehlers, N. (1997) Keratocyte migration and peptide growth factors: the effect of PDGF, bFGF, EGF, IGF-I, aFGF and TGF-beta on human keratocyte migration in a collagen gel, *Curr Eye Res*, 16, 605-13.
- Ang, L. P. and Tan, D. T. (2004) Ocular surface stem cells and disease: current concepts and clinical applications, *Ann Acad Med Singapore*, 33, 576-80.
- Ang, L. P., Tan, D. T., Cajucom-Uy, H., et al. (2004) Reconstruction of the ocular surface by transplantation of a serum free cultivated conjunctival tissue equivalent, *Ann Acad Med Singapore*, 33, S55-6.
- Arffa, R. (1991) Grayson's Diseases of the Cornea, *Mosby St. Louis*, 25-32.
- Arpitha, P., Prajna, N. V., Srinivasan, M. and Muthukkaruppan, V. (2005) High expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: implications on epithelial stem cells, *Invest Ophthalmol Vis Sci*, 46, 3631-6.

- Baharvand, H., Heidari, M., Ebrahimi, M., et al. (2007) Proteomic analysis of epithelium-denuded human amniotic membrane as a limbal stem cell niche, *Mol Vis*, 13, 1711-21.
- Balasubramanian, S., Jasty, S., Sitalakshmi, G., et al. (2008) Influence of feeder layer on the expression of stem cell markers in cultured limbal corneal epithelial cells, *Indian J Med Res*, 128, 616-22.
- Ban, Y., Cooper, L. J., Fullwood, N. J., et al. (2003) Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting, *Exp Eye Res*, 76, 735-43.
- Barbaro, V., Ferrari, S., Fasolo, A., et al. (2009) Reconstruction of a human hemicornea through natural scaffolds compatible with the growth of corneal epithelial stem cells and stromal keratocytes, *Mol Vis*, 15, 2084-93.
- Barbaro, V., Testa, A., Di Iorio, E., et al. (2007) C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells, *J Cell Biol*, 177, 1037-49.
- Barnard, Z., Apel, A. J. and Harkin, D. G. (2001) Phenotypic analyses of limbal epithelial cell cultures derived from donor corneoscleral rims, *Clin Experiment Ophthalmol*, 29, 138-42.
- Barrandon, Y. and Green, H. (1985) Cell size as a determinant of the clone-forming ability of human keratinocytes, *Proc Natl Acad Sci U S A*, 82, 5390-4.
- Barrandon, Y. and Green, H. (1987) Three clonal types of keratinocyte with different capacities for multiplication, *Proc Natl Acad Sci U S A*, 84, 2302-6.
- Barreca, A., De Luca, M., Del Monte, P., et al. (1992) In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors, *J Cell Physiol*, 151, 262-8.
- Beales, M. P., Funderburgh, J. L., Jester, J. V. and Hassell, J. R. (1999) Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture, *Invest Ophthalmol Vis Sci*, 40, 1658-63.
- Benchaouir, R., Picot, J., Greppo, N., et al. (2007) Combination of quantification and observation methods for study of "Side Population" cells in their "in vitro" microenvironment, *Cytometry A*, 71, 251-7.
- Benhabbour, S. R., Sheardown, H. and Adronov, A. (2008) Cell adhesion and proliferation on hydrophilic dendritically modified surfaces, *Biomaterials*, 29, 4177-86.
- Beuerman, R. W. and Pedroza, L. (1996) Ultrastructure of the human cornea, *Microsc Res Tech*, 33, 320-35.
- Bickenbach, J. R. (2005) Isolation, characterization, and culture of epithelial stem cells, *Methods Mol Biol*, 289, 97-102.

- Blouin, R., Swierenga, S. H. and Marceau, N. (1992) Evidence for post-transcriptional regulation of cytokeratin gene expression in a rat liver epithelial cell line, *Biochem Cell Biol*, 70, 1-9.
- Bron, A. J. (1973) Vortex patterns of the corneal epithelium, *Trans Ophthalmol Soc U K*, 93, 455-72.
- Buck, R. C. (1979) Cell migration in repair of mouse corneal epithelium, *Invest Ophthalmol Vis Sci*, 18, 767-84.
- Buck, R. C. (1985) Measurement of centripetal migration of normal corneal epithelial cells in the mouse, *Invest Ophthalmol Vis Sci*, 26, 1296-9.
- Budak, M. T., Alpdogan, O. S., Zhou, M., et al. (2005) Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells, *J Cell Sci*, 118, 1715-24.
- Burroughs, J., Gupta, P., Blazar, B. R. and Verfaillie, C. M. (1994) Diffusible factors from the murine cell line M2-10B4 support human in vitro hematopoiesis, *Exp Hematol*, 22, 1095-101.
- Buschke, W. (1949) Morphologic changes in cells of corneal epithelium in wound healing, *Arch Ophthal*, 41, 306-16.
- Buss, D. G., Giuliano, E. A., Sharma, A. and Mohan, R. R. (2010) Isolation and cultivation of equine corneal keratocytes, fibroblasts and myofibroblasts, *Vet Ophthalmol*, 13, 37-42.
- Caballero, M., Liton, P. B., Challa, P., et al. (2004) Effects of donor age on proteasome activity and senescence in trabecular meshwork cells, *Biochem Biophys Res Commun*, 323, 1048-54.
- Campagnoli, C., Roberts, I. A., Kumar, S., et al. (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow, *Blood*, 98, 2396-402.
- Chaloin-Dufau, C., Sun, T. T. and Dhouailly, D. (1990) Appearance of the keratin pair K3/K12 during embryonic and adult corneal epithelial differentiation in the chick and in the rabbit, *Cell Differ Dev*, 32, 97-108.
- Chanas, S. A., Collinson, J. M., Ramaesh, T., et al. (2009) Effects of elevated Pax6 expression and genetic background on mouse eye development, *Invest Ophthalmol Vis Sci*, 50, 4045-59.
- Charo, I. F., Nannizzi, L., Smith, J. W. and Cheresch, D. A. (1990) The vitronectin receptor alpha v beta 3 binds fibronectin and acts in concert with alpha 5 beta 1 in promoting cellular attachment and spreading on fibronectin, *J Cell Biol*, 111, 2795-800.
- Chee, K. Y., Kicic, A. and Wiffen, S. J. (2006) Limbal stem cells: the search for a marker, *Clin Experiment Ophthalmol*, 34, 64-73.

- Chen, B., Mi, S., Wright, B. and Connon, C. J. (2010) Differentiation status of limbal epithelial cells cultured on intact and denuded amniotic membrane before and after air-lifting, *Tissue Eng Part A*, 16, 2721-9.
- Chen, J., Diacovo, T. G., Grenache, D. G., et al. (2002) The alpha(2) integrin subunit-deficient mouse: a multifaceted phenotype including defects of branching morphogenesis and hemostasis, *Am J Pathol*, 161, 337-44.
- Chen, J. J. and Tseng, S. C. (1990) Corneal epithelial wound healing in partial limbal deficiency, *Invest Ophthalmol Vis Sci*, 31, 1301-14.
- Chen, J. J. and Tseng, S. C. (1991) Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium, *Invest Ophthalmol Vis Sci*, 32, 2219-33.
- Chen, W., Ishikawa, M., Yamaki, K. and Sakuragi, S. (2003) Wistar rat palpebral conjunctiva contains more slow-cycling stem cells that have larger proliferative capacity: implication for conjunctival epithelial homeostasis, *Jpn J Ophthalmol*, 47, 119-28.
- Chen, W. L., Lin, C. T., Ko, P. S., et al. (2009) In vivo confocal microscopic findings of corneal wound healing after corneal epithelial debridement in diabetic vitrectomy, *Ophthalmology*, 116, 1038-47.
- Chen, W. Y., Mui, M. M., Kao, W. W., et al. (1994) Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: expression of K12 keratin is restricted to corneal epithelium, *Curr Eye Res*, 13, 765-78.
- Chen, Y. T., Li, W., Hayashida, Y., et al. (2007) Human amniotic epithelial cells as novel feeder layers for promoting ex vivo expansion of limbal epithelial progenitor cells, *Stem Cells*, 25, 1995-2005.
- Chen, Z., de Paiva, C. S., Luo, L., et al. (2004) Characterization of putative stem cell phenotype in human limbal epithelia, *Stem Cells*, 22, 355-66.
- Cheng, C. C., Wang, D. Y., Kao, M. H. and Chen, J. K. (2009) The growth-promoting effect of KGF on limbal epithelial cells is mediated by upregulation of DeltaNp63alpha through the p38 pathway, *J Cell Sci*, 122, 4473-80.
- Choong, P. F., Mok, P. L., Cheong, S. K. and Then, K. Y. (2007) Mesenchymal stromal cell-like characteristics of corneal keratocytes, *Cytotherapy*, 9, 252-8.
- Chrzanowska-Wodnicka, M. and Burridge, K. (1994) Tyrosine phosphorylation is involved in reorganization of the actin cytoskeleton in response to serum or LPA stimulation, *J Cell Sci*, 107 ( Pt 12), 3643-54.
- Claudinot, S., Nicolas, M., Oshima, H., et al. (2005) Long-term renewal of hair follicles from clonogenic multipotent stem cells, *Proc Natl Acad Sci U S A*, 102, 14677-82.

- Colabelli Gisoldi, R. A., Pocobelli, A., Villani, C. M., et al. (2010) Evaluation of molecular markers in corneal regeneration by means of autologous cultures of limbal cells and keratoplasty, *Cornea*, 29, 715-22.
- Collinson, J. M., Chanas, S. A., Hill, R. E. and West, J. D. (2004) Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6(+/-) mouse, *Invest Ophthalmol Vis Sci*, 45, 1101-8.
- Cooper, D. and Sun, T. T. (1986) Monoclonal antibody analysis of bovine epithelial keratins. Specific pairs as defined by coexpression, *J Biol Chem*, 261, 4646-54.
- Cooper, L. J., Fullwood, N. J., Koizumi, N., et al. (2004) An investigation of removed cultivated epithelial transplants in patients after allocultivated corneal epithelial transplantation, *Cornea*, 23, 235-42.
- Cotsarelis, G., Cheng, S. Z., Dong, G., et al. (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells, *Cell*, 57, 201-9.
- Cristovam, P. C., Gloria, M. A., Melo, G. B. and Gomes, J. A. (2008) [Importance of 3T3 feeder layer to establish epithelial cultures from cell suspension obtained from corneo-scleral rims], *Arq Bras Oftalmol*, 71, 689-94.
- Daniels, J. T., Harris, A. R. and Mason, C. (2006a) Corneal epithelial stem cells in health and disease, *Stem Cell Rev*, 2, 247-54.
- Daniels, J. T., Secker, G. A., Shortt, A. J., et al. (2006b) Stem cell therapy delivery: treading the regulatory tightrope, *Regen Med*, 1, 715-9.
- Davanger, M. and Evensen, A. (1971) Role of the pericorneal papillary structure in renewal of corneal epithelium, *Nature*, 229, 560-1.
- Daya, S. M. and Ilari, F. A. (2001) Living related conjunctival limbal allograft for the treatment of stem cell deficiency, *Ophthalmology*, 108, 126-33; discussion 133-4.
- De Luca, M., Pellegrini, G. and Green, H. (2006) Regeneration of squamous epithelia from stem cells of cultured grafts, *Regen Med*, 1, 45-57.
- de Paiva, C. S., Chen, Z., Corrales, R. M., et al. (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells, *Stem Cells*, 23, 63-73.
- Deshpande, P., Notara, M., Bullett, N., et al. (2009) Development of a surface-modified contact lens for the transfer of cultured limbal epithelial cells to the cornea for ocular surface diseases, *Tissue Eng Part A*, 15, 2889-902.
- Di Girolamo, N., Bosch, M., Zamora, K., et al. (2009) A contact lens-based technique for expansion and transplantation of autologous epithelial progenitors for ocular surface reconstruction, *Transplantation*, 87, 1571-8.

- Di Iorio, E., Barbaro, V., Ruzza, A., et al. (2005) Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration, *Proc Natl Acad Sci U S A*, 102, 9523-8.
- Di Leonardo, A., Linke, S. P., Clarkin, K. and Wahl, G. M. (1994) DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts, *Genes Dev*, 8, 2540-51.
- Diebold, Y., Rios, J. D., Hodges, R. R., et al. (2001) Presence of nerves and their receptors in mouse and human conjunctival goblet cells, *Invest Ophthalmol Vis Sci*, 42, 2270-82.
- Dietlein, T. S., Jacobi, P. C., Paulsson, M., et al. (1997) [Variability of laminin isoforms in the limbus region of the eye], *Klin Monbl Augenheilkd*, 211, 188-91.
- Dimri, G. P. and Campisi, J. (1994) Molecular and cell biology of replicative senescence, *Cold Spring Harb Symp Quant Biol*, 59, 67-73.
- Dimri, G. P., Lee, X., Basile, G., et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo, *Proc Natl Acad Sci U S A*, 92, 9363-7.
- Dong, Y., Roos, M., Gruijters, T., et al. (1994) Differential expression of two gap junction proteins in corneal epithelium, *Eur J Cell Biol*, 64, 95-100.
- Donisi, P. M., Rama, P., Fasolo, A. and Ponzin, D. (2003) Analysis of limbal stem cell deficiency by corneal impression cytology, *Cornea*, 22, 533-8.
- Dravida, S., Gaddipati, S., Griffith, M., et al. (2008) A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation, *J Tissue Eng Regen Med*, 2, 263-71.
- Dravida, S., Pal, R., Khanna, A., et al. (2005) The transdifferentiation potential of limbal fibroblast-like cells, *Brain Res Dev Brain Res*, 160, 239-51.
- Du, Y., Chen, J., Funderburgh, J. L., et al. (2003) Functional reconstruction of rabbit corneal epithelium by human limbal cells cultured on amniotic membrane, *Mol Vis*, 9, 635-43.
- Du, Y., Funderburgh, M. L., Mann, M. M., et al. (2005) Multipotent stem cells in human corneal stroma, *Stem Cells*, 23, 1266-75.
- Du, Y., Sundarraj, N., Funderburgh, M. L., et al. (2007) Secretion and organization of a cornea-like tissue in vitro by stem cells from human corneal stroma, *Invest Ophthalmol Vis Sci*, 48, 5038-45.
- Dua, H. S. (1998) The conjunctiva in corneal epithelial wound healing, *Br J Ophthalmol*, 82, 1407-11.
- Dua, H. S. and Forrester, J. V. (1987) Clinical patterns of corneal epithelial wound healing, *Am J Ophthalmol*, 104, 481-9.

- Dua, H. S. and Forrester, J. V. (1990) The corneoscleral limbus in human corneal epithelial wound healing, *Am J Ophthalmol*, 110, 646-56.
- Dua, H. S., Gomes, J. A. and Singh, A. (1994) Corneal epithelial wound healing, *Br J Ophthalmol*, 78, 401-8.
- Dua, H. S., Joseph, A., Shanmuganathan, V. A. and Jones, R. E. (2003) Stem cell differentiation and the effects of deficiency, *Eye (Lond)*, 17, 877-85.
- Dua, H. S., Shanmuganathan, V. A., Powell-Richards, A. O., et al. (2005) Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche, *Br J Ophthalmol*, 89, 529-32.
- Ebato, B., Friend, J. and Thoft, R. A. (1987) Comparison of central and peripheral human corneal epithelium in tissue culture, *Invest Ophthalmol Vis Sci*, 28, 1450-6.
- Ebato, B., Friend, J. and Thoft, R. A. (1988) Comparison of limbal and peripheral human corneal epithelium in tissue culture, *Invest Ophthalmol Vis Sci*, 29, 1533-7.
- Egbert, P. R., Lauber, S. and Maurice, D. M. (1977) A simple conjunctival biopsy, *Am J Ophthalmol*, 84, 798-801.
- Eggl, P., Boulton, M. and Marshall, J. (1989) Growth characteristics of central and peripheral bovine corneal epithelial cells in vitro, *Graefes Arch Clin Exp Ophthalmol*, 227, 263-70.
- Ehlers, N. (1970) Some comparative studies on the mammalian corneal epithelium, *Acta Ophthalmol (Copenh)*, 48, 821-8.
- Ehmann, U. K., Stevenson, M. A., Calderwood, S. K. and DeVries, J. T. (1998) Physical connections between feeder cells and recipient normal mammary epithelial cells, *Exp Cell Res*, 243, 76-86.
- Endres, M., Leinhase, I., Kaps, C., et al. (2005) Changes in the gene expression pattern of cytokeratins in human respiratory epithelial cells during culture, *Eur Arch Otorhinolaryngol*, 262, 390-6.
- Espana, E. M., He, H., Kawakita, T., et al. (2003a) Human keratocytes cultured on amniotic membrane stroma preserve morphology and express keratocan, *Invest Ophthalmol Vis Sci*, 44, 5136-41.
- Espana, E. M., Ti, S. E., Grueterich, M., et al. (2003b) Corneal stromal changes following reconstruction by ex vivo expanded limbal epithelial cells in rabbits with total limbal stem cell deficiency, *Br J Ophthalmol*, 87, 1509-14.
- Fernandes, M., Sridhar, M. S., Sangwan, V. S. and Rao, G. N. (2005) Amniotic membrane transplantation for ocular surface reconstruction, *Cornea*, 24, 643-53.
- Figueira, E. C., Di Girolamo, N., Coroneo, M. T. and Wakefield, D. (2007) The phenotype of limbal epithelial stem cells, *Invest Ophthalmol Vis Sci*, 48, 144-56.

- Filenius, S., Hormia, M., Rissanen, J., et al. (2001) Laminin synthesis and the adhesion characteristics of immortalized human corneal epithelial cells to laminin isoforms, *Exp Eye Res*, 72, 93-103.
- Filenius, S., Tervo, T. and Virtanen, I. (2003) Production of fibronectin and tenascin isoforms and their role in the adhesion of human immortalized corneal epithelial cells, *Invest Ophthalmol Vis Sci*, 44, 3317-25.
- Fini, M. E. and Stramer, B. M. (2005) How the cornea heals: cornea-specific repair mechanisms affecting surgical outcomes, *Cornea*, 24, S2-S11.
- Forbes, B. (2002) Pulmonary epithelial cell culture, *Methods Mol Biol*, 188, 65-75.
- Francis, D., Abberton, K., Thompson, E. and Daniell, M. (2009) Myogel supports the ex-vivo amplification of corneal epithelial cells, *Exp Eye Res*, 88, 339-46.
- Frisch, S. M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis, *J Cell Biol*, 124, 619-26.
- Fuchs, E. V., Coppock, S. M., Green, H. and Cleveland, D. W. (1981) Two distinct classes of keratin genes and their evolutionary significance, *Cell*, 27, 75-84.
- Fujikawa, L. S., Foster, C. S., Gipson, I. K. and Colvin, R. B. (1984) Basement membrane components in healing rabbit corneal epithelial wounds: immunofluorescence and ultrastructural studies, *J Cell Biol*, 98, 128-38.
- Fukuda, K., Chikama, T., Nakamura, M. and Nishida, T. (1999) Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva, *Cornea*, 18, 73-9.
- Funderburgh, M. L., Du, Y., Mann, M. M., et al. (2005) PAX6 expression identifies progenitor cells for corneal keratocytes, *Faseb J*, 19, 1371-3.
- Galindo, E. E., Theiss, C., Pauklin, M., et al. (2009) Correlation of cell cycle kinetics with p63 expression in human limbal epithelial cells expanded on intact human amniotic membrane, *Ophthalmic Res*, 41, 83-90.
- Garana, R. M., Petroll, W. M., Chen, W. T., et al. (1992) Radial keratotomy. II. Role of the myofibroblast in corneal wound contraction, *Invest Ophthalmol Vis Sci*, 33, 3271-82.
- Geerling, G., MacLennan, S. and Hartwig, D. (2004) Autologous serum eye drops for ocular surface disorders, *Br J Ophthalmol*, 88, 1467-74.
- Germain, L., Auger, F. A., Grandbois, E., et al. (1999) Reconstructed human cornea produced in vitro by tissue engineering, *Pathobiology*, 67, 140-7.
- Ghoubay-Benallaoua, D., Basli, E., Goldschmidt, P., et al. (2011) Human epithelial cell cultures from superficial limbal explants, *Mol Vis*, 17, 341-54.
- Gilger, B. (2005) *Equine Ophthalmology, Elsevier Saunders, St. Louis.*

- Green, H., Rheinwald, J. G. and Sun, T. T. (1977) Properties of an epithelial cell type in culture: the epidermal keratinocyte and its dependence on products of the fibroblast, *Prog Clin Biol Res*, 17, 493-500.
- Greenhalgh, D. A., Rothnagel, J. A. and Roop, D. R. (1994) Epidermis: an attractive target tissue for gene therapy, *J Invest Dermatol*, 103, 63S-69S.
- Grinnell, F. (2000) Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading, *Trends Cell Biol*, 10, 362-5.
- Grueterich, M., Espana, E. and Tseng, S. C. (2002a) Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane, *Invest Ophthalmol Vis Sci*, 43, 63-71.
- Grueterich, M., Espana, E. M., Touhami, A., et al. (2002b) Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency, *Ophthalmology*, 109, 1547-52.
- Grueterich, M., Espana, E. M. and Tseng, S. C. (2003a) Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche, *Surv Ophthalmol*, 48, 631-46.
- Grueterich, M., Espana, E. M. and Tseng, S. C. (2003b) Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without a 3T3 fibroblast feeder layer, *Invest Ophthalmol Vis Sci*, 44, 4230-6.
- Grushkin-Lerner, L. S., Kewalramani, R. and Trinkaus-Randall, V. (1997) Expression of integrin receptors on plasma membranes of primary corneal epithelial cells is matrix specific, *Exp Eye Res*, 64, 323-34.
- Grushkin-Lerner, L. S. and Trinkaus-Randall, V. (1991) Localization of integrin and syndecan in vivo in a corneal epithelial abrasion and keratectomy, *Curr Eye Res*, 10, 75-85.
- Gumbiner, B. M. (2000) Regulation of cadherin adhesive activity, *J Cell Biol*, 148, 399-404.
- Hague, A. and Jones, G. E. (2008) Cell motility assays, *Cell Biol Toxicol*, 24, 381-9.
- Hamada, R., Giraud, J. P., Graf, B. and Pouliquen, Y. (1972) [Analytical and statistical study of the lamellae, keratocytes and collagen fibrils of the central region of the normal human cornea. (Light and electron microscopy)], *Arch Ophthalmol Rev Gen Ophthalmol*, 32, 563-70.
- Han, B., Schwab, I. R., Madsen, T. K. and Isseroff, R. R. (2002) A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells, *Cornea*, 21, 505-10.
- Hanna, C., Bicknell, D. S. and O'Brien, J. E. (1961) Cell turnover in the adult human eye, *Arch Ophthalmol*, 65, 695-8.

- Hao, Y., Ma, D. H., Hwang, D. G., et al. (2000) Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane, *Cornea*, 19, 348-52.
- Hay, E. D., Linsenmayer, T. F., Trelstad, R. L. and von der Mark, K. (1979) Origin and distribution of collagens in the developing avian cornea, *Curr Top Eye Res*, 1, 1-35.
- Hayashi, I., Larner, J. and Sato, G. (1978) Hormonal growth control of cells in culture, *In Vitro*, 14, 23-30.
- Hayashida, Y., Nishida, K., Yamato, M., et al. (2005) Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface, *Invest Ophthalmol Vis Sci*, 46, 1632-9.
- Hazlett, L. D., Wells, P. A. and Berk, R. S. (1984) Scanning electron microscopy of the normal and experimentally infected ocular surface, *Scan Electron Microsc*, 1379-89.
- He, Y. G. and McCulley, J. P. (1991) Growing human corneal epithelium on collagen shield and subsequent transfer to denuded cornea in vitro, *Curr Eye Res*, 10, 851-63.
- Herman, W. K., Doughman, D. J. and Lindstrom, R. L. (1983) Conjunctival autograft transplantation for unilateral ocular surface diseases, *Ophthalmology*, 90, 1121-6.
- Higa, K., Shimmura, S., Kato, N., et al. (2007) Proliferation and differentiation of transplantable rabbit epithelial sheets engineered with or without an amniotic membrane carrier, *Invest Ophthalmol Vis Sci*, 48, 597-604.
- Higa, K., Shimmura, S., Miyashita, H., et al. (2009) N-cadherin in the maintenance of human corneal limbal epithelial progenitor cells in vitro, *Invest Ophthalmol Vis Sci*, 50, 4640-5.
- Holland, E. J. and Schwartz, G. S. (1996) The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system, *Cornea*, 15, 549-56.
- Homma, R., Yoshikawa, H., Takeno, M., et al. (2004) Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice, *Invest Ophthalmol Vis Sci*, 45, 4320-6.
- Hopkinson, A., McIntosh, R. S., Shanmuganathan, V., et al. (2006) Proteomic analysis of amniotic membrane prepared for human transplantation: characterization of proteins and clinical implications, *J Proteome Res*, 5, 2226-35.
- Horenstein, A. L., Sizzano, F., Lusso, R., et al. (2009) CD38 and CD157 ectoenzymes mark cell subsets in the human corneal limbus, *Mol Med*, 15, 76-84.
- Hsueh, Y. J., Wang, D. Y., Cheng, C. C. and Chen, J. K. (2004) Age-related expressions of p63 and other keratinocyte stem cell markers in rat cornea, *J Biomed Sci*, 11, 641-51.

- Hu, C., Ding, Y., Chen, J., et al. (2009) Basic fibroblast growth factor stimulates epithelial cell growth and epithelial wound healing in canine corneas, *Vet Ophthalmol*, 12, 170-5.
- Huang, A. J. and Tseng, S. C. (1991) Corneal epithelial wound healing in the absence of limbal epithelium, *Invest Ophthalmol Vis Sci*, 32, 96-105.
- Huang, Y., Sen, T., Nagpal, J., et al. (2008) ATM kinase is a master switch for the Delta Np63 alpha phosphorylation/degradation in human head and neck squamous cell carcinoma cells upon DNA damage, *Cell Cycle*, 7, 2846-55.
- Huelsken, J., Vogel, R., Erdmann, B., et al. (2001) beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin, *Cell*, 105, 533-45.
- Ibaraki, N. (2002) Human lens epithelial cell culture, *Methods Mol Biol*, 188, 1-6.
- Ihanamaki, T., Pelliniemi, L. J. and Vuorio, E. (2004) Collagens and collagen-related matrix components in the human and mouse eye, *Prog Retin Eye Res*, 23, 403-34.
- In 't Anker, P. S., Scherjon, S. A., Kleijburg-van der Keur, C., et al. (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation, *Blood*, 102, 1548-9.
- Inatomi, T., Nakamura, T., Koizumi, N., et al. (2005) Current concepts and challenges in ocular surface reconstruction using cultivated mucosal epithelial transplantation, *Cornea*, 24, S32-S38.
- Inatomi, T., Nakamura, T., Koizumi, N., et al. (2006) Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation, *Am J Ophthalmol*, 141, 267-275.
- James, S. E., Rowe, A., Ilari, L., et al. (2001) The potential for eye bank limbal rings to generate cultured corneal epithelial allografts, *Cornea*, 20, 488-94.
- Jang, I. K., Ahn, J. I., Shin, J. S., et al. (2006) Transplantation of reconstructed corneal layer composed of corneal epithelium and fibroblasts on a lyophilized amniotic membrane to severely alkali-burned cornea, *Artif Organs*, 30, 424-31.
- Jay H. Krachmer, M. J. M., Edward J. Holland (2004) *Cornea*, 1.
- Jenkins, C., Tuft, S., Liu, C. and Buckley, R. (1993) Limbal transplantation in the management of chronic contact-lens-associated epitheliopathy, *Eye (Lond)*, 7 ( Pt 5), 629-33.
- Jensen, U. B., Lowell, S. and Watt, F. M. (1999) The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis, *Development*, 126, 2409-18.
- Jester, J. V., Moller-Pedersen, T., Huang, J., et al. (1999) The cellular basis of corneal transparency: evidence for 'corneal crystallins', *J Cell Sci*, 112 ( Pt 5), 613-22.

- Johnson, D. H., Bourne, W. M. and Campbell, R. J. (1982a) The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas, *Arch Ophthalmol*, 100, 1942-7.
- Johnson, D. H., Bourne, W. M. and Campbell, R. J. (1982b) The ultrastructure of Descemet's membrane. II. Aphakic bullous keratopathy, *Arch Ophthalmol*, 100, 1948-51.
- Jones, P. H., Harper, S. and Watt, F. M. (1995) Stem cell patterning and fate in human epidermis, *Cell*, 80, 83-93.
- Jones, P. H. and Watt, F. M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression, *Cell*, 73, 713-24.
- Joyce, N. C., Meklir, B., Joyce, S. J. and Zieske, J. D. (1996a) Cell cycle protein expression and proliferative status in human corneal cells, *Invest Ophthalmol Vis Sci*, 37, 645-55.
- Joyce, N. C., Navon, S. E., Roy, S. and Zieske, J. D. (1996b) Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ, *Invest Ophthalmol Vis Sci*, 37, 1566-75.
- Kandavel, G. R. and Chuck, R. S. (2005) Staining properties of deepithelialized human amniotic membrane, *Cornea*, 24, 853-6.
- Kang, Y. and Massague, J. (2004) Epithelial-mesenchymal transitions: twist in development and metastasis, *Cell*, 118, 277-9.
- Karamichos, D., Lakshman, N. and Petroll, W. M. (2007) Regulation of corneal fibroblast morphology and collagen reorganization by extracellular matrix mechanical properties, *Invest Ophthalmol Vis Sci*, 48, 5030-7.
- Kasper, M. (1992) Patterns of cytokeratins and vimentin in guinea pig and mouse eye tissue: evidence for regional variations in intermediate filament expression in limbal epithelium, *Acta Histochem*, 93, 319-32.
- Kasper, M., Moll, R., Stosiek, P. and Karsten, U. (1988) Patterns of cytokeratin and vimentin expression in the human eye, *Histochemistry*, 89, 369-77.
- Kasper, M., Stosiek, P. and Lane, B. (1992) Cytokeratin and vimentin heterogeneity in human cornea, *Acta Histochem*, 93, 371-81.
- Kaufman, P. L. and Lutjen-Drecoll, E. (1975) Total iridectomy in the primate in vivo: surgical technique and postoperative anatomy, *Invest Ophthalmol*, 14, 766-71.
- Kawakita, T., Espana, E. M., He, H., et al. (2005) Intrastromal invasion by limbal epithelial cells is mediated by epithelial-mesenchymal transition activated by air exposure, *Am J Pathol*, 167, 381-93.

- Kawasaki, S., Tanioka, H., Yamasaki, K., et al. (2006) Expression and tissue distribution of p63 isoforms in human ocular surface epithelia, *Exp Eye Res*, 82, 293-9.
- Kaye, D. B. (1980) Epithelial response in penetrating keratoplasty, *Am J Ophthalmol*, 89, 381-7.
- Kazuo Tsubota, S. C. G. T. a. M. L. N. (2002) Anatomy and Physiology of the Ocular Surface, *Ocular Surface Disease Medical and Surgical Management*, Part-1, 3-15.
- Kenyon, K. R. and Tseng, S. C. (1989) Limbal autograft transplantation for ocular surface disorders, *Ophthalmology*, 96, 709-22; discussion 722-3.
- Kessing, S. V. (1968) Mucous gland system of the conjunctiva. A quantitative normal anatomical study, *Acta Ophthalmol (Copenh)*, Suppl 95:1+.
- Kim, H. S., Jun Song, X., de Paiva, C. S., et al. (2004) Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures, *Exp Eye Res*, 79, 41-9.
- Kim, J. C. and Tseng, S. C. (1995) Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas, *Cornea*, 14, 473-84.
- Kim, M., Turnquist, H., Jackson, J., et al. (2002) The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells, *Clin Cancer Res*, 8, 22-8.
- Kim, M. K., Lee, J. L., Oh, J. Y., et al. (2008) Efficient cultivation conditions for human limbal epithelial cells, *J Korean Med Sci*, 23, 864-9.
- Kim, M. K., Lee, J. L., Shin, K. S., et al. (2006) Isolation of putative corneal epithelial stem cells from cultured limbal tissue, *Korean J Ophthalmol*, 20, 55-61.
- Kim, W. J., Mohan, R. R. and Wilson, S. E. (1999) Effect of PDGF, IL-1alpha, and BMP2/4 on corneal fibroblast chemotaxis: expression of the platelet-derived growth factor system in the cornea, *Invest Ophthalmol Vis Sci*, 40, 1364-72.
- Kinoshita, S., Friend, J. and Thoft, R. A. (1981) Sex chromatin of donor corneal epithelium in rabbits, *Invest Ophthalmol Vis Sci*, 21, 434-41.
- Kinoshita, S., Koizumi, N. and Nakamura, T. (2004) Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction, *Exp Eye Res*, 78, 483-91.
- Kinoshita, S. and Nakamura, T. (2004) Development of cultivated mucosal epithelial sheet transplantation for ocular surface reconstruction, *Artif Organs*, 28, 22-7.
- Kiritoshi, A., SundarRaj, N. and Thoft, R. A. (1991) Differentiation in cultured limbal epithelium as defined by keratin expression, *Invest Ophthalmol Vis Sci*, 32, 3073-7.
- Kivela, T. and Uusitalo, M. (1998) Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye, *Prog Retin Eye Res*, 17, 385-428.

- Knapp, A. C., Bosch, F. X., Hergt, M., et al. (1989) Cytokeratins and cytokeratin filaments in subpopulations of cultured human and rodent cells of nonepithelial origin: modes and patterns of formation, *Differentiation*, 42, 81-102.
- Koizumi, N., Cooper, L. J., Fullwood, N. J., et al. (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture, *Invest Ophthalmol Vis Sci*, 43, 2114-21.
- Koizumi, N., Inatomi, T., Quantock, A. J., et al. (2000) Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits, *Cornea*, 19, 65-71.
- Koizumi, N., Inatomi, T., Suzuki, T., et al. (2001a) Cultivated corneal epithelial stem cell transplantation in ocular surface disorders, *Ophthalmology*, 108, 1569-74.
- Koizumi, N., Inatomi, T., Suzuki, T., et al. (2001b) Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome, *Arch Ophthalmol*, 119, 298-300.
- Koizumi, N., Rigby, H., Fullwood, N. J., et al. (2007) Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells, *Graefes Arch Clin Exp Ophthalmol*, 245, 123-34.
- Kolli, S., Ahmad, S., Lako, M. and Figueiredo, F. (2010) Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency, *Stem Cells*, 28, 597-610.
- Kolli, S., Lako, M., Figueiredo, F., et al. (2008) Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane, *Regen Med*, 3, 329-42.
- Krishnan, S., Sudha, B. and Krishnakumar, S. (2010) Isoforms of p63 in corneal stem cells cultured on human amniotic membrane, *Biologicals*, 38, 570-6.
- Kruse, F. E. (1994) Stem cells and corneal epithelial regeneration, *Eye (Lond)*, 8 ( Pt 2), 170-83.
- Kruse, F. E., Chen, J. J., Tsai, R. J. and Tseng, S. C. (1990) Conjunctival transdifferentiation is due to the incomplete removal of limbal basal epithelium, *Invest Ophthalmol Vis Sci*, 31, 1903-13.
- Kruse, F. E. and Tseng, S. C. (1991) A serum-free clonal growth assay for limbal, peripheral, and central corneal epithelium, *Invest Ophthalmol Vis Sci*, 32, 2086-95.
- Kumagai, Y., Kurokawa, M. S., Ueno, H., et al. (2010) Induction of Corneal Epithelium-Like Cells From Cynomolgus Monkey Embryonic Stem Cells and Their Experimental Transplantation to Damaged Cornea, *Cornea*.
- Kurpakus, M. A., Maniaci, M. T. and Esco, M. (1994) Expression of keratins K12, K4 and K14 during development of ocular surface epithelium, *Curr Eye Res*, 13, 805-14.

- Kurpakus, M. A., Quaranta, V. and Jones, J. C. (1991) Surface relocation of alpha 6 beta 4 integrins and assembly of hemidesmosomes in an in vitro model of wound healing, *J Cell Biol*, 115, 1737-50.
- Kurpakus, M. A., Stock, E. L. and Jones, J. C. (1990) Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium, *Invest Ophthalmol Vis Sci*, 31, 448-56.
- Kurz, D. J., Decary, S., Hong, Y. and Erusalimsky, J. D. (2000) Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells, *J Cell Sci*, 113 ( Pt 20), 3613-22.
- Lajtha, L. G. (1979a) Stem cell concepts, *Differentiation*, 14, 23-34.
- Lajtha, L. G. (1979b) Stem cell concepts, *Nouv Rev Fr Hematol*, 21, 59-65.
- Latvala, T., Paallysaho, T., Tervo, K. and Tervo, T. (1996) Distribution of alpha 6 and beta 4 integrins following epithelial abrasion in the rabbit cornea, *Acta Ophthalmol Scand*, 74, 21-5.
- Lauffenburger, D. A. and Horwitz, A. F. (1996) Cell migration: a physically integrated molecular process, *Cell*, 84, 359-69.
- Lauweryns, B., van den Oord, J. J. and Missotten, L. (1993) The transitional zone between limbus and peripheral cornea. An immunohistochemical study, *Invest Ophthalmol Vis Sci*, 34, 1991-9.
- Lavker, R. M., Miller, S., Wilson, C., et al. (1993a) Hair follicle stem cells: their location, role in hair cycle, and involvement in skin tumor formation, *J Invest Dermatol*, 101, 16S-26S.
- Lavker, R. M., Miller, S. J. and Sun, T. T. (1993b) Epithelial stem cells, hair follicles, and tumor formation, *Recent Results Cancer Res*, 128, 31-43.
- Lavker, R. M., Tseng, S. C. and Sun, T. T. (2004) Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle, *Exp Eye Res*, 78, 433-46.
- Lawrenson, J. G. and Ruskell, G. L. (1991) The structure of corpuscular nerve endings in the limbal conjunctiva of the human eye, *J Anat*, 177, 75-84.
- Lekhanont, K., Choubtum, L., Chuck, R. S., et al. (2009) A serum- and feeder-free technique of culturing human corneal epithelial stem cells on amniotic membrane, *Mol Vis*, 15, 1294-302.
- Levis, H. and Daniels, J. T. (2009) New technologies in limbal epithelial stem cell transplantation, *Curr Opin Biotechnol*, 20, 593-7.
- Li, A., Simmons, P. J. and Kaur, P. (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype, *Proc Natl Acad Sci U S A*, 95, 3902-7.

- Li, D. Q., Chen, Z., Song, X. J., et al. (2005a) Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness, *Exp Eye Res*, 80, 581-90.
- Li, S., Guan, J. L. and Chien, S. (2005b) Biochemistry and biomechanics of cell motility, *Annu Rev Biomed Eng*, 7, 105-50.
- Li, T. and Lu, L. (2005) Epidermal growth factor-induced proliferation requires down-regulation of Pax6 in corneal epithelial cells, *J Biol Chem*, 280, 12988-95.
- Li, W., Hayashida, Y., Chen, Y. T., et al. (2008) Air exposure induced squamous metaplasia of human limbal epithelium, *Invest Ophthalmol Vis Sci*, 49, 154-62.
- Li, W., He, H., Kuo, C. L., et al. (2006) Basement membrane dissolution and reassembly by limbal corneal epithelial cells expanded on amniotic membrane, *Invest Ophthalmol Vis Sci*, 47, 2381-9.
- Lim, L. S., Poh, R. W., Riau, A. K., et al. (2010) Biological and ultrastructural properties of acelagraft, a freeze-dried gamma-irradiated human amniotic membrane, *Arch Ophthalmol*, 128, 1303-10.
- Lindberg, K., Brown, M. E., Chaves, H. V., et al. (1993) In vitro propagation of human ocular surface epithelial cells for transplantation, *Invest Ophthalmol Vis Sci*, 34, 2672-9.
- Liu, C. Y., Zhu, G., Westerhausen-Larson, A., et al. (1993) Cornea-specific expression of K12 keratin during mouse development, *Curr Eye Res*, 12, 963-74.
- Liu, J. J., Kao, W. W. and Wilson, S. E. (1999) Corneal epithelium-specific mouse keratin K12 promoter, *Exp Eye Res*, 68, 295-301.
- Liu, S., Li, J., Wang, C., et al. (2006a) Human limbal progenitor cell characteristics are maintained in tissue culture, *Ann Acad Med Singapore*, 35, 80-6.
- Liu, Y., Yanai, R., Lu, Y., et al. (2006b) Promotion by fibronectin of collagen gel contraction mediated by human corneal fibroblasts, *Exp Eye Res*, 83, 1196-204.
- Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods*, 25, 402-8.
- Ljubimov, A. V., Burgeson, R. E., Butkowski, R. J., et al. (1995) Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms, *Lab Invest*, 72, 461-73.
- Lu, J. M., Zhou, Z. Y., Zhang, X. R., et al. (2010) A preliminary study of mesenchymal stem cell-like cells derived from murine corneal stroma, *Graefes Arch Clin Exp Ophthalmol*, 248, 1279-85.

- Lutjen-Drecoll, E. and Kaufman, P. L. (1986) Long-term timolol and epinephrine in monkeys. II. Morphological alterations in trabecular meshwork and ciliary muscle, *Trans Ophthalmol Soc U K*, 105 ( Pt 2), 196-207.
- Lutjen-Drecoll, E., Kaufman, P. L. and Eichhorn, M. (1986) Long-term timolol and epinephrine in monkeys. I. Functional morphology of the ciliary processes, *Trans Ophthalmol Soc U K*, 105 ( Pt 2), 180-95.
- Lyngholm, M., Vorum, H., Nielsen, K., et al. (2008) Differences in the protein expression in limbal versus central human corneal epithelium--a search for stem cell markers, *Exp Eye Res*, 87, 96-105.
- Maas-Szabowski, N., Shimotoyodome, A. and Fusenig, N. E. (1999) Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism, *J Cell Sci*, 112 ( Pt 12), 1843-53.
- Maciag, T., Nettore, R. E., Weinstein, R. and Gilchrist, B. A. (1981) An endocrine approach to the control of epidermal growth: serum-free cultivation of human keratinocytes, *Science*, 211, 1452-4.
- Madhira, S. L., Vemuganti, G., Bhaduri, A., et al. (2008) Culture and characterization of oral mucosal epithelial cells on human amniotic membrane for ocular surface reconstruction, *Mol Vis*, 14, 189-96.
- Madri, J. A., Pratt, B. M., Yurchenco, P. D. and Furthmayr, H. (1984) The ultrastructural organization and architecture of basement membranes, *Ciba Found Symp*, 108, 6-24.
- Majo, F., Rochat, A., Nicolas, M., et al. (2008) Oligopotent stem cells are distributed throughout the mammalian ocular surface, *Nature*, 456, 250-4.
- Mann, I. (1944) A Study of Epithelial Regeneration in the Living Eye, *Br J Ophthalmol*, 28, 26-40.
- Marcelo, C. L. (1979) Differential effects of cAMP and cGMP on in vitro epidermal cell growth, *Exp Cell Res*, 120, 201-10.
- Marshall, G. E., Konstas, A. G. and Lee, W. R. (1993) Collagens in ocular tissues, *Br J Ophthalmol*, 77, 515-24.
- Masur, S. K., Cheung, J. K. and Antohi, S. (1993) Identification of integrins in cultured corneal fibroblasts and in isolated keratocytes, *Invest Ophthalmol Vis Sci*, 34, 2690-8.
- Matic, M., Petrov, I. N., Chen, S., et al. (1997) Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity, *Differentiation*, 61, 251-60.
- Matsumoto, B. (2002) Cell Biological applications of Confocal Microscopy, *Methods in Cell Biology*, 70.
- Maurice, D. M. (1972) The location of the fluid pump in the cornea, *J Physiol*, 221, 43-54.

- Mazzotta, C., Traversi, C., Baiocchi, S., et al. (2008) Corneal healing after riboflavin ultraviolet-A collagen cross-linking determined by confocal laser scanning microscopy in vivo: early and late modifications, *Am J Ophthalmol*, 146, 527-533.
- McGowan, S. L., Edelhauser, H. F., Pfister, R. R. and Whikehart, D. R. (2007) Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas, *Mol Vis*, 13, 1984-2000.
- McIntosh Ambrose, W., Salahuddin, A., So, S., et al. (2009) Collagen Vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers, *J Biomed Mater Res B Appl Biomater*, 90, 818-31.
- McLaughlin, B. J., Caldwell, R. B., Sasaki, Y. and Wood, T. O. (1985) Freeze-fracture quantitative comparison of rabbit corneal epithelial and endothelial membranes, *Curr Eye Res*, 4, 951-61.
- Meller, D., Pires, R. T. and Tseng, S. C. (2002) Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures, *Br J Ophthalmol*, 86, 463-71.
- Menichella, D. M., Goodenough, D. A., Sirkowski, E., et al. (2003) Connexins are critical for normal myelination in the CNS, *J Neurosci*, 23, 5963-73.
- Meyer-Blazejewska, E. A., Kruse, F. E., Bitterer, K., et al. (2010) Preservation of the limbal stem cell phenotype by appropriate culture techniques, *Invest Ophthalmol Vis Sci*, 51, 765-74.
- Michel, M., Torok, N., Godbout, M. J., et al. (1996) Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage, *J Cell Sci*, 109 ( Pt 5), 1017-28.
- Miller, S. J., Sun, T. T. and Lavker, R. M. (1993) Hair follicles, stem cells, and skin cancer, *J Invest Dermatol*, 100, 288S-294S.
- Mimura, T., Yamagami, S., Yokoo, S., et al. (2010) Selective isolation of young cells from human corneal endothelium by the sphere-forming assay, *Tissue Eng Part C Methods*, 16, 803-12.
- Minami, Y., Sugihara, H. and Oono, S. (1993) Reconstruction of cornea in three-dimensional collagen gel matrix culture, *Invest Ophthalmol Vis Sci*, 34, 2316-24.
- Miyashita, H., Shimmura, S., Higa, K., et al. (2008) A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations, *Tissue Eng Part A*, 14, 1275-82.
- Moll, R., Franke, W. W., Schiller, D. L., et al. (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells, *Cell*, 31, 11-24.

- Moore, J. E., McMullen, C. B., Mahon, G. and Adamis, A. P. (2002) The corneal epithelial stem cell, *DNA Cell Biol*, 21, 443-51.
- Morishige, N., Ko, J. A., Morita, Y. and Nishida, T. (2010) Expression of semaphorin 3A in the rat corneal epithelium during wound healing, *Biochem Biophys Res Commun*, 395, 451-7.
- Morris, R. J., Coulter, K., Tryson, K. and Steinberg, S. R. (1997) Evidence that cutaneous carcinogen-initiated epithelial cells from mice are quiescent rather than actively cycling, *Cancer Res*, 57, 3436-43.
- Muller, L. J., Pels, L. and Vrensen, G. F. (1995) Novel aspects of the ultrastructural organization of human corneal keratocytes, *Invest Ophthalmol Vis Sci*, 36, 2557-67.
- Murakami, J., Nishida, T. and Otori, T. (1992) Coordinated appearance of beta 1 integrins and fibronectin during corneal wound healing, *J Lab Clin Med*, 120, 86-93.
- Nagasaki, T. and Zhao, J. (2005) Uniform distribution of epithelial stem cells in the bulbar conjunctiva, *Invest Ophthalmol Vis Sci*, 46, 126-32.
- Nakagawa, S., Nishida, T., Kodama, Y. and Itoi, M. (1990) Spreading of cultured corneal epithelial cells on fibronectin and other extracellular matrices, *Cornea*, 9, 125-30.
- Nakamura, T., Inatomi, T., Sekiyama, E., et al. (2006a) Novel clinical application of sterilized, freeze-dried amniotic membrane to treat patients with pterygium, *Acta Ophthalmol Scand*, 84, 401-5.
- Nakamura, T., Inatomi, T., Sotozono, C., et al. (2004a) Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders, *Br J Ophthalmol*, 88, 1280-4.
- Nakamura, T., Inatomi, T., Sotozono, C., et al. (2006b) Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease, *Ophthalmology*, 113, 1765-72.
- Nakamura, T., Inatomi, T., Sotozono, C., et al. (2004b) Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease, *Acta Ophthalmol Scand*, 82, 468-71.
- Nakamura, T. and Kinoshita, S. (2003) Ocular surface reconstruction using cultivated mucosal epithelial stem cells, *Cornea*, 22, S75-80.
- Nakamura, T., Koizumi, N., Tsuzuki, M., et al. (2003) Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease, *Cornea*, 22, 70-1.
- Nakamura, T., Yoshitani, M., Rigby, H., et al. (2004c) Sterilized, freeze-dried amniotic membrane: a useful substrate for ocular surface reconstruction, *Invest Ophthalmol Vis Sci*, 45, 93-9.

- Niknejad, H., Peirovi, H., Jorjani, M., et al. (2008) Properties of the amniotic membrane for potential use in tissue engineering, *Eur Cell Mater*, 15, 88-99.
- Nishida, K., Yamato, M., Hayashida, Y., et al. (2004a) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface, *Transplantation*, 77, 379-85.
- Nishida, K., Yamato, M., Hayashida, Y., et al. (2004b) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium, *N Engl J Med*, 351, 1187-96.
- Nishida, T., Nakamura, M., Murakami, J., et al. (1992) Epidermal growth factor stimulates corneal epithelial cell attachment to fibronectin through a fibronectin receptor system, *Invest Ophthalmol Vis Sci*, 33, 2464-9.
- Noort, W. A., Kruisselbrink, A. B., in't Anker, P. S., et al. (2002) Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice, *Exp Hematol*, 30, 870-8.
- Notara, M., Haddow, D. B., MacNeil, S. and Daniels, J. T. (2007) A xenobiotic-free culture system for human limbal epithelial stem cells, *Regen Med*, 2, 919-27.
- Onguchi, T., Han, K. Y., Chang, J. H. and Azar, D. T. (2009) Membrane type-1 matrix metalloproteinase potentiates basic fibroblast growth factor-induced corneal neovascularization, *Am J Pathol*, 174, 1564-71.
- O'Sullivan, F. and Clynes, M. (2007) Limbal stem cells, a review of their identification and culture for clinical use, *Cytotechnology*, 53, 101-6.
- Paallysaho, T., Tervo, K., Tervo, T., et al. (1992) Distribution of integrins alpha 6 and beta 4 in the rabbit corneal epithelium after anterior keratectomy, *Cornea*, 11, 523-8.
- Pajoohesh-Ganji, A., Pal-Ghosh, S., Simmens, S. J. and Stepp, M. A. (2006) Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse, *Stem Cells*, 24, 1075-86.
- Pal-Ghosh, S., Tadvalkar, G., Jurjus, R. A., et al. (2008) BALB/c and C57BL6 mouse strains vary in their ability to heal corneal epithelial debridement wounds, *Exp Eye Res*, 87, 478-86.
- Papini, S., Rosellini, A., Nardi, M., et al. (2005) Selective growth and expansion of human corneal epithelial basal stem cells in a three-dimensional-organ culture, *Differentiation*, 73, 61-8.
- Parnigotto, P. P., Bassani, V., Gottardo, A., et al. (1996) Growth, morphology, morphometry and keratin patterns of bovine corneal epithelial cells cultured in vitro, *Ann Anat*, 178, 545-51.

- Parsa, R., Yang, A., McKeon, F. and Green, H. (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes, *J Invest Dermatol*, 113, 1099-105.
- Pellegrini, G., Dellambra, E., Golisano, O., et al. (2001) p63 identifies keratinocyte stem cells, *Proc Natl Acad Sci U S A*, 98, 3156-61.
- Pellegrini, G., Golisano, O., Paterna, P., et al. (1999a) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface, *J Cell Biol*, 145, 769-82.
- Pellegrini, G., Ranno, R., Stracuzzi, G., et al. (1999b) The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin, *Transplantation*, 68, 868-79.
- Pellegrini, G., Traverso, C. E., Franzi, A. T., et al. (1997) Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium, *Lancet*, 349, 990-3.
- Perrella, G., Brusini, P., Spelat, R., et al. (2007) Expression of haematopoietic stem cell markers, CD133 and CD34 on human corneal keratocytes, *Br J Ophthalmol*, 91, 94-9.
- Pessina, A., Bonomi, A., Baglio, C., et al. (2008) Microbiological risk assessment in stem cell manipulation, *Crit Rev Microbiol*, 34, 1-12.
- Pessina, A., Bonomi, A., Sisto, F., et al. (2010) CD45+/CD133+ positive cells expanded from umbilical cord blood expressing PDX-1 and markers of pluripotency, *Cell Biol Int*, 34, 783-90.
- Pfister, R. R. (1973) The normal surface of corneal epithelium: a scanning electron microscopic study, *Invest Ophthalmol*, 12, 654-68.
- Pittenger, M. F., Mackay, A. M., Beck, S. C., et al. (1999) Multilineage potential of adult human mesenchymal stem cells, *Science*, 284, 143-7.
- Pitz, S. and Moll, R. (2002) Intermediate-filament expression in ocular tissue, *Prog Retin Eye Res*, 21, 241-62.
- Polisetty, N., Fatima, A., Madhira, S. L., et al. (2008) Mesenchymal cells from limbal stroma of human eye, *Mol Vis*, 14, 431-42.
- Poon, A. C., Geerling, G., Dart, J. K., et al. (2001) Autologous serum eyedrops for dry eyes and epithelial defects: clinical and in vitro toxicity studies, *Br J Ophthalmol*, 85, 1188-97.
- Powell, D. W. (1981) Barrier function of epithelia, *Am J Physiol*, 241, G275-88.
- Puangricharern, V. and Tseng, S. C. (1995) Cytologic evidence of corneal diseases with limbal stem cell deficiency, *Ophthalmology*, 102, 1476-85.

- Puck, T. T., Marcus, P. I. and Cieciura, S. J. (1956) Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer, *J Exp Med*, 103, 273-83.
- Quinlan, R. A., Schiller, D. L., Hatzfeld, M., et al. (1985) Patterns of expression and organization of cytokeratin intermediate filaments, *Ann N Y Acad Sci*, 455, 282-306.
- Raeder, S., Utheim, T. P., Utheim, O. A., et al. (2007) Effect of limbal explant orientation on the histology, phenotype, ultrastructure and barrier function of cultured limbal epithelial cells, *Acta Ophthalmol Scand*, 85, 377-86.
- Rama, P., Bonini, S., Lambiase, A., et al. (2001) Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency, *Transplantation*, 72, 1478-85.
- Revoltella, R. P., Papini, S., Rosellini, A. and Micheli, M. (2007) Epithelial stem cells of the eye surface, *Cell Prolif*, 40, 445-61.
- Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells, *Cell*, 6, 331-43.
- Rheinwald, J. G. and Green, H. (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes, *Nature*, 265, 421-4.
- Robles, S. J. and Adami, G. R. (1998) Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts, *Oncogene*, 16, 1113-23.
- Rochat, A., Kobayashi, K. and Barrandon, Y. (1994) Location of stem cells of human hair follicles by clonal analysis, *Cell*, 76, 1063-73.
- Roelen, B. A. and Dijke, P. (2003) Controlling mesenchymal stem cell differentiation by TGFβ family members, *J Orthop Sci*, 8, 740-8.
- Romano, A. C., Espana, E. M., Yoo, S. H., et al. (2003) Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry, *Invest Ophthalmol Vis Sci*, 44, 5125-9.
- Romano, R. A., Birkaya, B. and Sinha, S. (2006) Defining the regulatory elements in the proximal promoter of DeltaNp63 in keratinocytes: Potential roles for Sp1/Sp3, NF-κB, and p63, *J Invest Dermatol*, 126, 1469-79.
- Romanov, Y. A., Svintsitskaya, V. A. and Smirnov, V. N. (2003) Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord, *Stem Cells*, 21, 105-10.
- Rothnagel, J. A., Greenhalgh, D. A., Gagne, T. A., et al. (1993) Identification of a calcium-inducible, epidermal-specific regulatory element in the 3'-flanking region of the human keratin 1 gene, *J Invest Dermatol*, 101, 506-13.

- Sangwan, V. S., Burman, S., Tejwani, S., et al. (2007) Amniotic membrane transplantation: a review of current indications in the management of ophthalmic disorders, *Indian J Ophthalmol*, 55, 251-60.
- Sangwan, V. S., Matalia, H. P., Vemuganti, G. K., et al. (2006) Clinical outcome of autologous cultivated limbal epithelium transplantation, *Indian J Ophthalmol*, 54, 29-34.
- Sangwan, V. S., Murthy, S. I., Vemuganti, G. K., et al. (2005) Cultivated corneal epithelial transplantation for severe ocular surface disease in vernal keratoconjunctivitis, *Cornea*, 24, 426-30.
- Sangwan, V. S., Vemuganti, G. K., Iftekhar, G., et al. (2003a) Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application, *Cornea*, 22, 478-81.
- Sangwan, V. S., Vemuganti, G. K., Singh, S. and Balasubramanian, D. (2003b) Successful reconstruction of damaged ocular outer surface in humans using limbal and conjunctival stem cell culture methods, *Biosci Rep*, 23, 169-74.
- Schermer, A., Galvin, S. and Sun, T. T. (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells, *J Cell Biol*, 103, 49-62.
- Schlotzer-Schrehardt, U., Dietrich, T., Saito, K., et al. (2007) Characterization of extracellular matrix components in the limbal epithelial stem cell compartment, *Exp Eye Res*, 85, 845-60.
- Schlotzer-Schrehardt, U. and Kruse, F. E. (2005) Identification and characterization of limbal stem cells, *Exp Eye Res*, 81, 247-64.
- Schneider, I. C. and Haugh, J. M. (2005) Quantitative elucidation of a distinct spatial gradient-sensing mechanism in fibroblasts, *J Cell Biol*, 171, 883-92.
- Schneider, I. C. and Haugh, J. M. (2006) Mechanisms of gradient sensing and chemotaxis: conserved pathways, diverse regulation, *Cell Cycle*, 5, 1130-4.
- Schramek, H., Feifel, E., Healy, E. and Pollack, V. (1997) Constitutively active mutant of the mitogen-activated protein kinase kinase MEK1 induces epithelial dedifferentiation and growth inhibition in madin-darby canine kidney-C7 cells, *J Biol Chem*, 272, 11426-33.
- Schwab, I. R. (1999) Cultured corneal epithelia for ocular surface disease, *Trans Am Ophthalmol Soc*, 97, 891-986.
- Schwab, I. R., Reyes, M. and Isseroff, R. R. (2000a) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease, *Cornea*, 19, 421-6.

- Schwab, I. R., Reyes, M. and Isseroff, R. R. (2000b) Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease(1), *Am J Ophthalmol*, 130, 543-4.
- Schweizer, J. and Winter, H. (1983) Keratin biosynthesis in normal mouse epithelia and in squamous cell carcinomas. mRNA-dependent alterations of the primary structure of distinct keratin subunits in tumors, *J Biol Chem*, 258, 13268-72.
- Scott, R. A., Lauweryns, B., Snead, D. M., et al. (1997) E-cadherin distribution and epithelial basement membrane characteristics of the normal human conjunctiva and cornea, *Eye (Lond)*, 11 ( Pt 5), 607-12.
- Secker GA, D. J. (2009) Limbal epithelial stem cells of the cornea, *StemBook*, Cambridge (MA): Harvard Stem Cell Institute; 2008-.
- 2009 Jun 30.
- Seigel, G. M., Sun, W., Salvi, R., et al. (2003) Human corneal stem cells display functional neuronal properties, *Mol Vis*, 9, 159-63.
- Serrano, M., Lin, A. W., McCurrach, M. E., et al. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a, *Cell*, 88, 593-602.
- Setzer, P. Y., Nichols, B. A. and Dawson, C. R. (1987) Unusual structure of rat conjunctival epithelium. Light and electron microscopy, *Invest Ophthalmol Vis Sci*, 28, 531-7.
- Shanmuganathan, V. A., Foster, T., Kulkarni, B. B., et al. (2007) Morphological characteristics of the limbal epithelial crypt, *Br J Ophthalmol*, 91, 514-9.
- Shanmuganathan, V. A., Rotchford, A. P., Tullo, A. B., et al. (2006) Epithelial proliferative potential of organ cultured corneoscleral rims; implications for allo-limbal transplantation and eye banking, *Br J Ophthalmol*, 90, 55-8.
- Sharifi, A. M., Darabi, R. and Jadidi, K. (2010) Isolation, culture, characterization and optimization of human corneal stem cells, *Biocell*, 34, 53-5.
- Shimazaki, J., Aiba, M., Goto, E., et al. (2002) Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders, *Ophthalmology*, 109, 1285-90.
- Shimazaki, J., Yang, H. Y. and Tsubota, K. (1997) Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns, *Ophthalmology*, 104, 2068-76.
- Shimmura, S., Shimazaki, J., Ohashi, Y. and Tsubota, K. (2001) Antiinflammatory effects of amniotic membrane transplantation in ocular surface disorders, *Cornea*, 20, 408-13.

- Shortt, A. J., Secker, G. A., Munro, P. M., et al. (2007) Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells, *Stem Cells*, 25, 1402-9.
- Shortt, A. J., Secker, G. A., Rajan, M. S., et al. (2008) Ex vivo expansion and transplantation of limbal epithelial stem cells, *Ophthalmology*, 115, 1989-97.
- Silvestri, F., Banavali, S., Savignano, C., et al. (1993) CD34+ cell selection: focus on immunomagnetic beads and chymopapain, *Int J Artif Organs*, 16 Suppl 5, 96-101.
- Silvestri, F., Banavali, S., Yin, M., et al. (1992) Comparison of two methods for concentrating CD34+ cells from patients with acute non-lymphocytic leukemia, *Leuk Lymphoma*, 8, 389-96.
- Sitalakshmi, G., Sudha, B., Madhavan, H. N., et al. (2009) Ex vivo cultivation of corneal limbal epithelial cells in a thermoreversible polymer (Mebiol Gel) and their transplantation in rabbits: an animal model, *Tissue Eng Part A*, 15, 407-15.
- Sivak, J. M. and Fini, M. E. (2002) MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology, *Prog Retin Eye Res*, 21, 1-14.
- Smola, H., Thiekotter, G. and Fusenig, N. E. (1993) Mutual induction of growth factor gene expression by epidermal-dermal cell interaction, *J Cell Biol*, 122, 417-29.
- Solomon, A., Rosenblatt, M., Monroy, D., et al. (2001) Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix, *Br J Ophthalmol*, 85, 444-9.
- Sonnenberg, A., Calafat, J., Janssen, H., et al. (1991) Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion, *J Cell Biol*, 113, 907-17.
- Sosnova, M., Bradl, M. and Forrester, J. V. (2005) CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers, *Stem Cells*, 23, 507-15.
- Spurr, S. J. and Gipson, I. K. (1985) Isolation of corneal epithelium with Dispase II or EDTA. Effects on the basement membrane zone, *Invest Ophthalmol Vis Sci*, 26, 818-27.
- Sridhar, M. S., Bansal, A. K., Sangwan, V. S. and Rao, G. N. (2000) Amniotic membrane transplantation in acute chemical and thermal injury, *Am J Ophthalmol*, 130, 134-7.
- Sridhar, M. S., Sangwan, V. S., Bansal, A. K. and Rao, G. N. (2001) Amniotic membrane transplantation in the management of shield ulcers of vernal keratoconjunctivitis, *Ophthalmology*, 108, 1218-22.
- Stepp, M. A., Spurr-Michaud, S. and Gipson, I. K. (1993) Integrins in the wounded and unwounded stratified squamous epithelium of the cornea, *Invest Ophthalmol Vis Sci*, 34, 1829-44.

- Steuhl, K. P. and Thiel, H. J. (1987) Histochemical and morphological study of the regenerating corneal epithelium after limbus-to-limbus denudation, *Graefes Arch Clin Exp Ophthalmol*, 225, 53-8.
- Streilein, J. W. (2003) New thoughts on the immunology of corneal transplantation, *Eye (Lond)*, 17, 943-8.
- Sudha, B., Sitalakshmi, G., Iyer, G. K. and Krishnakumar, S. (2008) Putative stem cell markers in limbal epithelial cells cultured on intact & denuded human amniotic membrane, *Indian J Med Res*, 128, 149-56.
- Sugrue, S. P. and Zieske, J. D. (1997) ZO1 in corneal epithelium: association to the zonula occludens and adherens junctions, *Exp Eye Res*, 64, 11-20.
- Sun, C. C., Cheng, C. Y., Chien, C. S., et al. (2005) Role of matrix metalloproteinase-9 in ex vivo expansion of human limbal epithelial cells cultured on human amniotic membrane, *Invest Ophthalmol Vis Sci*, 46, 808-15.
- Sun, L., Sun, T. T. and Lavker, R. M. (2000) CLED: a calcium-linked protein associated with early epithelial differentiation, *Exp Cell Res*, 259, 96-106.
- Sun, T. T., Eichner, R., Nelson, W. G., et al. (1983) Keratin classes: molecular markers for different types of epithelial differentiation, *J Invest Dermatol*, 81, 109s-15s.
- Sun, T. T. and Green, H. (1977) Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states, *Nature*, 269, 489-93.
- Sun, T. T., Tseng, S. C. and Lavker, R. M. Location of corneal epithelial stem cells, *Nature*, 463, E10-1; discussion E11.
- Suzuki, K., Tanaka, T., Enoki, M. and Nishida, T. (2000) Coordinated reassembly of the basement membrane and junctional proteins during corneal epithelial wound healing, *Invest Ophthalmol Vis Sci*, 41, 2495-500.
- Szabowski, A., Maas-Szabowski, N., Andrecht, S., et al. (2000) c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin, *Cell*, 103, 745-55.
- Szerenyi, K. D., Wang, X., Gabrielian, K. and McDonnell, P. J. (1994) Keratocyte loss and repopulation of anterior corneal stroma after de-epithelialization, *Arch Ophthalmol*, 112, 973-6.
- Szurman, P., Warga, M., Grisanti, S., et al. (2006) Sutureless amniotic membrane fixation using fibrin glue for ocular surface reconstruction in a rabbit model, *Cornea*, 25, 460-6.
- Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator, *Science*, 251, 1451-5.

- Talbot, M., Carrier, P., Giasson, C. J., et al. (2006) Autologous transplantation of rabbit limbal epithelia cultured on fibrin gels for ocular surface reconstruction, *Mol Vis*, 12, 65-75.
- Talmi, Y. P., Sigler, L., Inge, E., et al. (1991) Antibacterial properties of human amniotic membranes, *Placenta*, 12, 285-8.
- Tan, D. T., Ang, L. P. and Beuerman, R. W. (2004) Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent, *Transplantation*, 77, 1729-34.
- Tanaka, M., Ohnishi, Y. and Kuwabara, T. (1983) Membrane structure of corneal epithelium: freeze-fracture observation, *Jpn J Ophthalmol*, 27, 434-43.
- Tanifuji-Terai, N., Terai, K., Hayashi, Y., et al. (2006) Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth, *Invest Ophthalmol Vis Sci*, 47, 545-51.
- Tanioka, H., Kawasaki, S., Yamasaki, K., et al. (2006) Establishment of a cultivated human conjunctival epithelium as an alternative tissue source for autologous corneal epithelial transplantation, *Invest Ophthalmol Vis Sci*, 47, 3820-7.
- Tervo, K., Tervo, T., van Setten, G. B. and Virtanen, I. (1991) Integrins in human corneal epithelium, *Cornea*, 10, 461-5.
- Thiery, J. P. (2002) Epithelial-mesenchymal transitions in tumour progression, *Nat Rev Cancer*, 2, 442-54.
- Thoft, R. A. and Friend, J. (1983) The X, Y, Z hypothesis of corneal epithelial maintenance, *Invest Ophthalmol Vis Sci*, 24, 1442-3.
- Tondreau, T., Lagneaux, L., Dejeneffe, M., et al. (2004) Isolation of BM mesenchymal stem cells by plastic adhesion or negative selection: phenotype, proliferation kinetics and differentiation potential, *Cytotherapy*, 6, 372-9.
- Touhami, A., Grueterich, M. and Tseng, S. C. (2002) The role of NGF signaling in human limbal epithelium expanded by amniotic membrane culture, *Invest Ophthalmol Vis Sci*, 43, 987-94.
- Tsai, R. J., Li, L. and Chen, J. (2000a) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells(1), *Am J Ophthalmol*, 130, 543.
- Tsai, R. J., Li, L. M. and Chen, J. K. (2000b) Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells, *N Engl J Med*, 343, 86-93.
- Tsai, R. J., Sun, T. T. and Tseng, S. C. (1990) Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits, *Ophthalmology*, 97, 446-55.

- Tseng, S. C. (1989) Concept and application of limbal stem cells, *Eye (Lond)*, 3 ( Pt 2), 141-57.
- Tseng, S. C. (2000) Significant impact of limbal epithelial stem cells, *Indian J Ophthalmol*, 48, 79-81.
- Tseng, S. C., Hirst, L. W., Farazdaghi, M. and Green, W. R. (1984) Goblet cell density and vascularization during conjunctival transdifferentiation, *Invest Ophthalmol Vis Sci*, 25, 1168-76.
- Tseng, S. C., Kruse, F. E., Merritt, J. and Li, D. Q. (1996) Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media, *Curr Eye Res*, 15, 973-84.
- Tseng, S. C., Li, D. Q. and Ma, X. (1999) Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix, *J Cell Physiol*, 179, 325-35.
- Tseng, S. C., Prabhasawat, P., Barton, K., et al. (1998) Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency, *Arch Ophthalmol*, 116, 431-41.
- Tsubota, K., Satake, Y., Kaido, M., et al. (1999) Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation, *N Engl J Med*, 340, 1697-703.
- Tsubota, K., Satake, Y., Ohyama, M., et al. (1996) Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome, *Am J Ophthalmol*, 122, 38-52.
- Tsubota, K., Toda, I., Saito, H., et al. (1995) Reconstruction of the corneal epithelium by limbal allograft transplantation for severe ocular surface disorders, *Ophthalmology*, 102, 1486-96.
- Tsukita, S. and Furuse, M. (1999) Occludin and claudins in tight-junction strands: leading or supporting players?, *Trends Cell Biol*, 9, 268-73.
- Uchida, S., Yokoo, S., Yanagi, Y., et al. (2005) Sphere formation and expression of neural proteins by human corneal stromal cells in vitro, *Invest Ophthalmol Vis Sci*, 46, 1620-5.
- Van Buskirk, E. M. (1989) The anatomy of the limbus, *Eye (Lond)*, 3 ( Pt 2), 101-8.
- Varghese, V. M., Prasad, T. and Kumary, T. V. (2010) Optimization of culture conditions for an efficient xeno-feeder free limbal cell culture system towards ocular surface regeneration, *Microsc Res Tech*.

- Vascotto, S. G. and Griffith, M. (2006) Localization of candidate stem and progenitor cell markers within the human cornea, limbus, and bulbar conjunctiva in vivo and in cell culture, *Anat Rec A Discov Mol Cell Evol Biol*, 288, 921-31.
- Vemuganti, G. K., Kashyap, S., Sangwan, V. S. and Singh, S. (2004) Ex-vivo potential of cadaveric and fresh limbal tissues to regenerate cultured epithelium, *Indian J Ophthalmol*, 52, 113-20.
- Wang, D. Y., Hsueh, Y. J., Yang, V. C. and Chen, J. K. (2003) Propagation and phenotypic preservation of rabbit limbal epithelial cells on amniotic membrane, *Invest Ophthalmol Vis Sci*, 44, 4698-704.
- Wang, J., Lin, A. and Lu, L. (2010) Effect of EGF-induced HDAC6 activation on corneal epithelial wound healing, *Invest Ophthalmol Vis Sci*, 51, 2943-8.
- Waring, G. O., 3rd, Bourne, W. M., Edelhauser, H. F. and Kenyon, K. R. (1982) The corneal endothelium. Normal and pathologic structure and function, *Ophthalmology*, 89, 531-90.
- Watanabe, K., Nishida, K., Yamato, M., et al. (2004) Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2, *FEBS Lett*, 565, 6-10.
- Watt, F. M. (1989) Terminal differentiation of epidermal keratinocytes, *Curr Opin Cell Biol*, 1, 1107-15.
- Watt, F. M. and Jones, P. H. (1993) Expression and function of the keratinocyte integrins, *Dev Suppl*, 185-92.
- Wayner, E. A., Orlando, R. A. and Cheresch, D. A. (1991) Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface, *J Cell Biol*, 113, 919-29.
- Wei, Z. G., Cotsarelis, G., Sun, T. T. and Lavker, R. M. (1995) Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis, *Invest Ophthalmol Vis Sci*, 36, 236-46.
- Wei, Z. G., Wu, R. L., Lavker, R. M. and Sun, T. T. (1993) In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia. Implications on conjunctival epithelial transdifferentiation and stem cells, *Invest Ophthalmol Vis Sci*, 34, 1814-28.
- Whikehart, D. R., Parikh, C. H., Vaughn, A. V., et al. (2005) Evidence suggesting the existence of stem cells for the human corneal endothelium, *Mol Vis*, 11, 816-24.
- Wilson, S. E., Mohan, R. R., Ambrosio, R., Jr., et al. (2001) The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells, *Prog Retin Eye Res*, 20, 625-37.

- Wilson, S. E., Walker, J. W., Chwang, E. L. and He, Y. G. (1993) Hepatocyte growth factor, keratinocyte growth factor, their receptors, fibroblast growth factor receptor-2, and the cells of the cornea, *Invest Ophthalmol Vis Sci*, 34, 2544-61.
- Winter, H. and Schweizer, J. (1983) Keratin synthesis in normal mouse epithelia and in squamous cell carcinomas: evidence in tumors for masked mRNA species coding for high molecular weight keratin polypeptides, *Proc Natl Acad Sci U S A*, 80, 6480-4.
- Winter, H., Schweizer, J. and Goerttler, K. (1983) Keratin polypeptide composition as a biochemical tool for the discrimination of benign and malignant epithelial lesions in man, *Arch Dermatol Res*, 275, 27-34.
- Wirtschafter, J. D., Ketcham, J. M., Weinstock, R. J., et al. (1999) Mucocutaneous junction as the major source of replacement palpebral conjunctival epithelial cells, *Invest Ophthalmol Vis Sci*, 40, 3138-46.
- Wolosin, J. M., Budak, M. T. and Akinci, M. A. (2004) Ocular surface epithelial and stem cell development, *Int J Dev Biol*, 48, 981-91.
- Wolosin, J. M., Xiong, X., Schutte, M., et al. (2000) Stem cells and differentiation stages in the limbo-corneal epithelium, *Prog Retin Eye Res*, 19, 223-55.
- Xu, L., Overbeek, P. A. and Reneker, L. W. (2002) Systematic analysis of E-, N- and P-cadherin expression in mouse eye development, *Exp Eye Res*, 74, 753-60.
- Yaeger, P. C., Stiles, C. D. and Rollins, B. J. (1991) Human keratinocyte growth-promoting activity on the surface of fibroblasts, *J Cell Physiol*, 149, 110-6.
- Yamagami, S., Ebihara, N., Usui, T., et al. (2006) Bone marrow-derived cells in normal human corneal stroma, *Arch Ophthalmol*, 124, 62-9.
- Yamagami, S., Yokoo, S., Mimura, T., et al. (2007) Distribution of precursors in human corneal stromal cells and endothelial cells, *Ophthalmology*, 114, 433-9.
- Yamaya, M., Hosoda, M., Suzuki, T., et al. (2002) Human airway epithelial cell culture, *Methods Mol Biol*, 188, 7-16.
- Yang, A., Kaghad, M., Wang, Y., et al. (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities, *Mol Cell*, 2, 305-16.
- Yang, A. and McKeon, F. (2000) P63 and P73: P53 mimics, menaces and more, *Nat Rev Mol Cell Biol*, 1, 199-207.
- Yang, A., Schweitzer, R., Sun, D., et al. (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development, *Nature*, 398, 714-8.
- Yang, X., Qu, L., Wang, X., et al. (2007) Plasticity of epidermal adult stem cells derived from adult goat ear skin, *Mol Reprod Dev*, 74, 386-96.

- Ye, J., Lee, S. Y., Kook, K. H. and Yao, K. (2008) Bone marrow-derived progenitor cells promote corneal wound healing following alkali injury, *Graefes Arch Clin Exp Ophthalmol*, 246, 217-22.
- Yeung, A. M., Tint, N. L., Kulkarni, B. B., et al. (2009) Infant limbus: an immunohistological study, *Exp Eye Res*, 88, 1161-4.
- Yokoo, S., Yamagami, S., Usui, T., et al. (2008) Human corneal epithelial equivalents for ocular surface reconstruction in a complete serum-free culture system without unknown factors, *Invest Ophthalmol Vis Sci*, 49, 2438-43.
- Yokoo, S., Yamagami, S., Yanagi, Y., et al. (2005) Human corneal endothelial cell precursors isolated by sphere-forming assay, *Invest Ophthalmol Vis Sci*, 46, 1626-31.
- Yoshida, S., Shimmura, S., Kawakita, T., et al. (2006) Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces, *Invest Ophthalmol Vis Sci*, 47, 4780-6.
- Yoshida, S., Shimmura, S., Shimazaki, J., et al. (2005) Serum-free spheroid culture of mouse corneal keratocytes, *Invest Ophthalmol Vis Sci*, 46, 1653-8.
- Yoshida, Y., Ban, Y. and Kinoshita, S. (2009) Tight junction transmembrane protein claudin subtype expression and distribution in human corneal and conjunctival epithelium, *Invest Ophthalmol Vis Sci*, 50, 2103-8.
- Yoshioka, R., Shiraishi, A., Kobayashi, T., et al. Corneal epithelial wound healing impaired in keratinocyte-specific HB-EGF-deficient mice in vivo and in vitro, *Invest Ophthalmol Vis Sci*, 51, 5630-9.
- Young, A. L., Cheng, A. C., Ng, H. K., et al. (2004) The use of autologous serum tears in persistent corneal epithelial defects, *Eye (Lond)*, 18, 609-14.
- Zhang, X., Sun, H., Tang, X., et al. (2005) Comparison of cell-suspension and explant culture of rabbit limbal epithelial cells, *Exp Eye Res*, 80, 227-33.
- Zhou, M., Li, X. M. and Lavker, R. M. (2006) Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells. A comparison of limbal and corneal epithelial basal cells, *J Biol Chem*, 281, 19600-9.
- Zhu, J., Woods, D., McMahon, M. and Bishop, J. M. (1998) Senescence of human fibroblasts induced by oncogenic Raf, *Genes Dev*, 12, 2997-3007.
- Zieske, J. D., Higashijima, S. C., Spurr-Michaud, S. J. and Gipson, I. K. (1987) Biosynthetic responses of the rabbit cornea to a keratectomy wound, *Invest Ophthalmol Vis Sci*, 28, 1668-77.
- Zieske, J. D., Hutcheon, A. E., Guo, X., et al. (2001) TGF-beta receptor types I and II are differentially expressed during corneal epithelial wound repair, *Invest Ophthalmol Vis Sci*, 42, 1465-71.

- Zieske, J. D. and Wasson, M. (1993) Regional variation in distribution of EGF receptor in developing and adult corneal epithelium, *J Cell Sci*, 106 ( Pt 1), 145-52.
- Zito-Abbad, E., Borderie, V. M., Baudrimont, M., et al. (2006) Corneal epithelial cultures generated from organ-cultured limbal tissue: factors influencing epithelial cell growth, *Curr Eye Res*, 31, 391-9.
- Zvaifler, N. J., Marinova-Mutafchieva, L., Adams, G., et al. (2000) Mesenchymal precursor cells in the blood of normal individuals, *Arthritis Res*, 2, 477-88.

# 8.0 Section

---

# Appendices

	<b>C<sub>T</sub></b>		
	<b>23 No-3T3</b>	<b>27 No-3T3</b>	<b>29 No-3T3</b>
<b>CK3</b>	33.83466667	30.465	32.087
<b>CK12</b>	34.95466667	32.66033333	27.18433333
<b>p63</b>	27.7215	28.04566667	27.59533333
<b>Integrinβ4</b>	24.86	25.90133333	24.609
<b>Integrinα6</b>	25.9865	27.62333333	25.61533333
<b>ZO1</b>	30.487	30.907	29.69266667
<b>B-actin</b>	24.943	25.185	24.2
	<b>23 Co-3T3</b>	<b>27 Co-3T3</b>	<b>29 Co-3T3</b>
<b>CK3</b>	32.84166667	30.29466667	31.334
<b>CK12</b>	33.513	32.878	30.12666667
<b>p63</b>	27.8045	27.97566667	28.57733333
<b>Integrinβ4</b>	24.1965	25.9	25.31633333
<b>Integrinα6</b>	25.9835	27.35533333	27.02766667
<b>ZO1</b>	29.8905	30.29066667	29.93533333
<b>B-actin</b>	24.00866667	25.20566667	24.608
	<b>23 Bot-3T3</b>	<b>27 Bot-3T3</b>	<b>29 Bot-3T3</b>
<b>CK3</b>	29.91133333	34.16366667	30.41133333
<b>CK12</b>	32.49	<b>35.892</b>	30.254
<b>p63</b>	26.9625	27.34433333	27.608
<b>Integrin β4</b>	24.9735	25.24066667	25.49066667
<b>Integrin α6</b>	26.7685	26.98466667	27.30066667
<b>ZO1</b>	29.8135	30.75133333	30.43366667
<b>B-actin</b>	24.764	24.93666667	24.80166667

**Appendices 1:** Raw C<sub>T</sub> values of three biological replicates, in condition A no-3T3, condition B Co-3T3 and condition C Bot-3T3. Any gene with C<sub>T</sub> value above 35 is excluded from analysis. (Indicated in red)

	<b>C<sub>T</sub></b>		
	<b>46 HAM No-3T3</b>	<b>47 HAM No-3T3</b>	<b>48 HAM No-3T3</b>
<b>CK3</b>	36.897	32.88833333	35.69366667
<b>CK12</b>	33.49966667	29.01233333	34.24066667
<b>p63</b>	27.1425	26.89366667	27.86466667
<b>Integrin β4</b>	24.55	24.92033333	25.72066667
<b>Integrin α6</b>	25.585	26.01066667	26.348
<b>ZO1</b>	29.1415	29.89966667	29.191
<b>B-actin</b>	23.97266667	24.651	24.555
	<b>46 HAM Co-3T3</b>	<b>47 HAM Co-3T3</b>	<b>48 HAM Co-3T3</b>
<b>CK3</b>	31.93266667	31.054	35.617
<b>CK12</b>	32.595	31.28966667	35.01733333
<b>p63</b>	29.55	28.917	27.78
<b>Integrin β4</b>	26.2955	25.32233333	23.948
<b>Integrin α6</b>	27.9045	26.80966667	25.606
<b>ZO1</b>	31.7515	29.75366667	29.91633333
<b>B-actin</b>	25.155	22.83033333	24.06366667
	<b>46 HAM Bot-3T3</b>	<b>47 HAM Bot-3T3</b>	<b>48 HAM Bot-3T3</b>
<b>CK3</b>	34.33866667	30.12233333	35.06366667
<b>CK12</b>	32.614	30.51633333	35.91233333
<b>p63</b>	27.998	27.63533333	28.456
<b>Integrin β4</b>	24.274	24.10766667	24.64366667
<b>Integrin α6</b>	26.337	25.83733333	25.95533333
<b>ZO1</b>	29.733	29.466	29.07233333
<b>B-actin</b>	24.64466667	23.79133333	23.864

**Appendices 2:** Raw C<sub>T</sub> values of three biological replicates, in condition D dHAM no-3T3, condition E dHAM Co-3T3 and condition F dHAM Bot-3T3. Any genes with CT value above 35 were excluded from analysis (Indicated in red).

	<b>Av C<sub>T</sub></b>	<b>Delta C<sub>T</sub></b>	<b>ΔΔC<sub>T</sub></b>	<b>RQ</b>
	<b>Condition A No-3T3</b>			
<b>CK3</b>	32.1288889	7.35288889		
<b>CK12</b>	31.5997778	6.82377778		
<b>p63</b>	27.7875	3.0115		
<b>Integrin β4</b>	25.1234444	0.34744444		
<b>Integrin α6</b>	26.4083889	1.63238889		
<b>ZO1</b>	30.3622222	5.58622222		
<b>B-actin</b>	24.776	0		

**Appendices 3:** The average C<sub>T</sub>. and Δ C<sub>T</sub> values of calibrated sample condition A no-3T3.

	<b>Condition B Co-3T3</b>	<b>ΔC<sub>T</sub></b>	<b>ΔΔ C<sub>T</sub></b>	<b>RQ</b>	<b>RQ with -1</b>
<b>CK3</b>	31.4901111	6.88266667	-0.47022222	1.3853228	
<b>CK12</b>	32.1725556	7.56511111	0.741333333	0.5981863	-1.6717201
<b>p63</b>	28.1191667	3.51172222	0.500222222	0.7069979	-1.4144314
<b>Integrin β4</b>	25.1376111	0.53016667	0.182722222	0.881039	-1.1350235
<b>Integrin α6</b>	26.7888333	2.18138889	0.549	0.6834937	-1.4630712
<b>ZO1</b>	30.0388333	5.43138889	-0.15483333	1.113293	
<b>B-actin</b>	24.6074444	0	0	1	

**Appendices 4:** The average C<sub>T</sub>. Δ C<sub>T</sub>, ΔΔ C<sub>T</sub>, and fold change (RQ) values of condition B Co-3T3 compared to calibrator sample condition A no-3T3.

	<b>Condition C Bot-3T3</b>	$\Delta C_T$	$\Delta\Delta C_T$	<b>RQ</b>	<b>RQ with -1</b>
<b>CK3</b>	31.4954444	6.66133333	-0.69155556	1.6150239	
<b>CK12</b>	31.372	6.53788889	-0.28588889	1.2191612	
<b>p63</b>	27.3049444	2.47083333	-0.54066667	1.4546446	
<b>Integrin <math>\beta</math>4</b>	25.2349444	0.40083333	0.053388889	0.96367	-1.0376996
<b>Integrin <math>\alpha</math>6</b>	27.0179444	2.18383333	0.551444444	0.6823366	-1.4655523
<b>ZO1</b>	30.3328333	5.49872222	-0.0875	1.0625274	
<b>B-actin</b>	24.8341111	0	0	1	

**Appendices 4:** The average  $C_T$ ,  $\Delta C_T$ ,  $\Delta\Delta C_T$ , and fold change (RQ) values of condition C Bot-3T3 compared to calibrator sample condition A no-3T3.

	<b>Condition D dHAM No-3T3</b>	$\Delta C_T$	$\Delta\Delta C_T$	<b>RQ</b>	<b>RQ with -1</b>
<b>CK3</b>	32.8883333	8.49544444	1.14255556	0.4529565	-2.2077175
<b>CK12</b>	32.2508889	7.858	1.03422222	0.488279	-2.0480092
<b>p63</b>	27.3002778	2.90738889	-0.10411111	1.0748319	
<b>Integrin <math>\beta</math>4</b>	25.0636667	0.67077778	0.323333333	0.7992211	-1.2512181
<b>Integrin <math>\alpha</math>6</b>	25.9812222	1.58833333	-0.04405556	1.031008	
<b>ZO1</b>	29.4107222	5.01783333	-0.56838889	1.4828667	
<b>B-actin</b>	24.3928889	0	0	1	

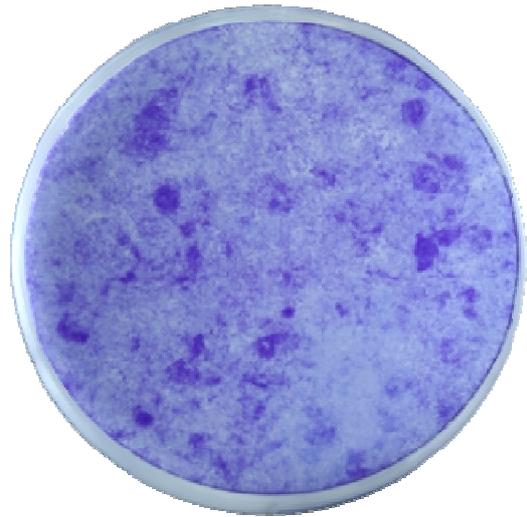
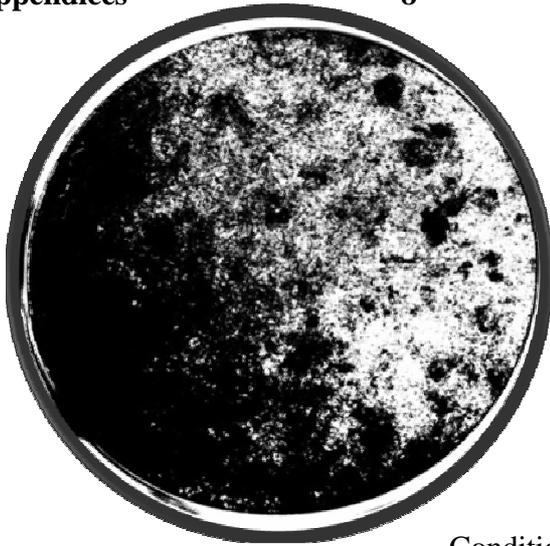
**Appendices 5:** The average  $C_T$ ,  $\Delta C_T$ ,  $\Delta\Delta C_T$ , and fold change (RQ) values of condition D dHAM no-3T3 compared to calibrator sample condition A no-3T3.

	<b>Condition E dHAM Co-3T3</b>	$\Delta C_T$	$\Delta\Delta C_T$	<b>RQ</b>	<b>RQ with -1</b>
<b>CK3</b>	31.4933333	7.477	0.124111111	0.9175692	-1.089836
<b>CK12</b>	31.9423333	7.926	1.102222222	0.4657985	-2.1468512
<b>p63</b>	28.749	4.73266667	1.721166667	0.3033033	-3.2970292
<b>Integrin <math>\beta</math>4</b>	25.1886111	1.17227778	0.824833333	0.5645474	-1.7713304
<b>Integrin <math>\alpha</math>6</b>	26.7733889	2.75705556	1.124666667	0.458608	-2.1805116
<b>ZO1</b>	30.4738333	6.4575	0.871277778	0.5466625	-1.8292824
<b>B-actin</b>	24.0163333	0	0	1	

**Appendices 6:** The average  $C_T$ ,  $\Delta C_T$ ,  $\Delta\Delta C_T$ , and fold change (RQ) values of condition E dHAM Co-3T3 compared to calibrator sample condition A no-3T3.

	<b>Condition F dHAM Bot-3T3</b>	$\Delta C_T$	$\Delta\Delta C_T$	<b>RQ</b>	<b>RQ with -1</b>
<b>CK3</b>	32.2305	8.1305	0.777611111	0.5833319	-1.7142899
<b>CK12</b>	31.5651667	7.46516667	0.641388889	0.6410955	-1.5598301
<b>p63</b>	28.0297778	3.92977778	0.918277778	0.5291403	-1.8898579
<b>Integrin <math>\beta</math>4</b>	24.3417778	0.24177778	-0.10566667	1.0759915	
<b>Integrin <math>\alpha</math>6</b>	26.0432222	1.94322222	0.310833333	0.806176	-1.240424
<b>ZO1</b>	29.4237778	5.32377778	-0.26244444	1.1995094	
<b>B-actin</b>	24.1	0	0	1	

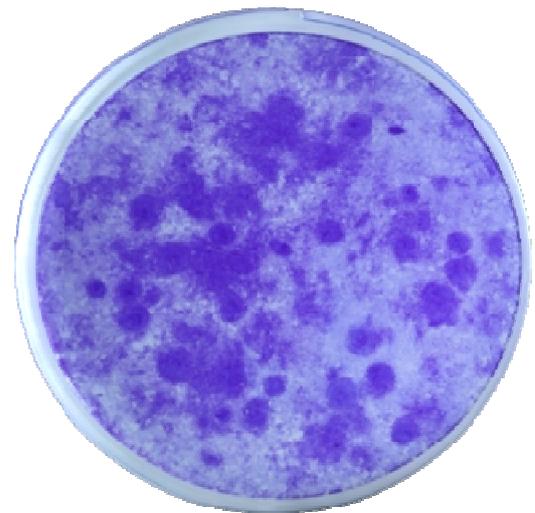
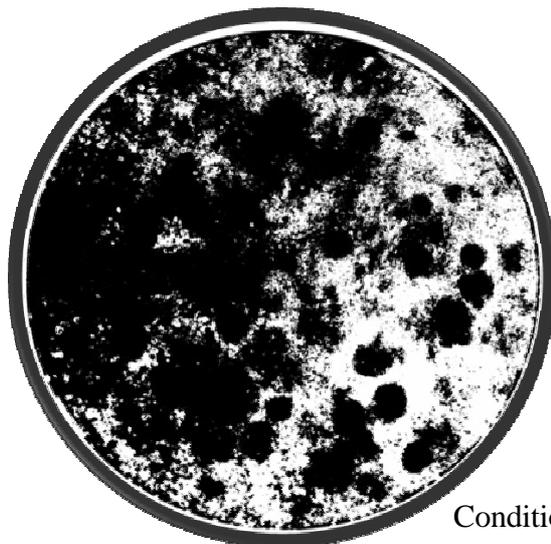
**Appendices 7:** The average  $C_T$ ,  $\Delta C_T$ ,  $\Delta\Delta C_T$ , and fold change (RQ) values of condition F dHAM Bot-3T3 compared to calibrator sample condition A no-3T3.



Condition A No-3T3

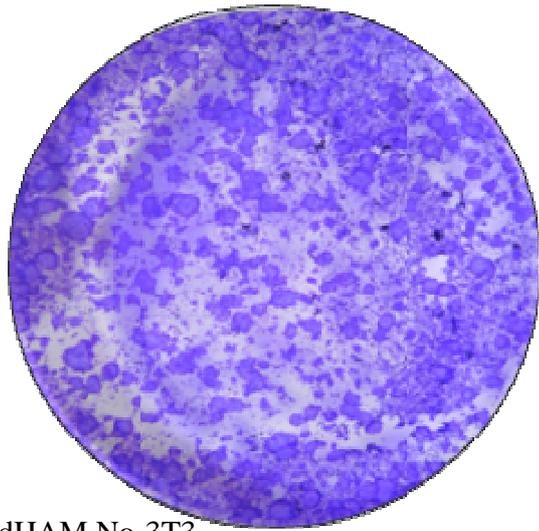
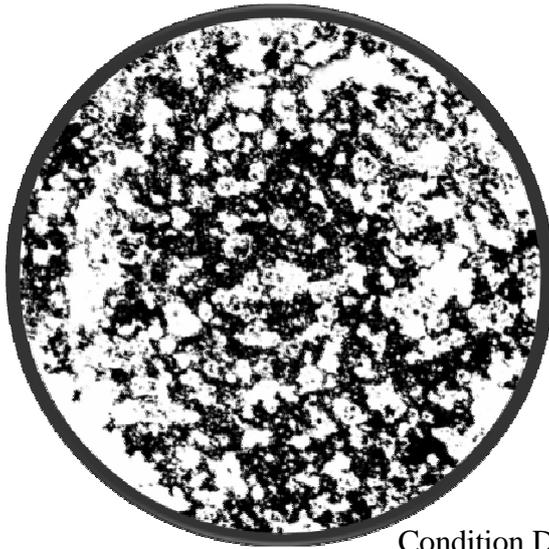


Condition B Co-3T3

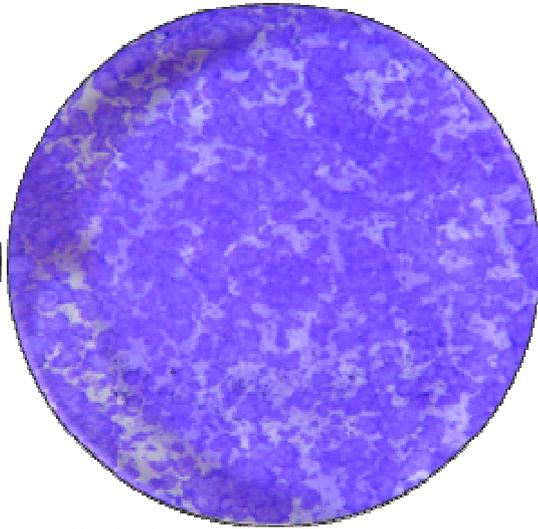
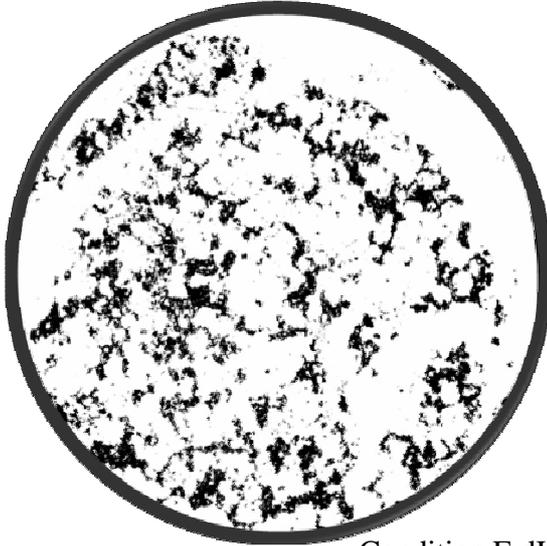


Condition C Bot-3T3

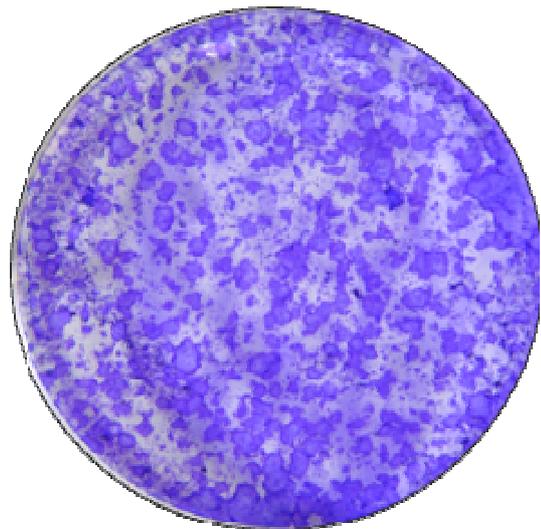
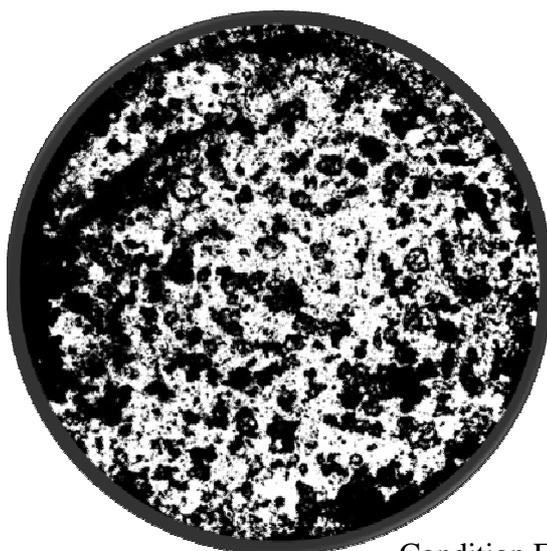
**Figure:** Binary (left) and original (right) images of CFE in conditions A, B and C



Condition D dHAM No-3T3



Condition E dHAM Co-3T3



Condition F dHAM Bot-3T3

**Figure:** Binary (left) and original (right) images of CFE in conditions D, E and F

## Appendices 9

### Project output:

#### Poster Presentations:

1. **Kishore. Katiki Reddy, Martin Clynes, Andra Bobart, Finbarr O'Sullivan, William. Power.** *NIH/3T3 feeder system to engineer corneal epithelial sheets with enhanced positive progenitor populations.* 21st Meeting of the European Society for Animal Cell Technology. June 7th-10th 2009, Dublin, Ireland.(Published in ESACT proceedings short paper).
2. **Kishore. Katiki Reddy, Willam Power, Andra. Bobart, Martin Clynes, Finbarr O'Sullivan.** *Culture, characterization and trans-differentiation of limbal "fibroblast-like" cells.* UK National Stem Cell Network (UKNSCN) Nottingham, July 12th-14th 2010.

#### Oral Presentation

1. Kishore. Katiki Reddy *et.al.* *Comparison of Cell Culture Conditions for Limbal-Corneal Cells.* 3<sup>rd</sup> Limbal Stem cell meeting, School of Pharmacy, University of Reading. September 10th 2010.
2. F. O'Sullivan, *et.al.* *Establishment of Limbal Stem Cell Culture As Treatment Option For Irish Patients With Corneal Epithelial Damage* Irish College of Ophthalmologists Annual Meeting May 2008.