Human limbus-derived stromal/epithelial stem cells to treat cornea pathology (a transcriptomic study)

Presented by

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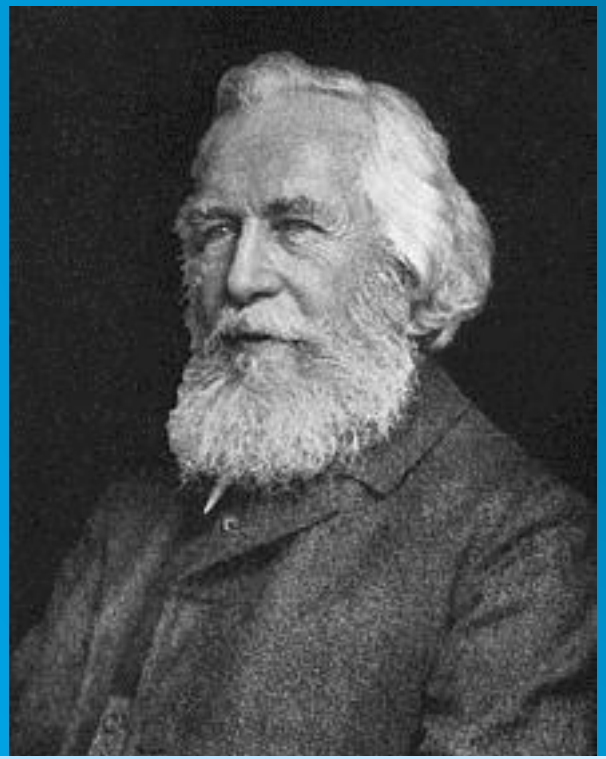
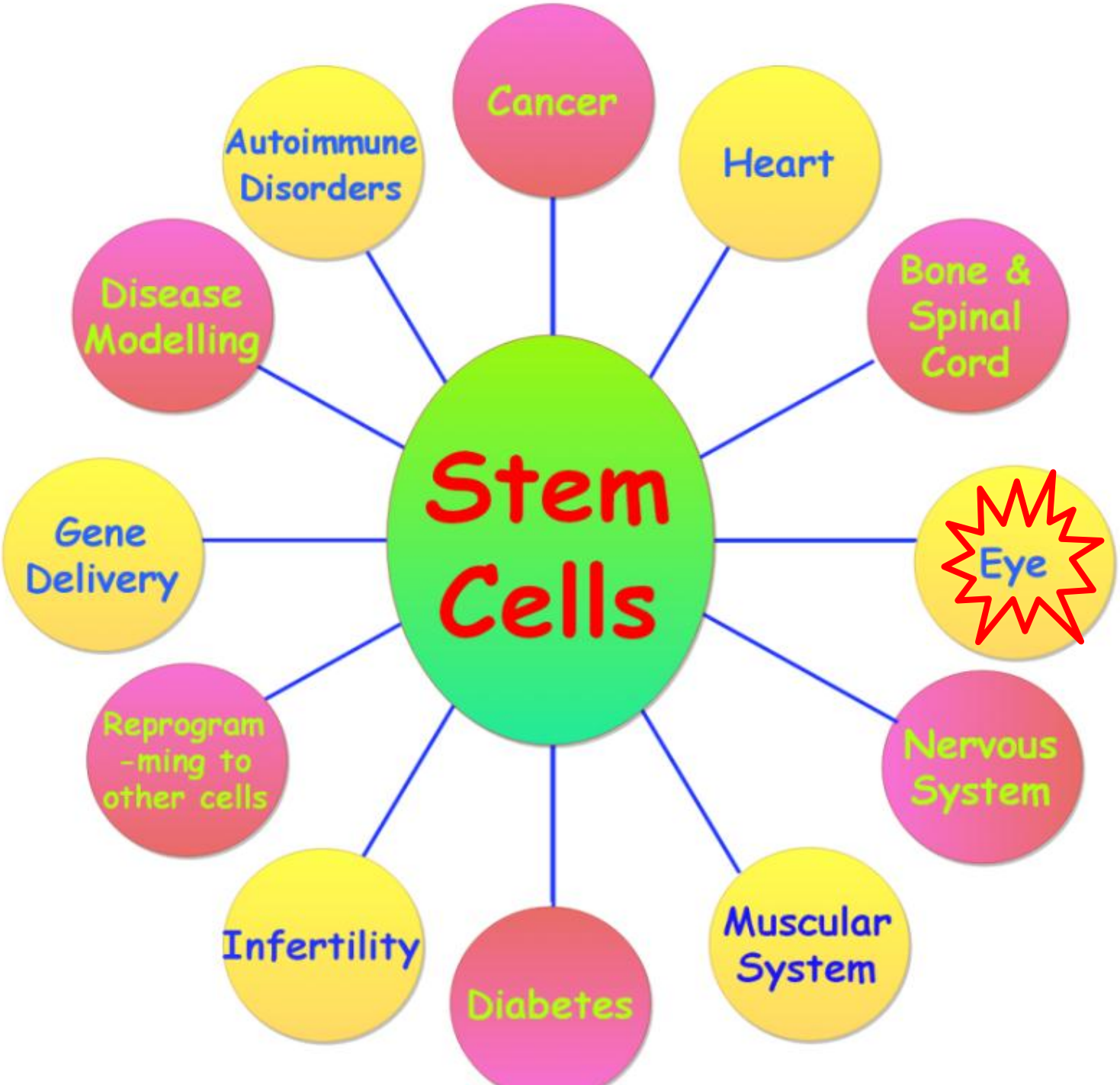
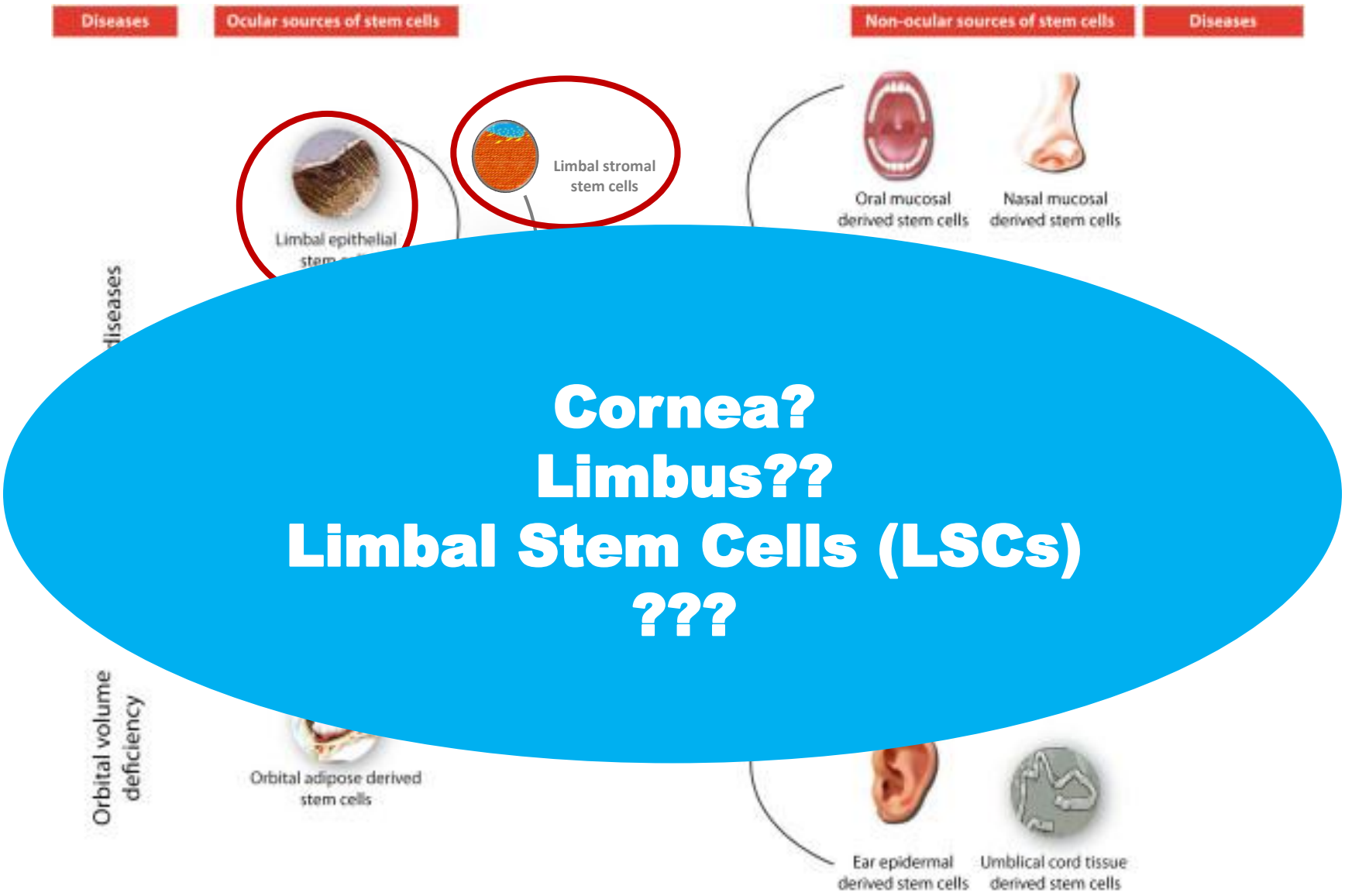
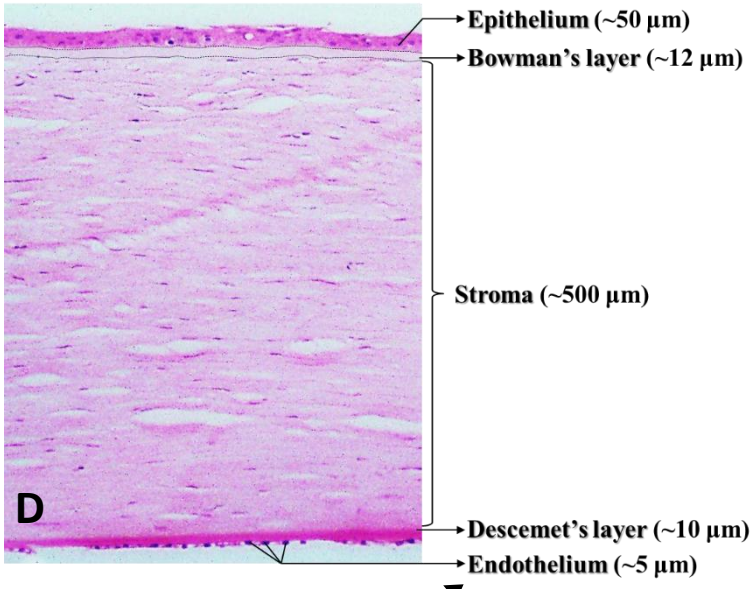


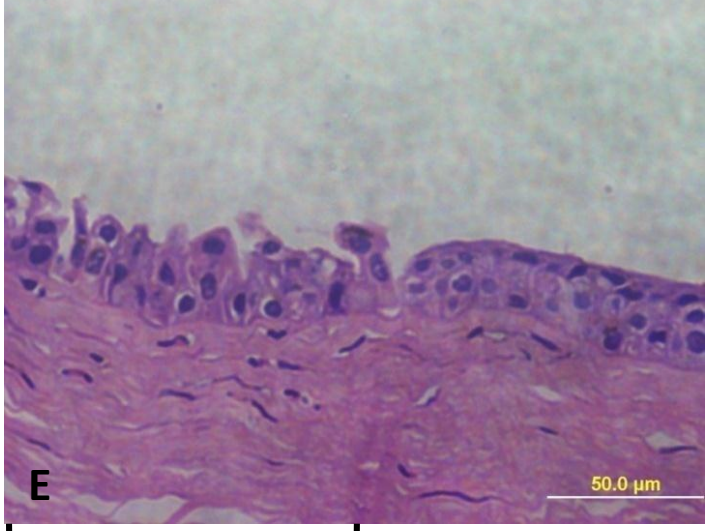
Ernst Heinrich Philipp August Haeckel (1834 –1919)
German biologist, naturalist, philosopher, physician, professor and marine biologist
For the first time used the *stammzelle* phrase to describe the fertilized egg which eventually gestates into an organism

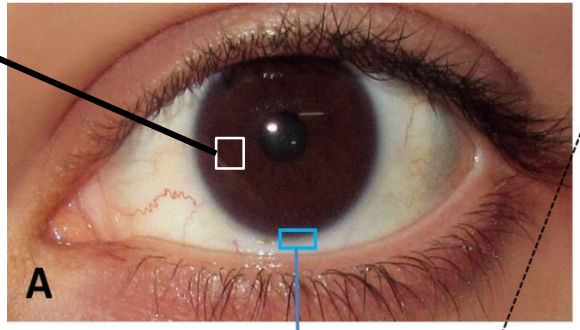

Stem Cells
Cancer
Heart
Bone & Spinal Cord
Eye
Nervous System
Muscular System
Diabetes
Infertility
Reprogram -ming to other cells
Gene Delivery
Disease Modelling
Autoimmune Disorders

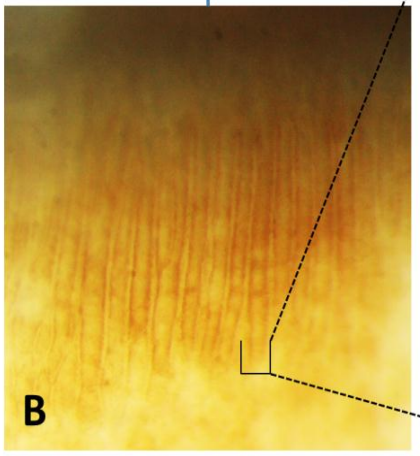


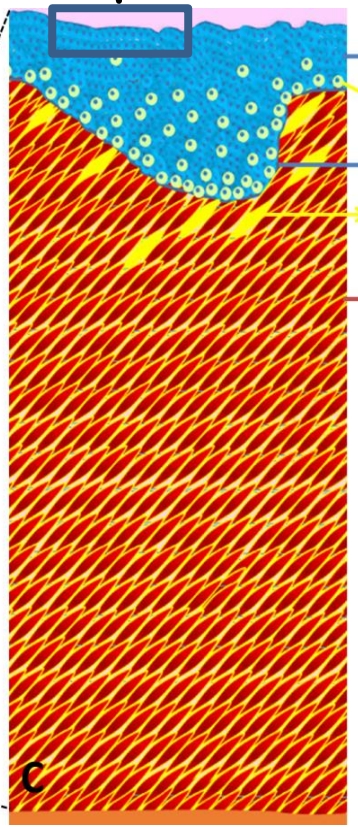
Status of ocular and non-ocular stem cell transplantation for anterior surface disorders of the eye











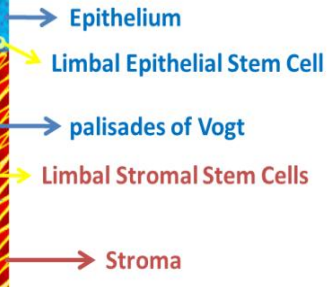


Figure: A) Localization of limbus in ocular surface B) Limbal palisades of Vogt C) Structure of limbus and location of limbal epithelial and stromal stem cells in palisades of Vogt D) Hematoxylin & eosin staining of the human Cornea with stratified epithelium and E) Limbus with unstratified squamous epithelium.

- **Such study will help in developing techniques to induce differentiation of cornea like cells reducing heavy demand on donor tissue for treating a wide range of blindness**

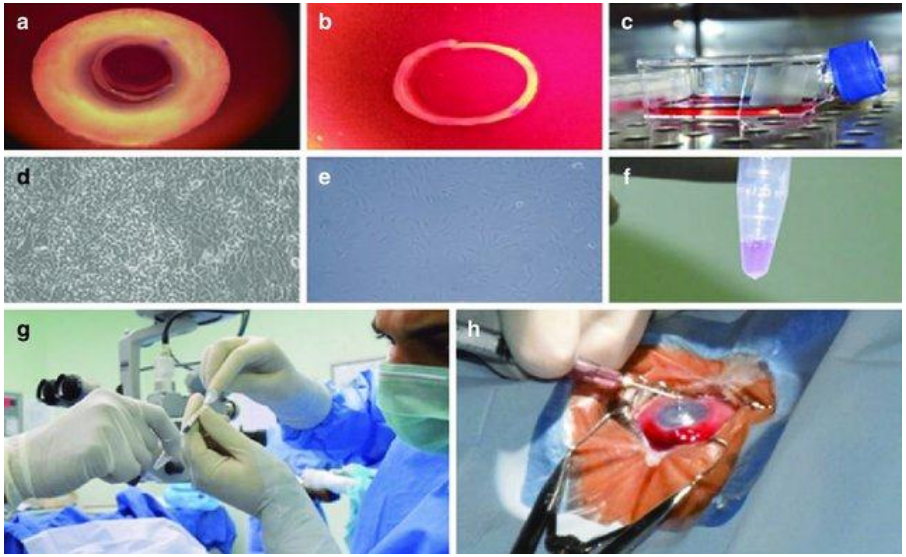


Figure: Events of tissue processing and application of the hLMSCs during transplantation. (a) Debris and unwanted layers of corneal tissues removed. (b) A 360° limbal rim dissected out. (c) Collagenase-digested limbal explants were cultured in T25 flasks. (d) Image of epithelial cells population after 14 days of culture. (e) Image of the pure stromal cell population which can be obtained after few passages. (f) Trypsinized cell pellet sent for transplantation. (g) Mixing of the hLMSCs with fibrin glue. (h) Cells with fibrin glue being applied onto the debrided cornea

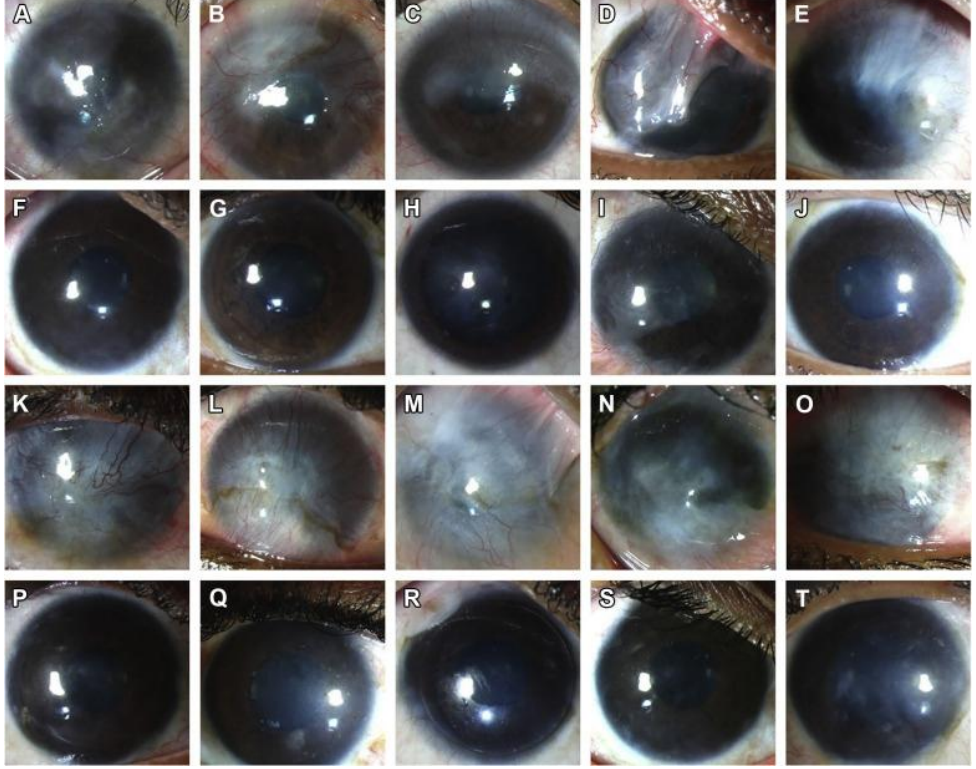


Figure: Slit-lamp photographs showing 2-year clinical outcomes of limbal epithelial/stromal transplantation. **A–J**, Patients with partial limbal stem cell deficiency (LSCD) after ocular burns: **(A–F)** preoperative photographs and **(F–J)** their corresponding 2-year postoperative photographs showing a completely epithelized and stable corneal surface. **K–U**, Eyes with total LSCD: **(K–O)** preoperative clinical photographs and **(P–T)** corresponding 2-year postoperative photographs after transplantaion showing excellent anatomic outcomes.

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+ Author Affiliations & Notes

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Human Limbus-derived Mesenchymal/Stromal Stem Cell Therapy for Superficial Corneal Pathologies: Two-Year Outcomes

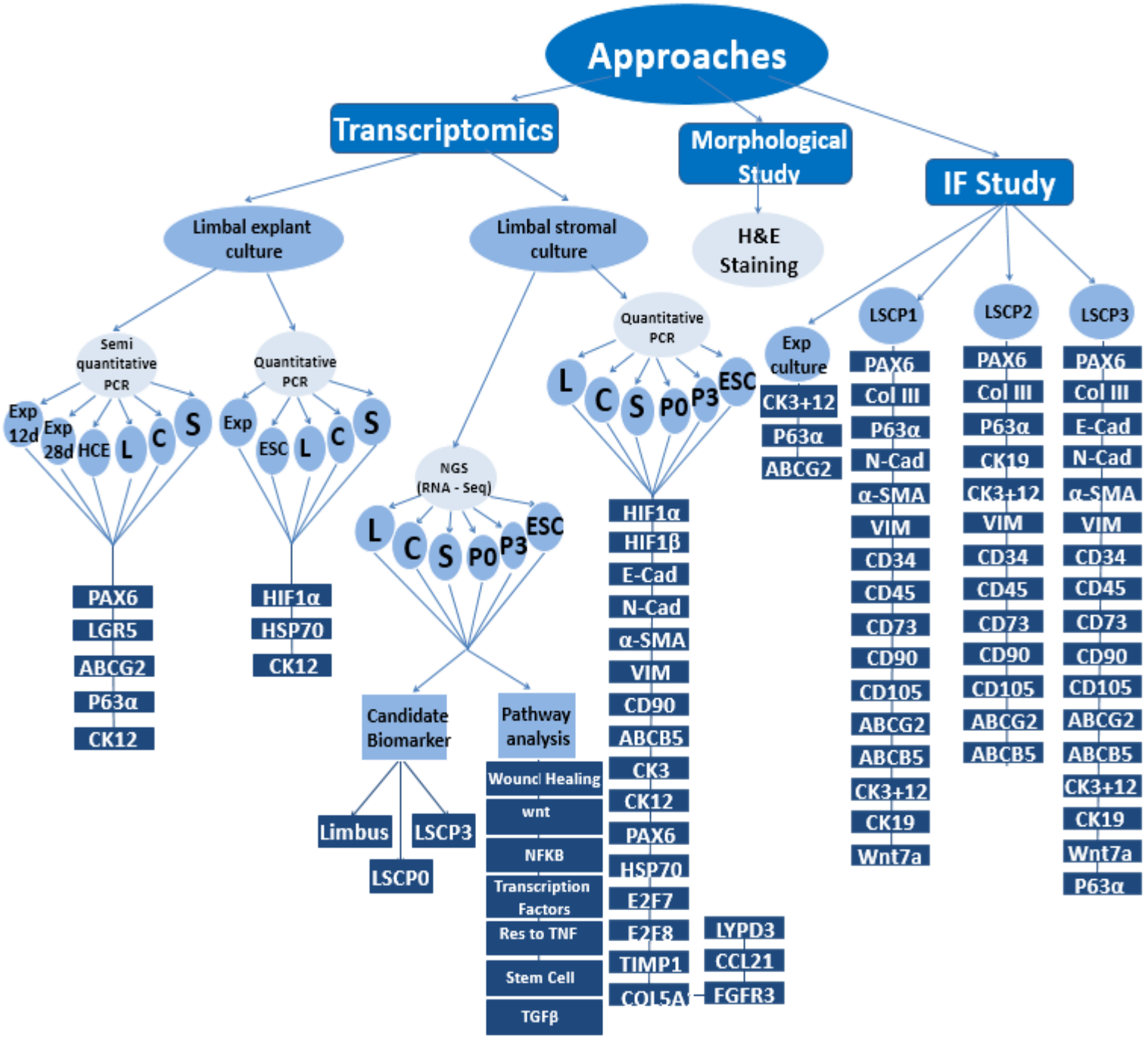
Sayan Basu; Mukesh Damala; Fateme Tavakkoli; Noopur Mitragotri; Vivek Singh

+ Author Affiliations & Notes

Investigative Ophthalmology & Visual Science July 2019, Vol.60, 4146.
doi:https://doi.org/

The number of samples collected from the Ramayamma International Eye Bank (RIEB)-LVPEI to carry out the different experiments

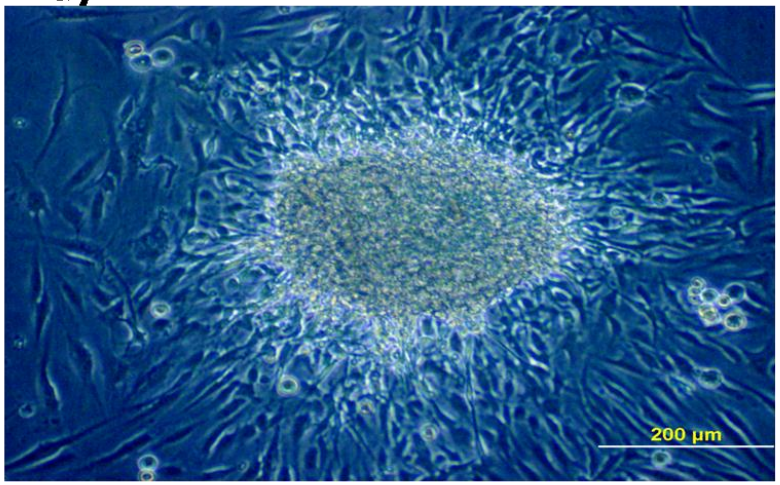
	Limbal Explant Culture (live biopsy)	Limbal Explant Culture (cadaver)	Limbal Stromal Culture	RNA Extraction from Limbal Tissue	RNA Extraction from Corneal Tissue	RNA Extraction from Scleral Tissue	H & E Staining	IF Study
Successful	2	4	53	31	25	24	5	10
Unsuccessful	0	0	22	12	41	13		
Total	2	4	75	43	66	37		
Total no of collected Samples	242							


Approaches
Transcriptomics
Morphological Study
IF Study
Limbal explant culture
Limbal stromal culture
H&E Staining
Semi quantitative PCR
Quantitative PCR
Quantitative PCR
Exp culture
LSCP1
LSCP2
LSCP3
Exp 12d
Exp 28d
HCE
L
C
S
Exp
ESC
L
C
S
L
C
S
P0
P3
ESC
CK3+12
P63α
ABCG2
PAX6
Col III
P63α
N-Cad
α-SMA
VIM
CD34
CD45
CD73
CD90
CD105
ABCG2
ABCB5
CK3+12
CK19
Wnt7a
PAX6
Col III
P63α
CK19
CK3+12
VIM
CD34
CD45
CD73
CD90
CD105
ABCG2
ABCB5
PAX6
Col III
E-Cad
N-Cad
α-SMA
VIM
CD34
CD45
CD73
CD90
CD105
ABCG2
ABCB5
CK3+12
CK19
Wnt7a
P63α
PAX6
LGR5
ABCG2
P63α
CK12
HIF1α
HSP70
CK12
NGS (RNA - Seq)
L
C
S
P0
P3
ESC
HIF1α
HIF1β
E-Cad
N-Cad
α-SMA
VIM
CD90
ABCB5
CK3
CK12
PAX6
HSP70
E2F7
E2F8
TIMP1
COL5A1
LYPD3
CCL21
FGFR3
Candidate Biomarker
Pathway analysis
Limbus
LSCP3
LSCP0
Wound Healing
wnt
NFKB
Transcription Factors
Res to TNF
Stem Cell
TGFβ

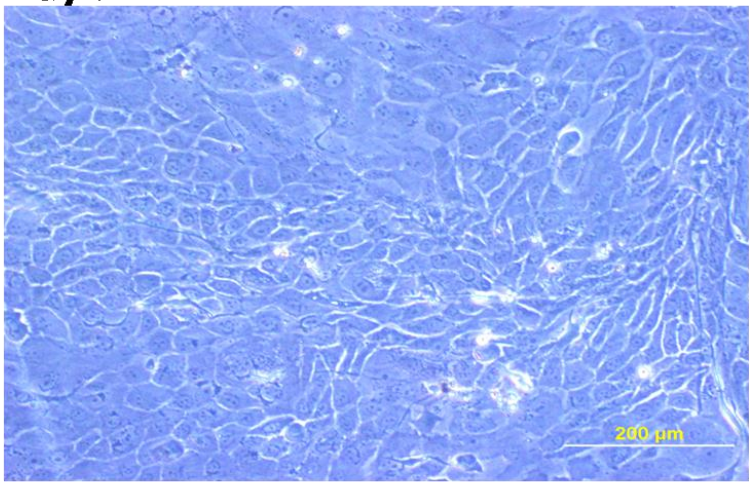
Results

Limbal Stromal Culture (LSC)

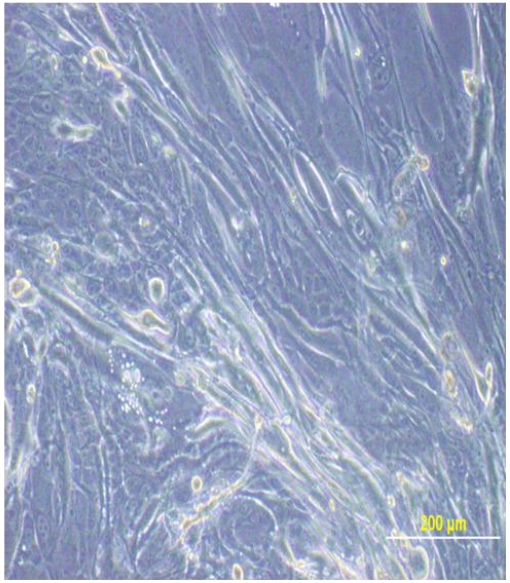
Day 2



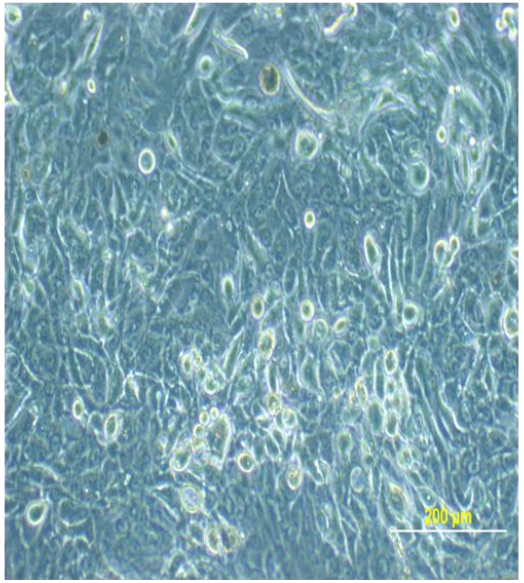
Day 7



Day 14



First passage



Second Passage

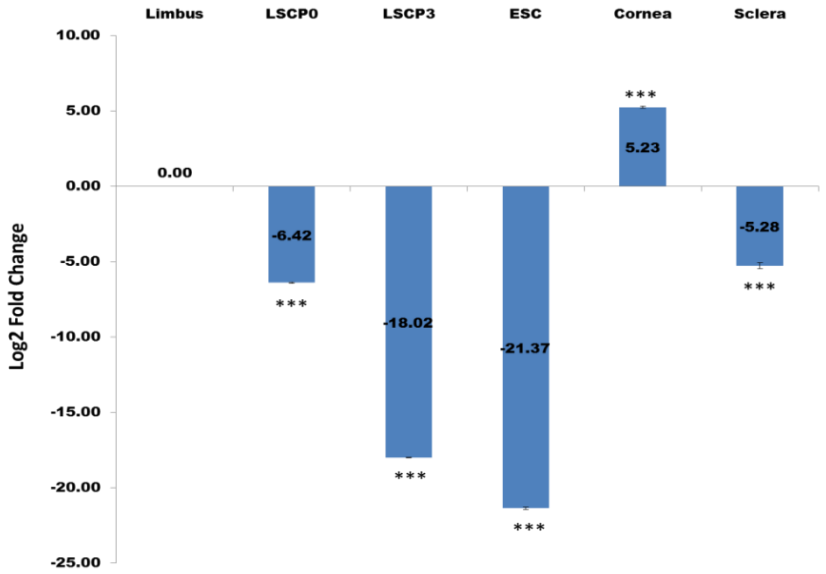
Human limbal tissue has been used to set up the limbal stromal culture. In this method (Funduberg technique) the limbal tissue was cut into small pieces, passed enzymatic digestion with Collagenase type IV for 16 hours then culture was set up via DMEM F12 Ham (1:1 ratio), 2% FBS+ human recombinant insulin + human recombinant epidermal growth factor.

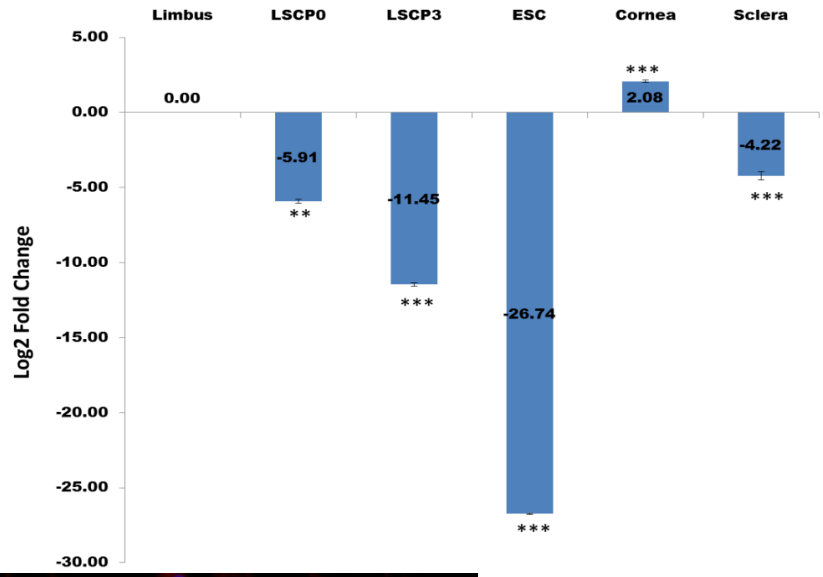
General Characterization

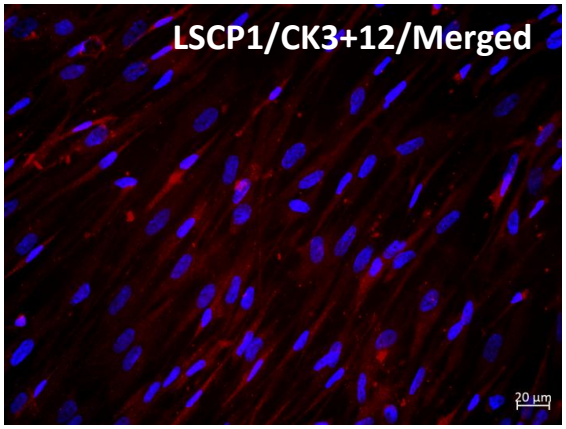
CK3 and CK12

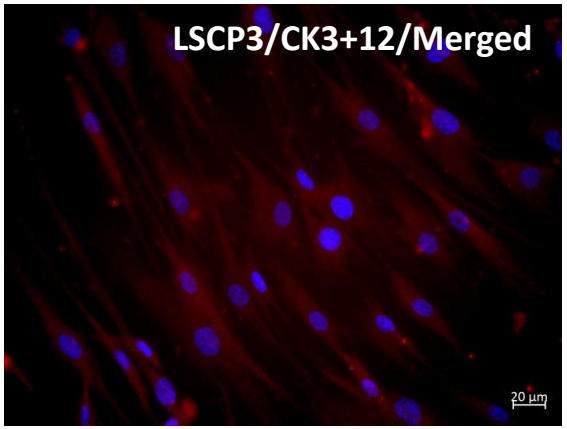
CK3/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	-6.6118	-9.02978	0	0	0
P-Value	2.93E-10	2.19E-11	0	0	0

CK12/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	-6.14758	-12.7499	0	-2.30755	-3.73115
P-Value	1.2E-09	0	0	0.134748	0.000156





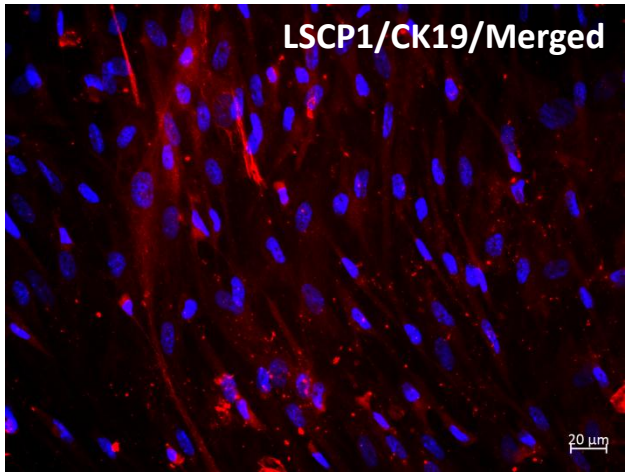


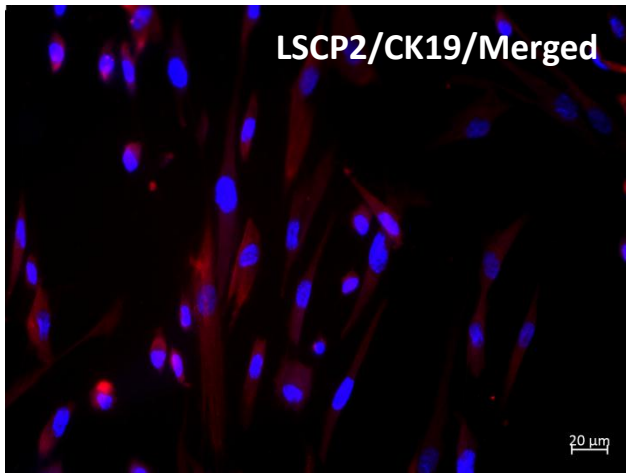


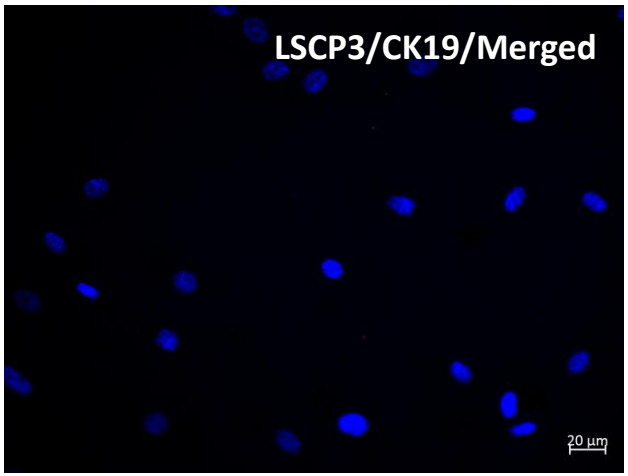
CK3+12 are the corneal epithelial marker(as differentiation marker)

CK19

NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	0	-3.3848	0	-6.03039	-6.13581
P-Value	0	0.020343	0	4.24E-13	2.45E-11



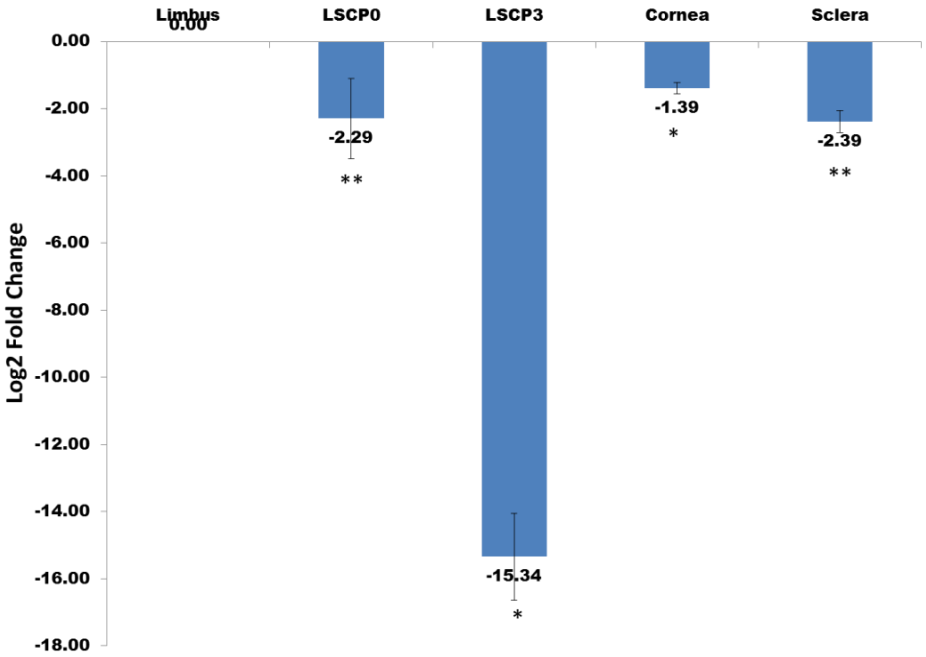


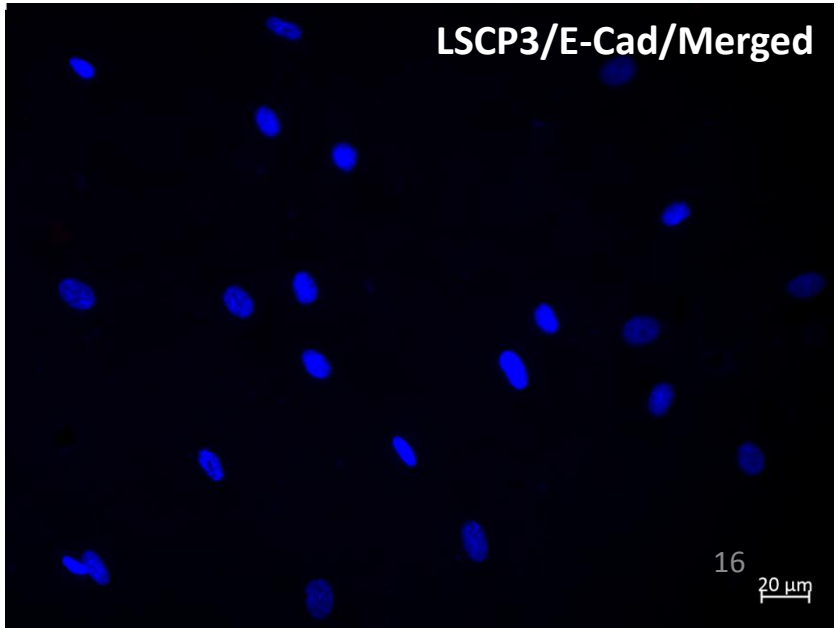


CK19 is limbal epithelial marker

E-Cadherin

E-Cadherin/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	0	-6.9773	0	-3.14949	-3.79405
P-Value	0	4.45E-09	0	0.000404	4.98E-05

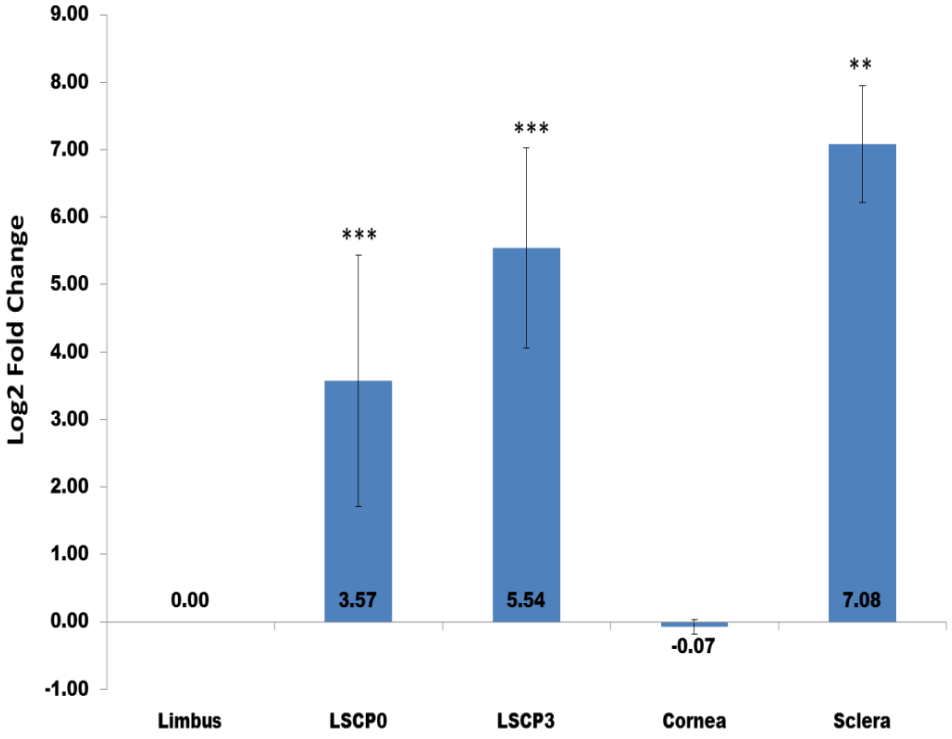




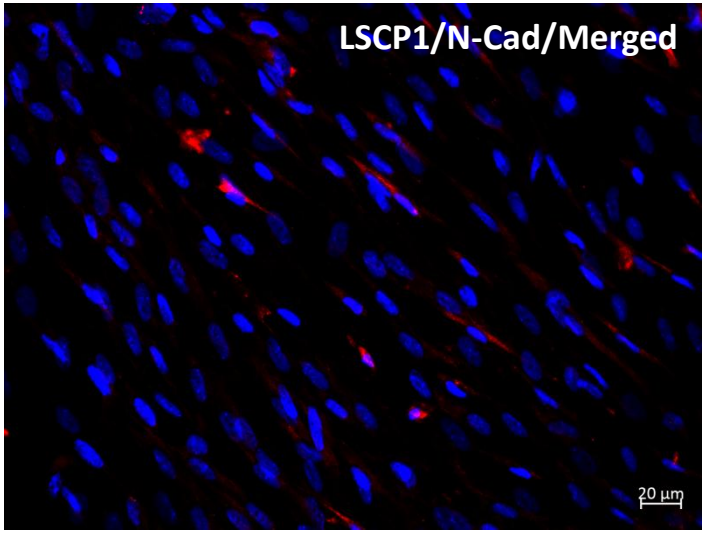
E-Cadherin is regulating the cell-cell adhesions, mobility and proliferation in epithelial cells

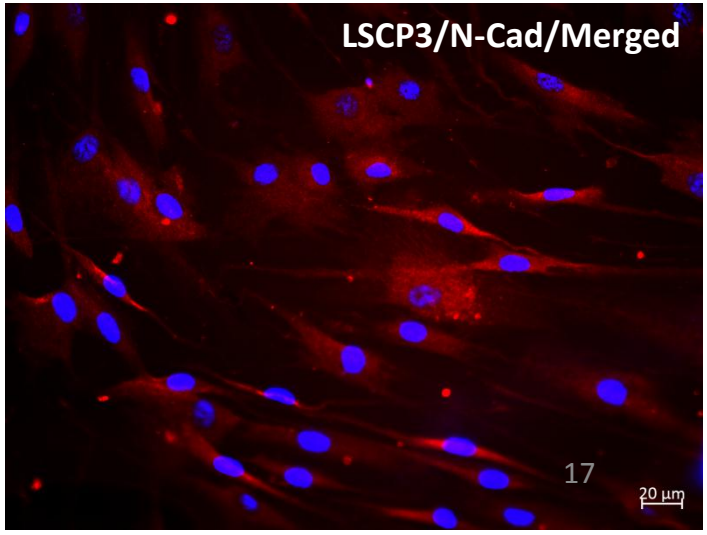
N-Cadherin

N-Cad/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	0	3.362117	0	0	0
P-Value	0	0.0106	0	0	0



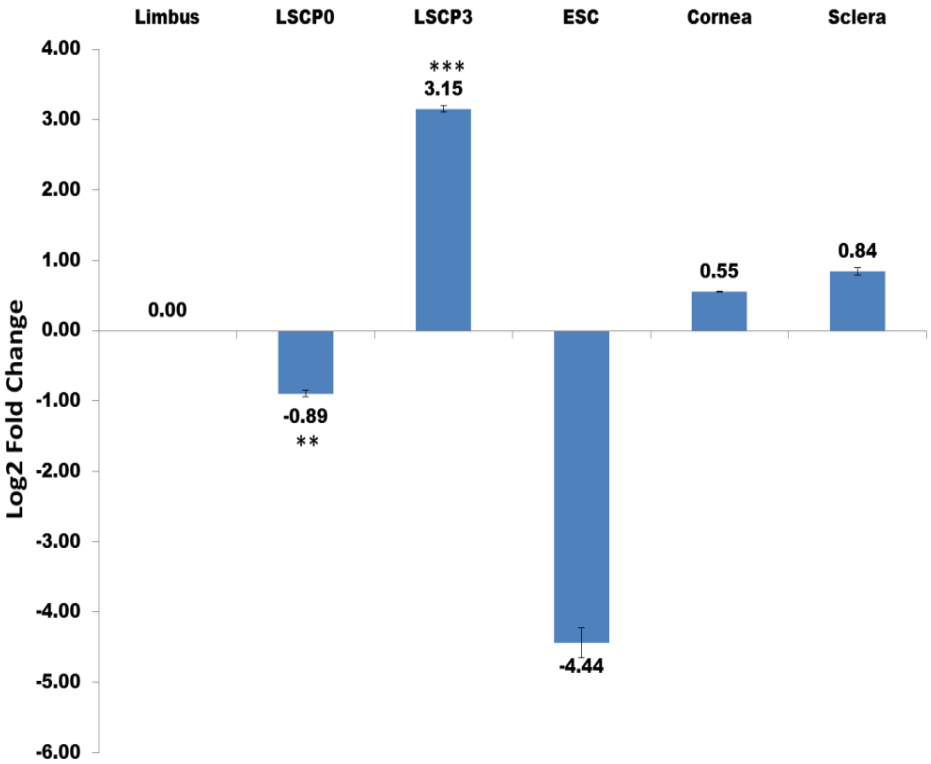
N-Cadherin is a prospective cell surface marker of human mesenchymal stem cells and function to mediate cell–cell adhesion but in inverse pattern of E-Cadherin

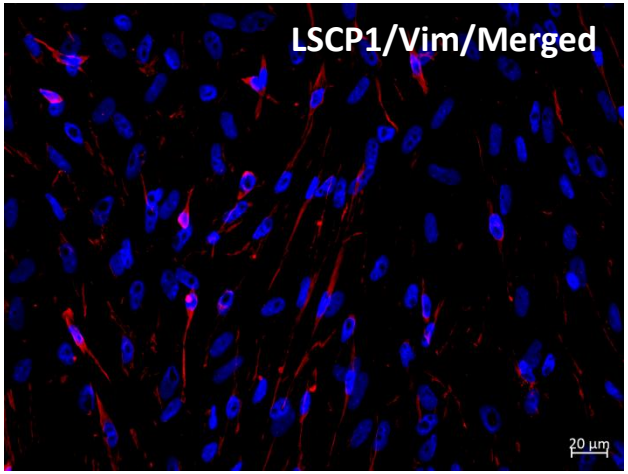


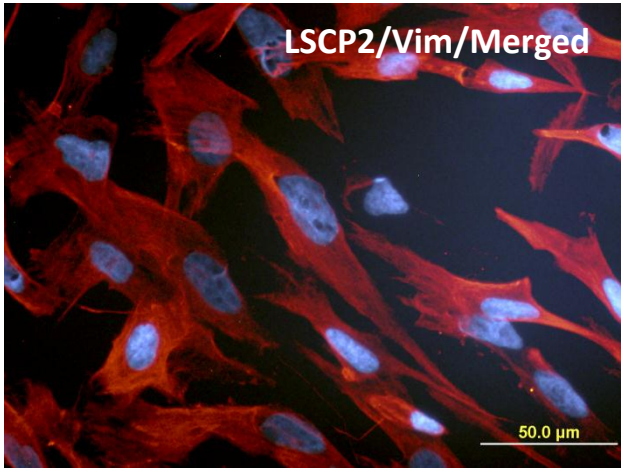


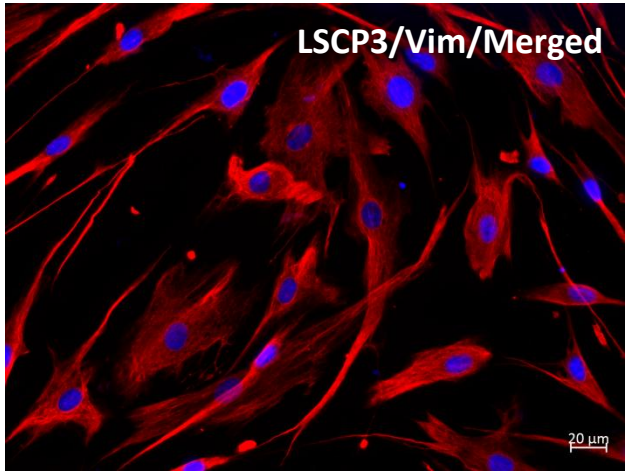
Vimentin

Vimentin/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	0	0	-3.60423	0	0
P-Value	0	0	0.02032	0	0



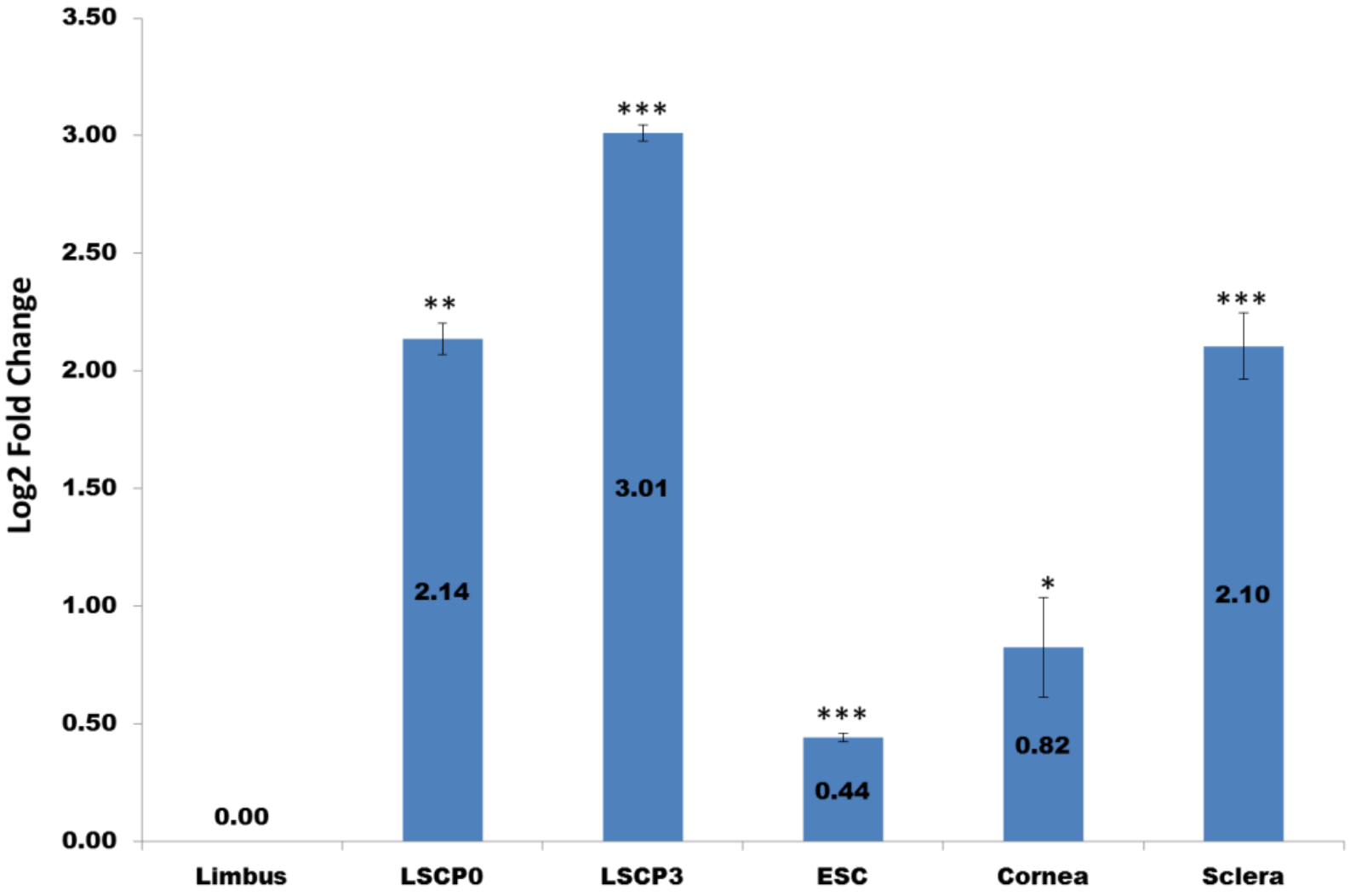






Vimentin is marker for mesenchymal cells

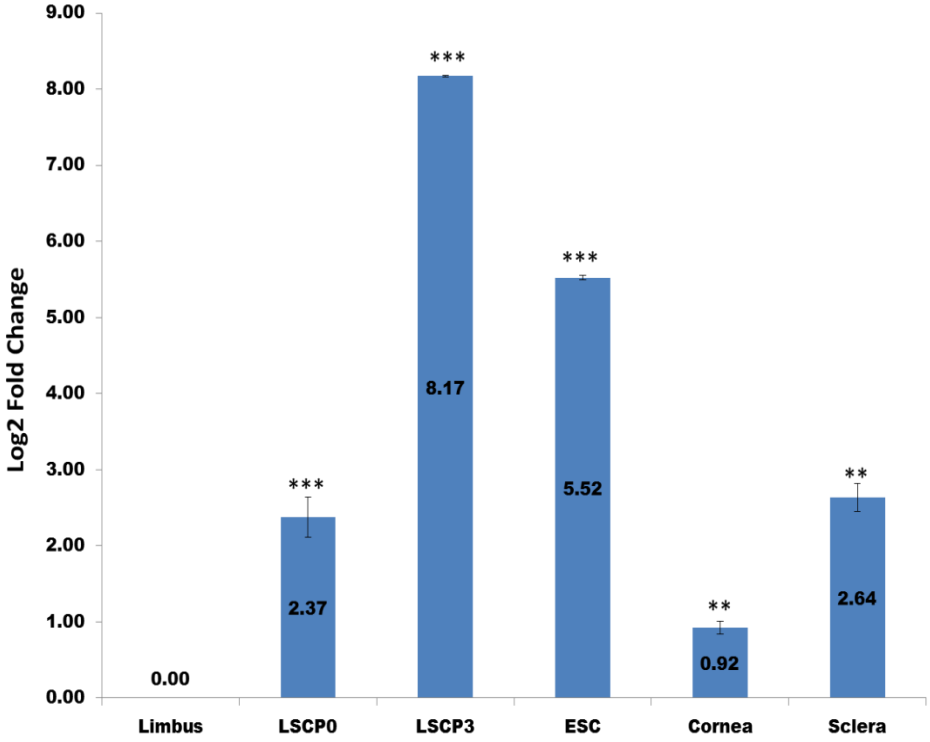
HIF1α

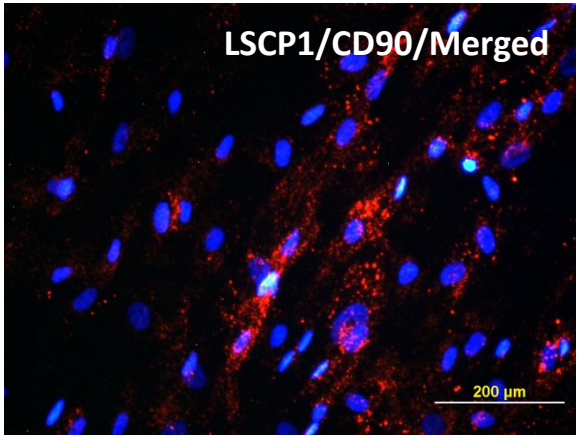


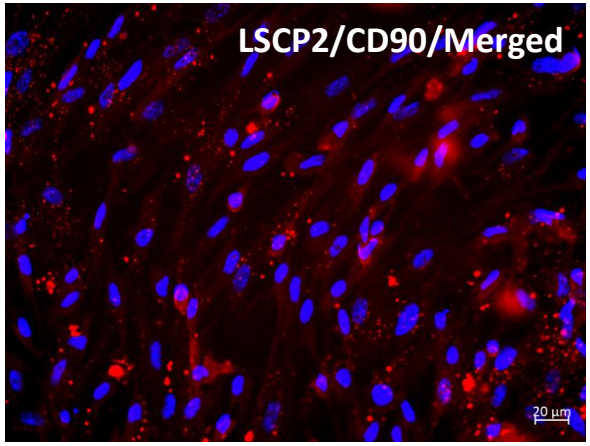
HIF1α is a general regulator for controlling the metabolic fate and potency in human mesenchymal stem cells

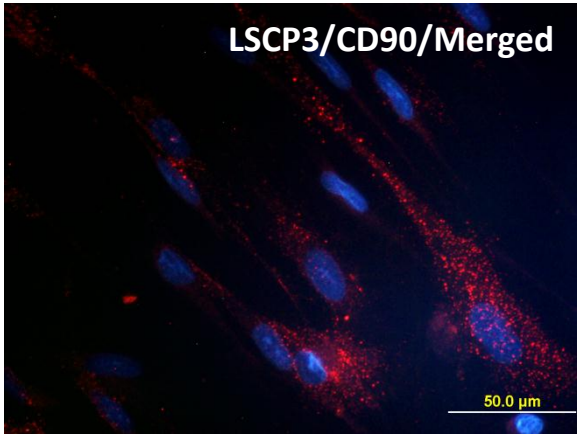
CD90

CK3/ NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	2.835907	7.771958	6.962016	0	0
P-Value	0.013702	1.21E-10	1.74E-07	0	0





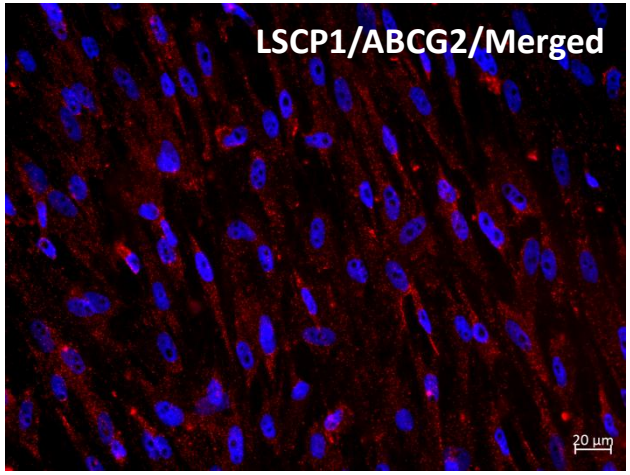


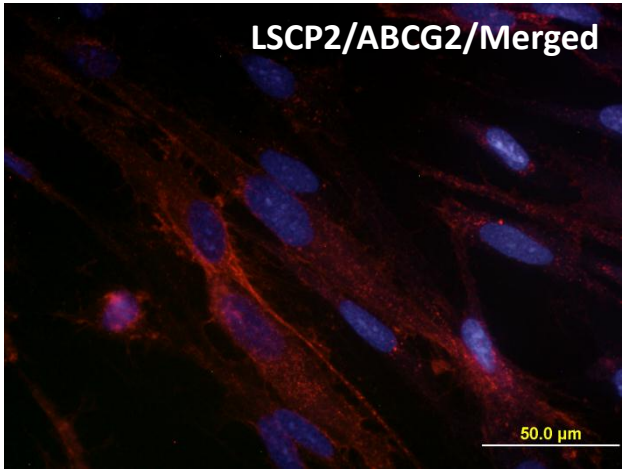


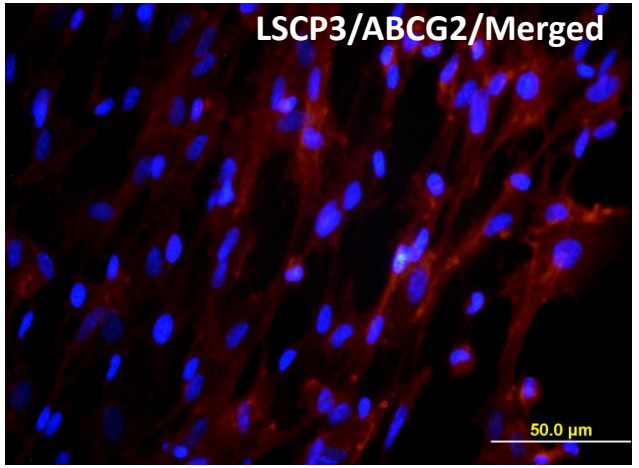
CD90 is cell surface glycoprotein and markedly expressed by limbal stromal cells

CD105

NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	0	0	-4.29919	0	0
P-Value	0	0	0.000663	0	0



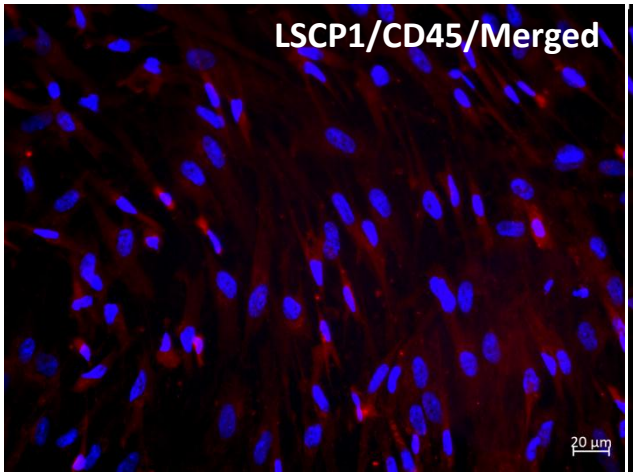


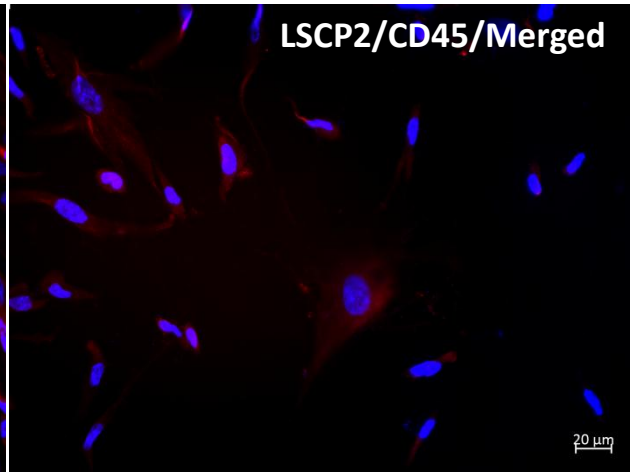


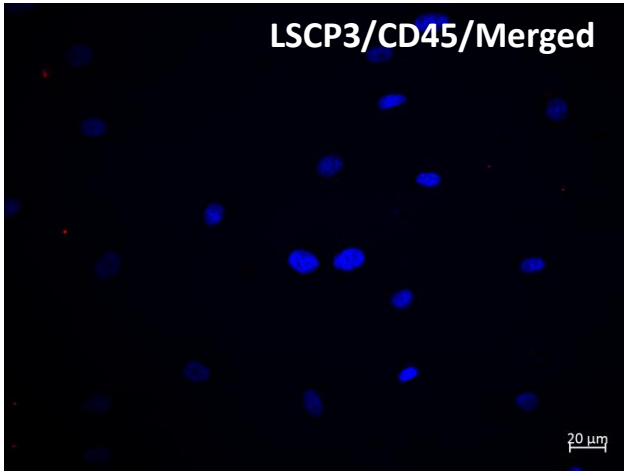
CD105 is a membrane glycoprotein located on cell surfaces

CD45

NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	-4.98238	-4.39103	-4.79702	-2.19772	2.259656
P-Value	6.14E-05	0.001186	0.00052	0.087053	0.086036



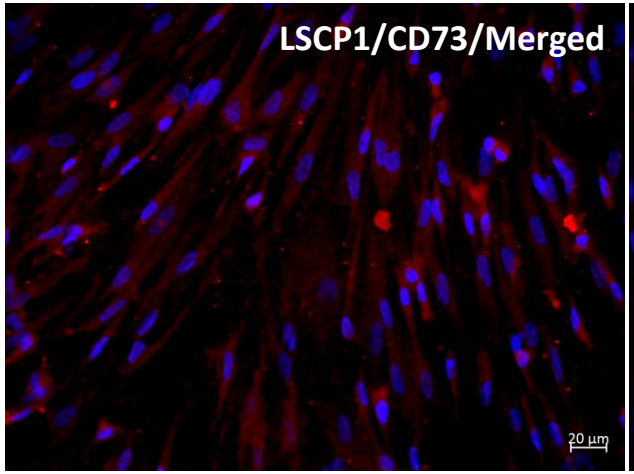


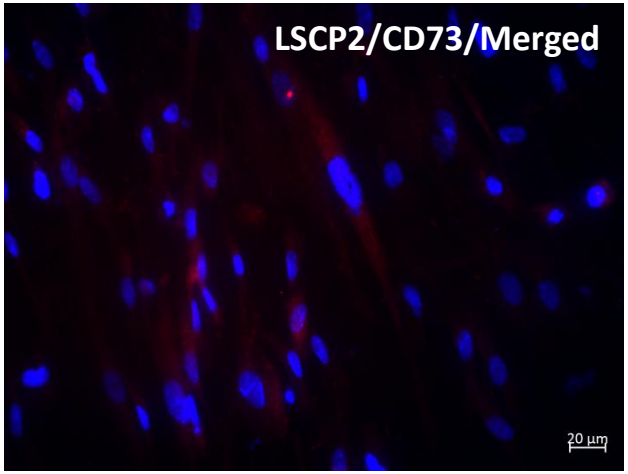


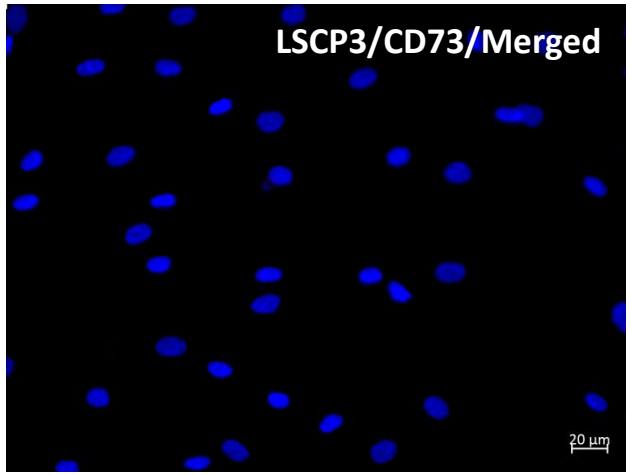
CD45 is hematopoietic marker

CD73

NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	4.268486	3.367869	-3.99038	2.350381	0
P-Value	1.39E-05	0.015336	0.001998	0.043913	0



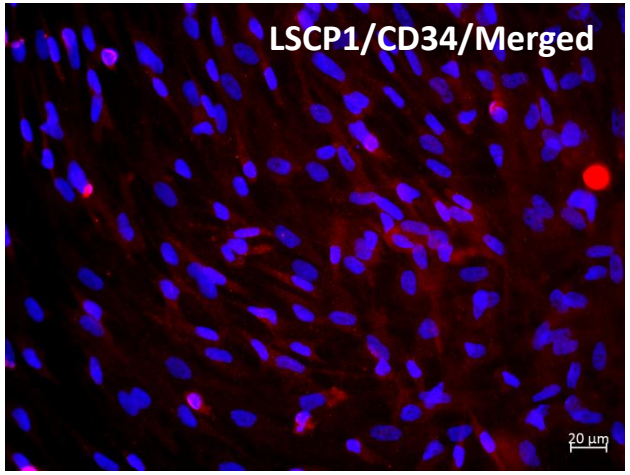


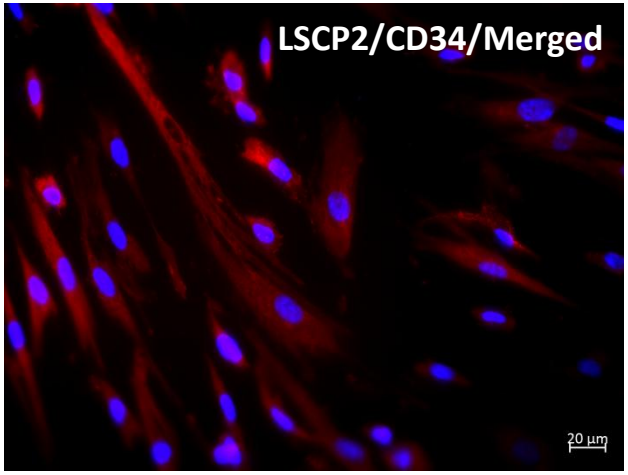


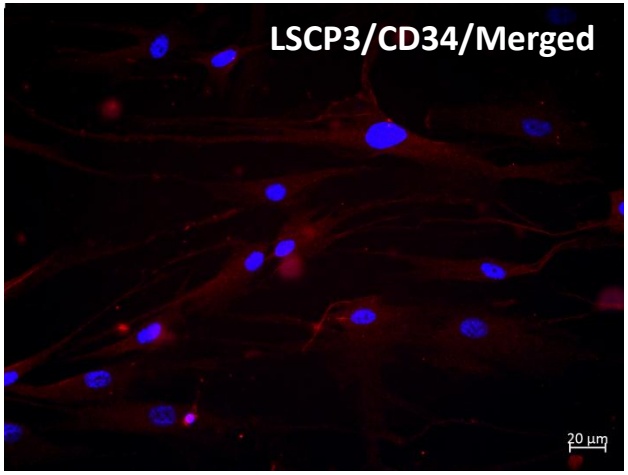
CD73 is a hematopoietic marker

CD34

NGS/ Vs. Limbus	LSCP0	LSCP3	ESC	Cornea	Sclera
Log2 FC	-7.13857	-5.78231	-8.37879	2.507431	0
P-Value	1.09E-10	1.01E-06	1.27E-09	0.029737	0

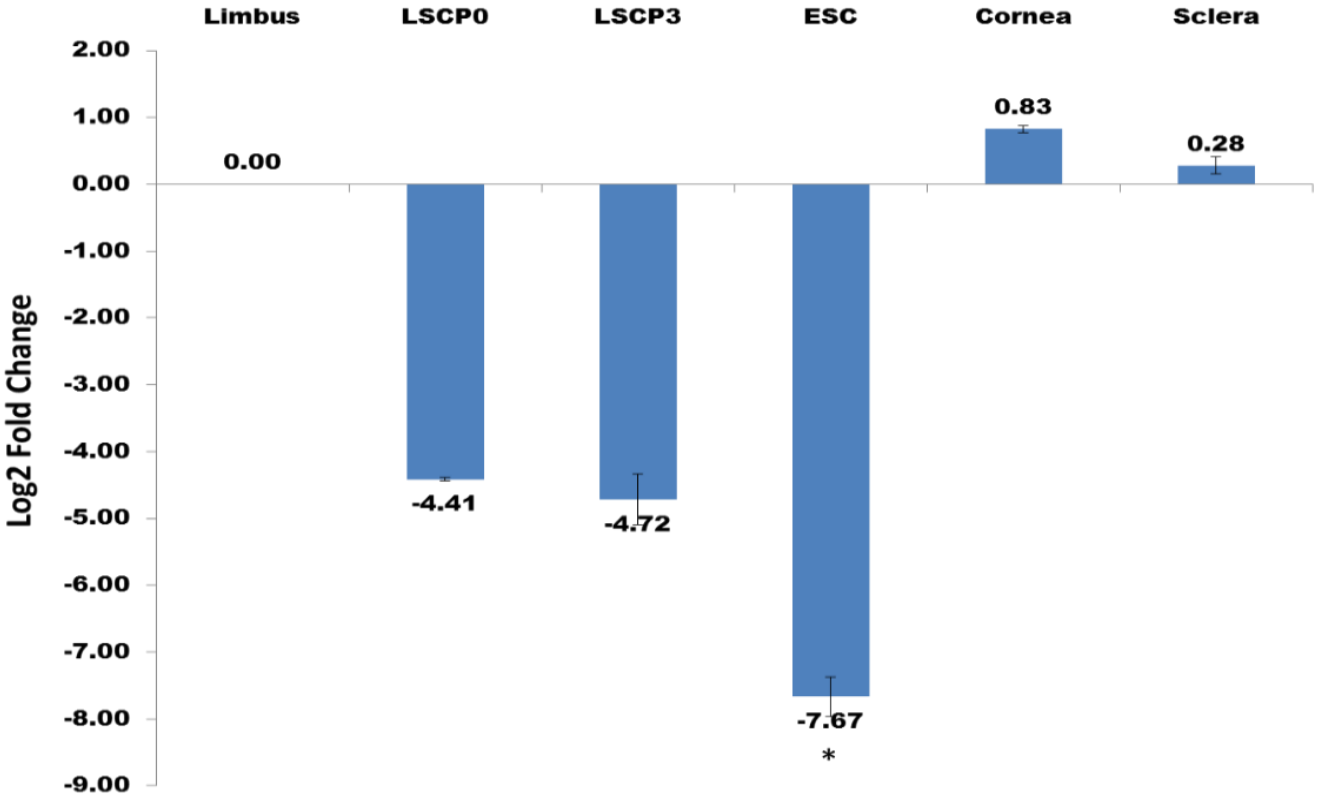


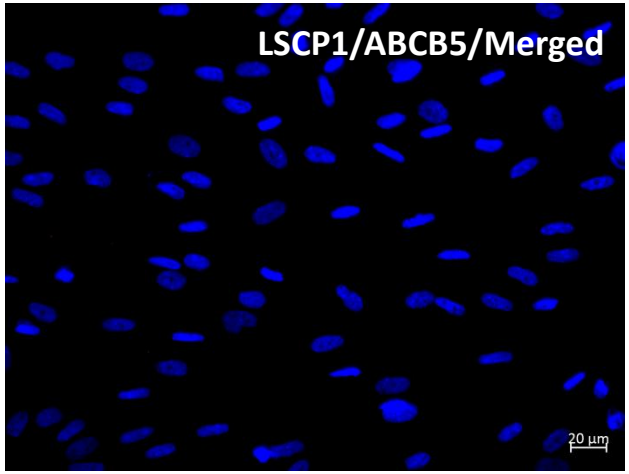


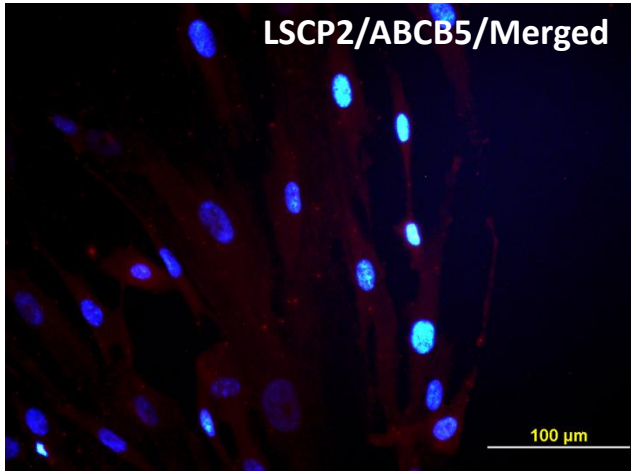


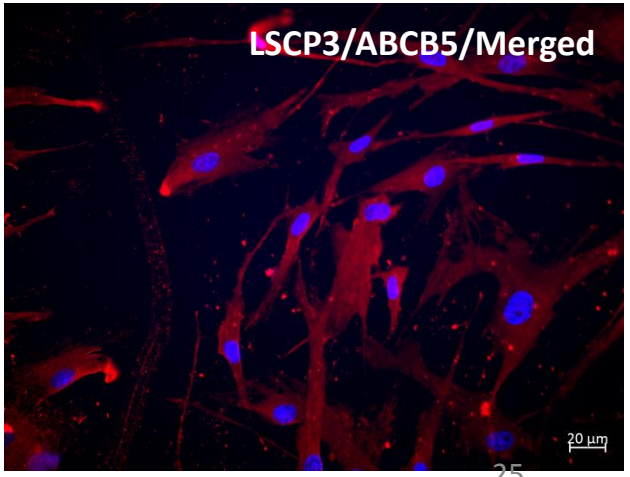
CD34 is hematopoietic proliferation marker

ABCB5





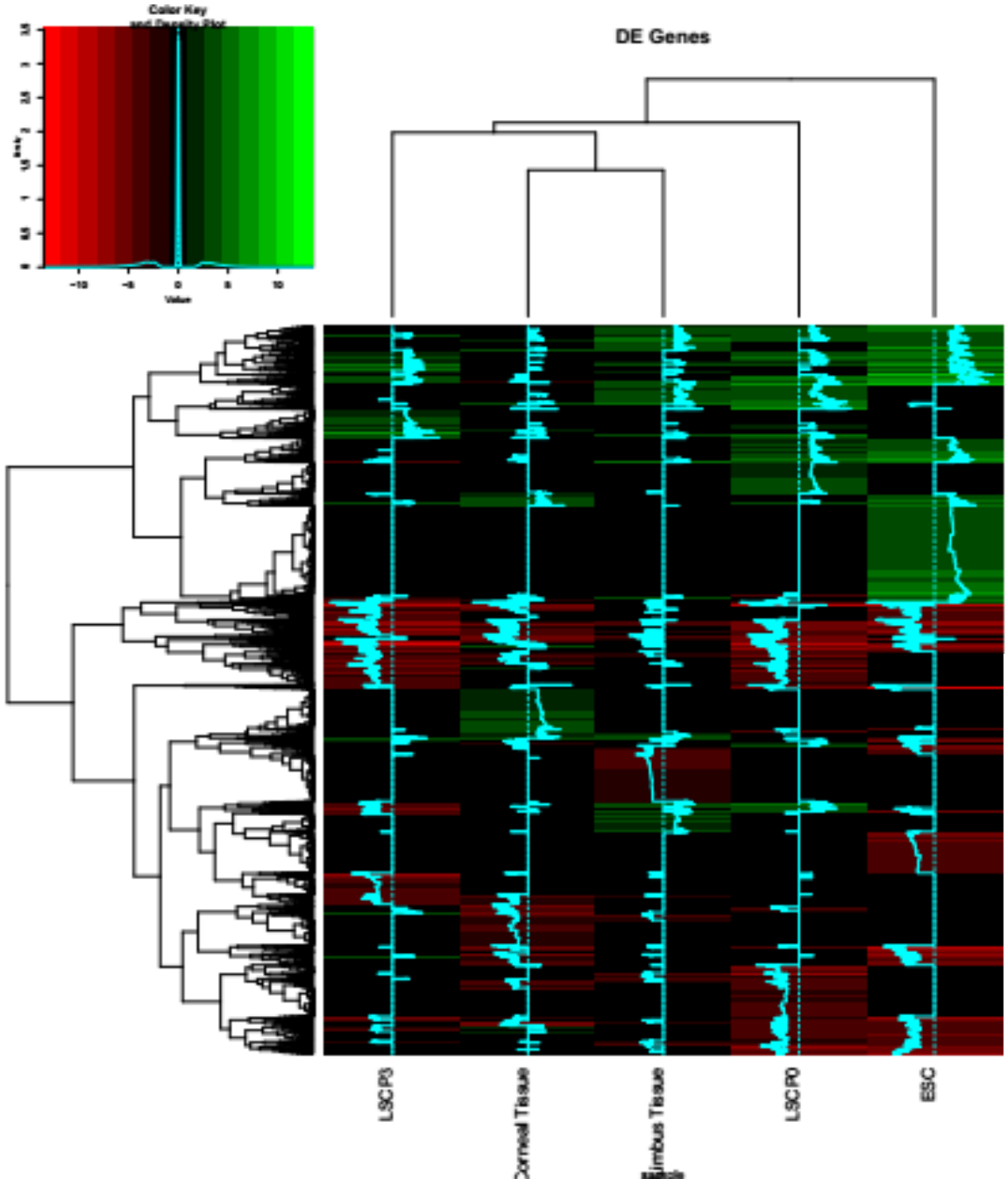




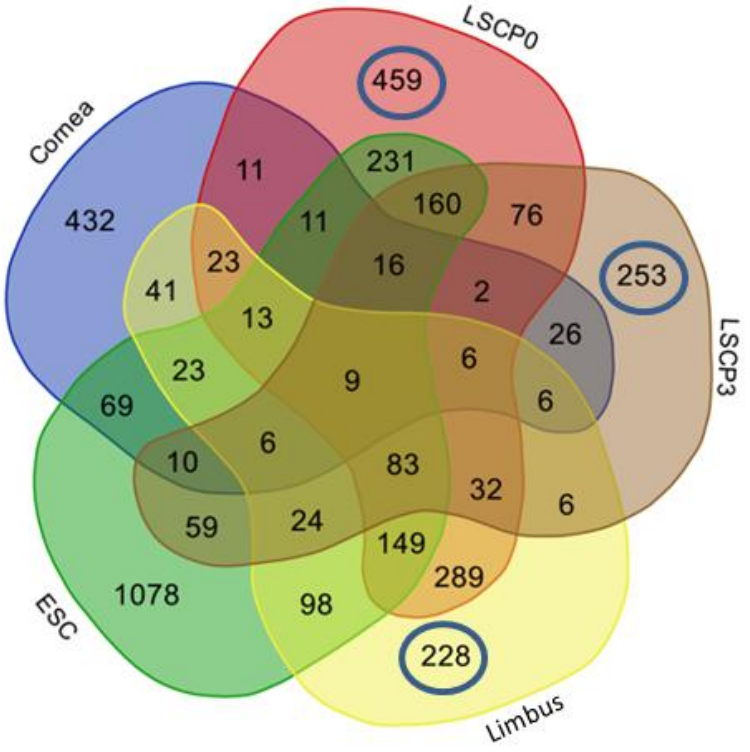
ABCB5 is a limbal stem cell gene required for corneal development and repair

- To find the limbal stem cell biomarker
- Pathway analysis

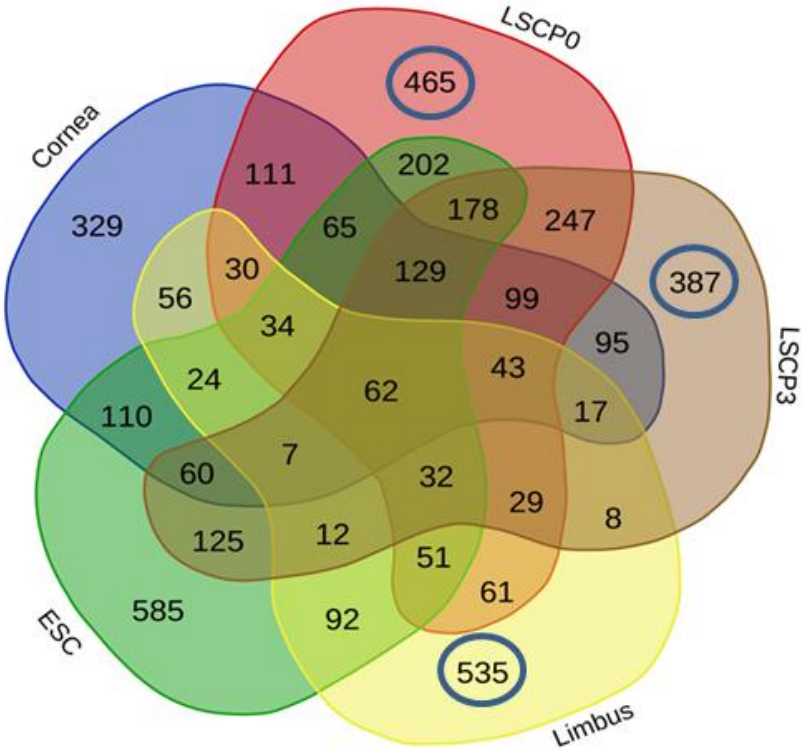
Characterization of limbal stem cell biomarkers



Out of 28000 genes detected by RNA Seq, 7800 genes were differentially expressed and heat map representing the expression status of these 7800 genes in LSCP3, Corneal Tissue, LSCP0 and Embryonic Stem Cell line (ESC) Vs. Sclera



Venn diagram – up regulated genes of LSCP0, LSCP3, ESC , Cornea and Limbus



Venn diagram – down regulated genes of LSCP0, LSCP3, ESC , Cornea and Limbus

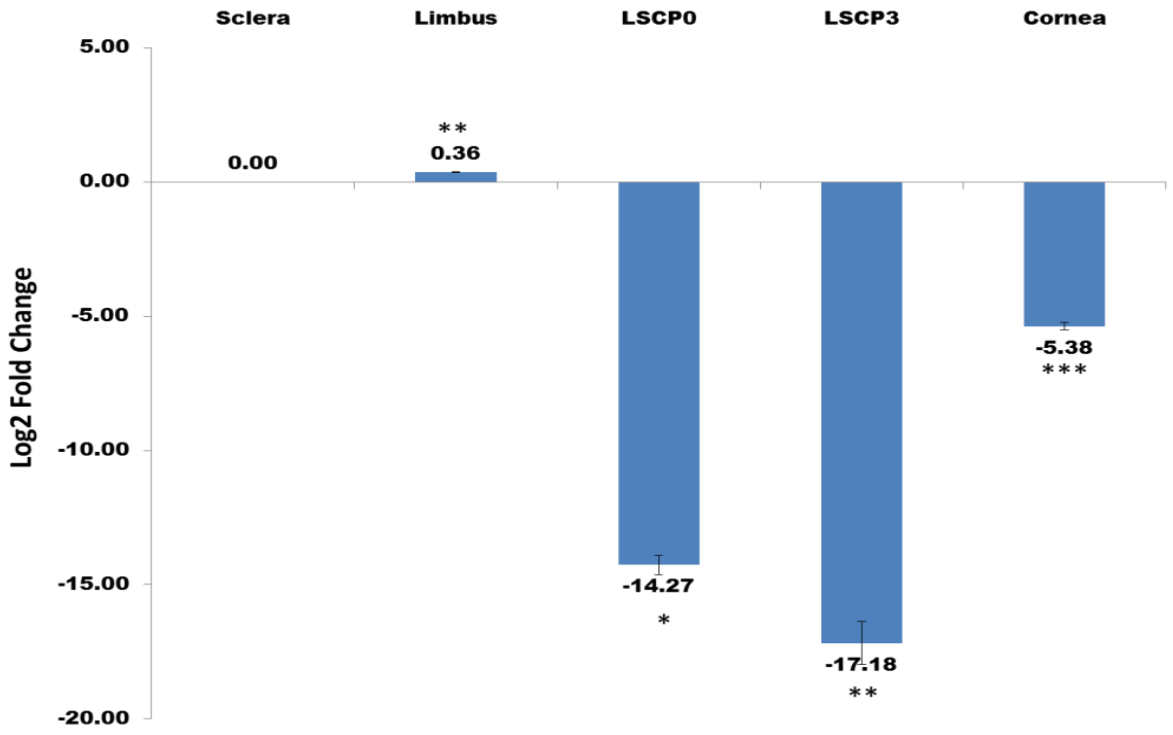
5 top most genes that exclusively up-regulated in limbus which they can be as candidate biomarkers for LSCs

Gene	Corneal Tissue	ESC	Limbus Tissue	LSCP0	LSCP3
CCL21	0	0	5.032894	0	0
FGFR3	0	0	4.656376	0	0
LYPD3	0	0	4.803375	0	0
FOXQ1	0	0	4.47607	0	0
B3GNT8	0	0	4.174022	0	0

Zero representing the no expression or not differentially expression for particular gene in particular sample with respect to sclera as control

CCL21

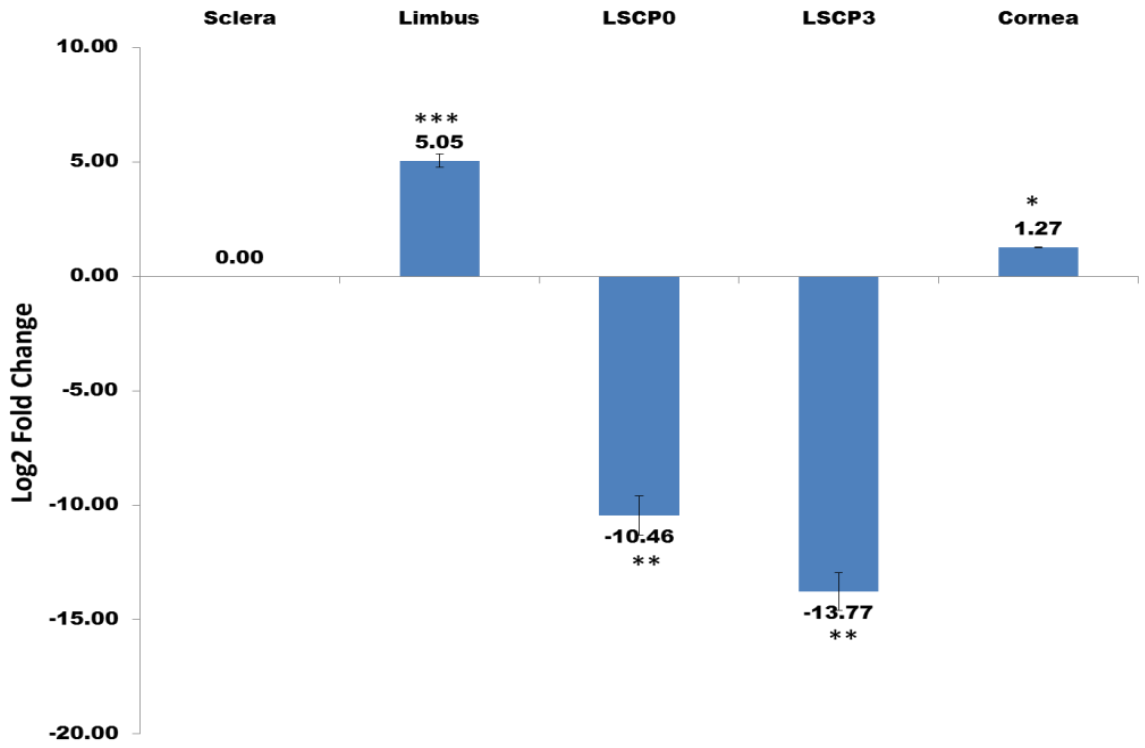
candidate biomarker for LSCs	Limbus Tissue	LSCP0	LSCP3	Corneal Tissue	ESC
CCL21	4.803375	-6.5418	-6.59216	-3.01806	-6.06383
P-Value	1.13E-07	3.24E-06	4.95E-06	0.000821	0.000546



CCL21 is a small cytokine belonging to the CC chemokine family. CCL21 elicits its effects by binding to a cell surface chemokine receptor known as CCR7.

FGFR3

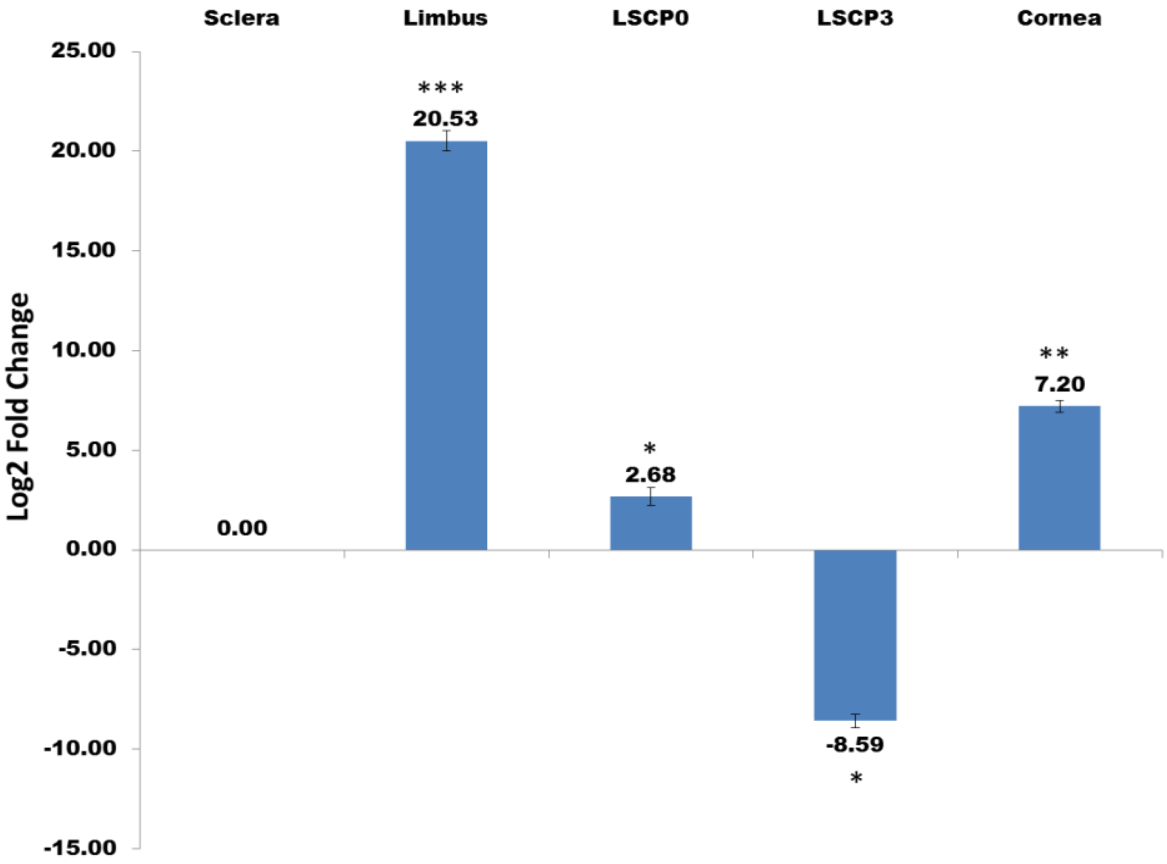
candidate biomarker for LSCs	Limbus Tissue	LSCP0	LSCP3	Corneal Tissue	ESC
FGFR3	4.47607	0.727706	-4.98873	0.786601	3.072642
P-Value	8.48E-07	0.899399	8.99E-05	0.900809	0.203429



FGFR3 acts as cell-surface receptor for fibroblast growth factors and plays an essential role in the regulation of cell proliferation, differentiation and apoptosis.

LYPD3

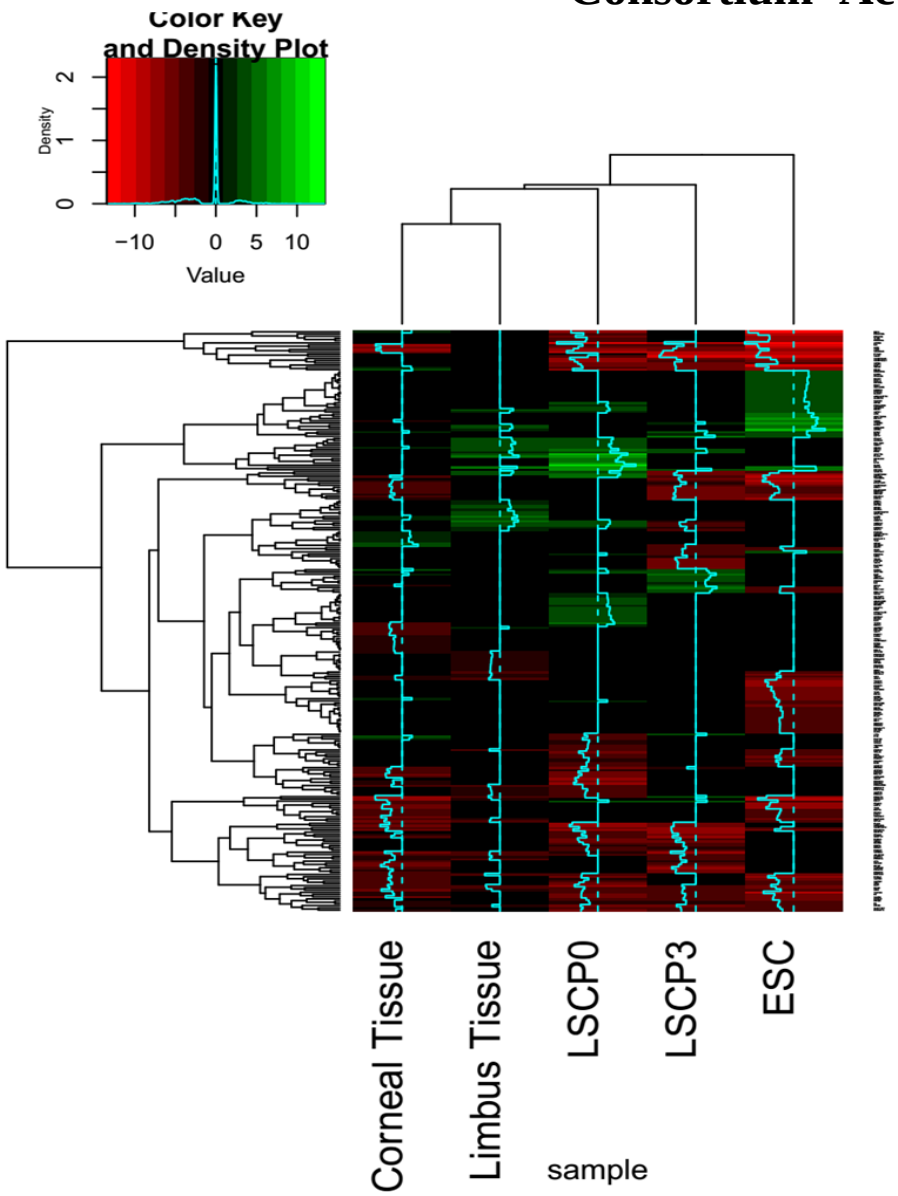
candidate biomarker for LSCs	Limbus Tissue	LSCP0	LSCP3	Corneal Tissue	ESC
LYPD3	4.174022	1.887923	-5.71745	0.554912	-2.06344
LYPD3	6.42E-06	0.539112	1.78E-06	0.95335	0.528598



LYPD3 Supports cell migration

wound healing pathway

Overall 261 genes were collected from AmiGO Gene Ontology - Gene Ontology Consortium- Accession GO:0042060

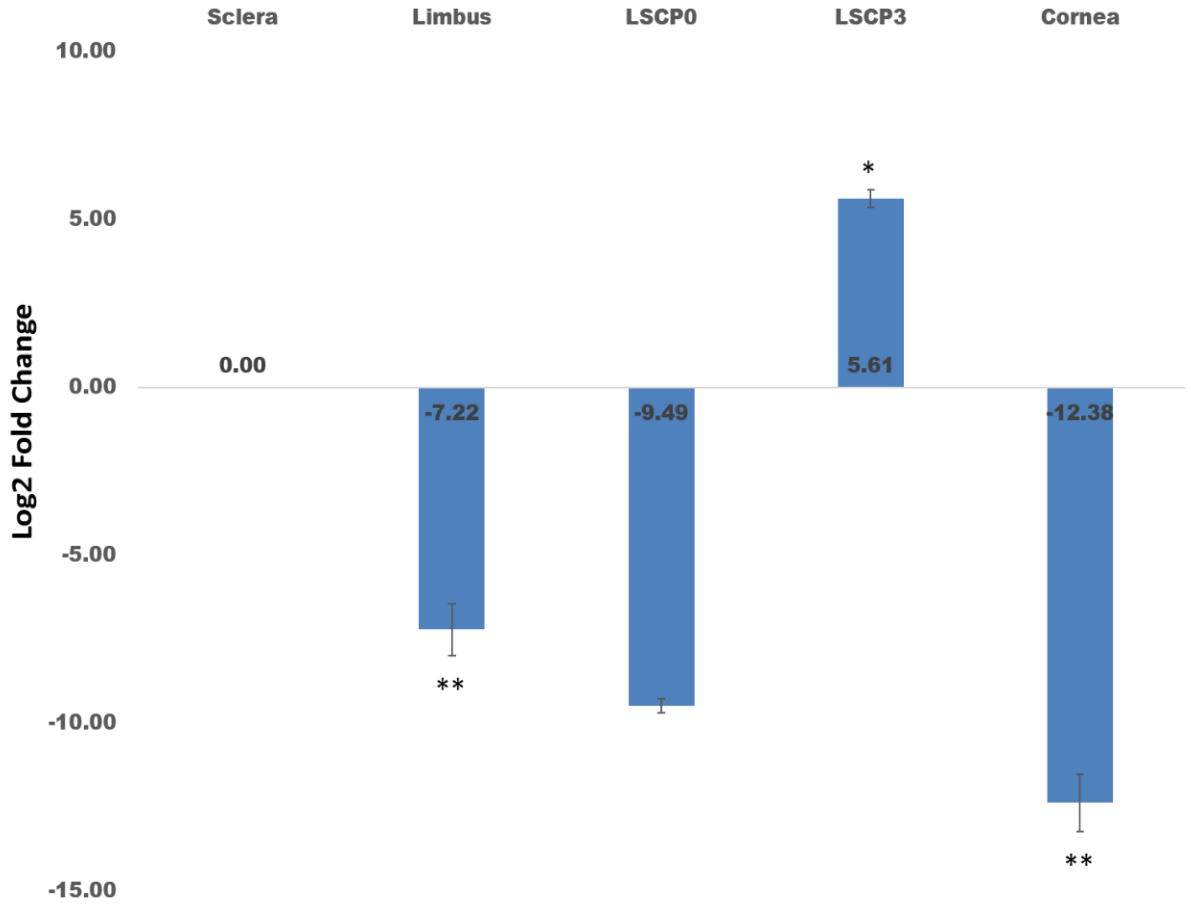


Up regulated 5 top most genes belongs to wound healing pathway in LSCP0 & LSCP3

Genes	Corneal Tissue	ESC	Limbus Tissue	LSCP0	LSCP3
TIMP1	0	0	0	0	4.03621
COL5A1	0	0	0	0	3.536237
PRKCA	0	0	0	0	2.717908
ILK	0	0	0	0	2.591918

TIMP1

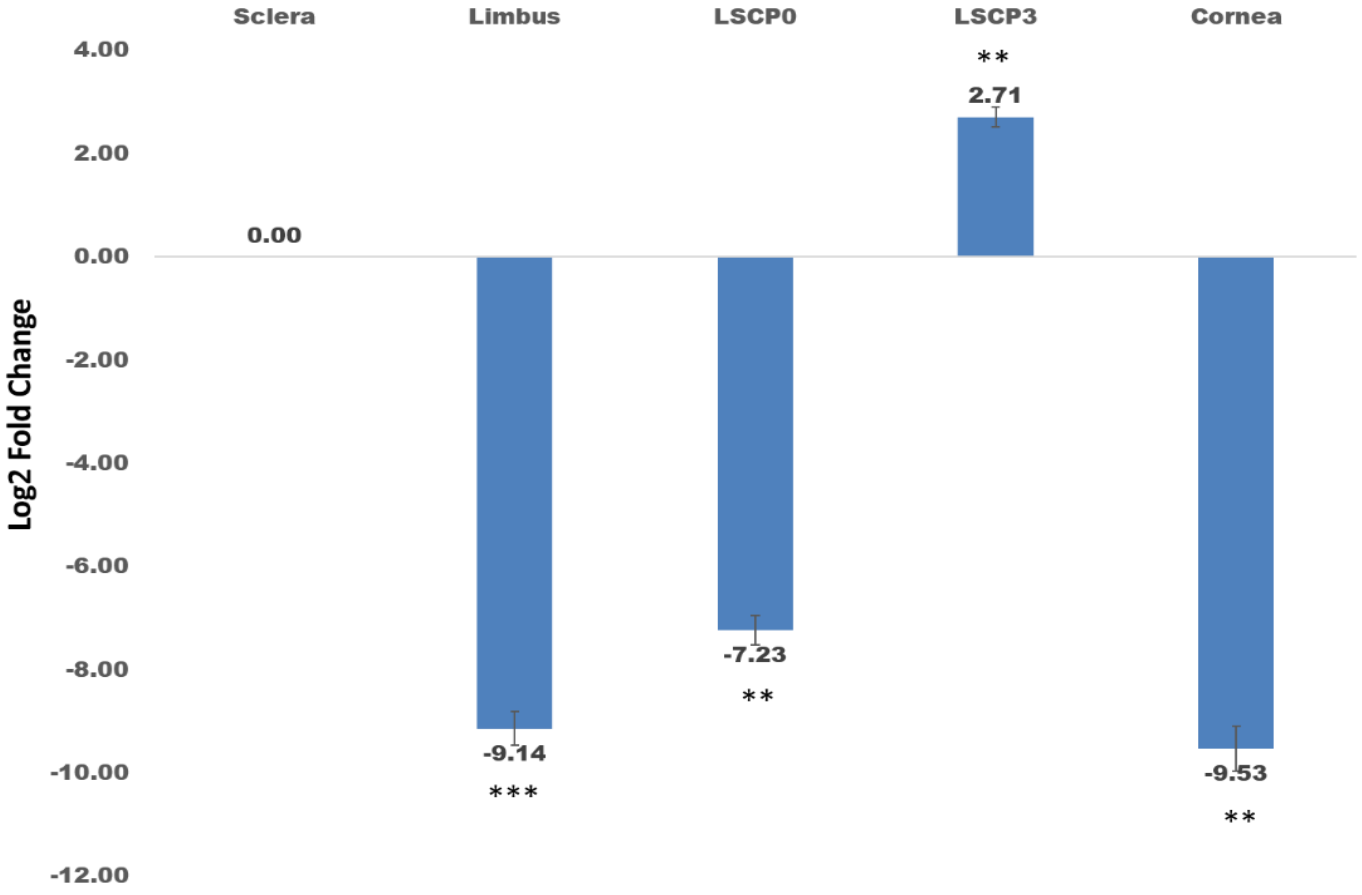
candidate biomarker for LSCs	Limbus Tissue	LSCP0	LSCP3	Corneal Tissue	ESC
TIMP1	-0.43101	-1.27099	4.03621	-0.19688	-2.39514
P-Value	0.986117	0.919315	0.002408	0.990759	0.62186



TIMP1 is metalloproteinase inhibitor and also functions as a growth factor that regulates cell differentiation, migration and cell death and activates cellular signaling cascades via CD63 and ITGB1.

COL5A1

candidate biomarker for LSCs	Limbus Tissue	LSCP0	LSCP3	Corneal Tissue	ESC
COL5A1	-0.47126	-0.74931	3.536237	0.66819	-2.50102
P-Value	0.974271	0.947473	0.011197	0.971249	0.458324



Type V collagen is a member of group I collagen (fibrillar forming collagen). It is a minor connective tissue component of nearly ubiquitous distribution. Type V collagen binds to DNA, heparan sulfate, thrombospondin, heparin, and insulin.

Conclusion

- **Present study indicates that the ABCG2, FGFR3, LYPD3, TIMP1, COL5A1, WNT7A and P63 have potential to be limbal stem cell biomarker (either be as limbal epithelial or limbal stromal biomarker)**

- **As in both late limbal explant culture and passage 3 of limbal stromal culture, epithelial markers (CK3, CK12, CK19, P63 and E-Cadherin) expression decreased and mesenchymal markers (N-Cadherin, Vimentin and HIF1α) expression increased, we can conclude that the age of culture is the key point for mesenchymal enrichment regardless of low serum condition and deepithelialization by passaging (as we assumed first to be the main causes of mesenchymal enrichment)**

- **According to high expression level of hematopoietic markers in LSCP0 which is accompanied with epithelial stem cells expression (as progenitor cells) and stromal stem cells expression (as niche) therefore we can infer that LSCP0 has multipotent stem cells characteristics which can undergo all 3 mesenchymal lineage**

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- Imanishi, J., et al., Growth factors: importance in wound healing and maintenance of transparency of the cornea. Prog Retin Eye Res, 2000. 19(1): p. 113-29.

List of Publications

1. Sachin Shukla, **Fatemeh Tavakkoli**, Vivek Singh and Virender Singh Sangwan., Mesenchymal stem cell therapy for corneal diseases. Expert Opinion on Orphan Drugs, 2016. 4(9): p. 917-926.

2. Mukesh Damala, Abhinav Reddy Kethiri, **Fatemeh Tavakkoli**, Enoch Raju, and Vivek Singh. The Basics of Stem Cells and Their Role in Vision. In book: Trends in Life Science Research, Nova Science Publishers, New York, 2018. (ISBN: 978-1-53613-241-0).

3. James Funderburgh , Sayan Basu , Mukesh Damala , **Fateme Tavakkoli** , Virender Sangwan , Vivek Singh, Limbal Stromal Stem Cell Therapy for Acute and Chronic Superficial Corneal Pathologies: One-Year Outcomes-Investigative Ophthalmology & Visual Science, 2018. **58**(8): p. 3371-3371.

4. Sachin Shukla, Swapna S Shanbhag, **Fatemeh Tavakkoli**, Shobhit Varma, Vivek Singh, Sayan Basu. Limbal Epithelial and Mesenchymal Stem Cell Therapy for Corneal Regeneration. Curr Eye Res. 2019 Jul 6. doi: 10.1080/02713683.2019.1639765. [Epub ahead of print], PMID: 31280624.

5. **Fatemeh Tavakkoli**, Amit Kumar Rai, Mukesh Damala, Virender Singh Sangwan, Sayan Basu, Vivek Singh. Limbal and Corneal Stem Cells in Wound Healing and Homeostasis. Manuscript under review (March 2020).

6. **Fatemeh Tavakkoli**, Mukesh Damala, Abhilash Gangadharan, Amit Kumar Rai, Debashish Dash, Sayan Basu, Virender Sangwan and Vivek Singh. TIMP1 and COL5A1 gene upregulation in limbal stromal stem cells: In silico and in vitro validation in corneal wound healing Manuscript under review (April 2020).

Thank You