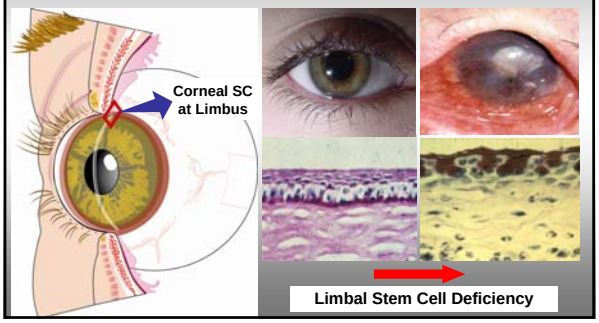
Dhanwantari Lecture Niche Regulation of Limbal Epithelial Stem Cells: *Lessons Learned from Amniotic Membrane in Promoting In Vivo and Ex Vivo Expansion*



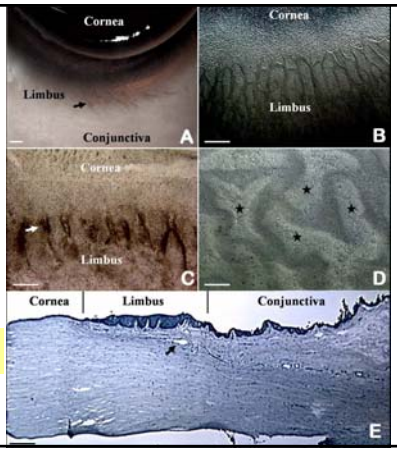
Acknowledgment

- **NIH, NEI; Ocular Surface Research Education Foundation; TissueTech, Inc.**
- **Fellows:** Daniel Meller, Martin Grueterich, Edgar Espana, Tetsuya Kawakita, Wei Li, Ying-Ting Chen, Yasutaka Hayashida, Hua He, Szu-Yu Chen





Human Limbus and Limbal SC Niche



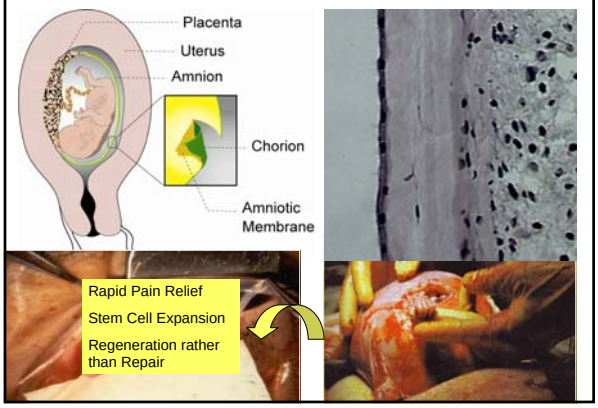
Espana et al, *IOVS*, 2003

Corneal Diseases with Limbal SC Deficiency

Destructive Loss of **Stem Cells**	Dysfunction of **Limbal Niche**
• Chemical (mustard) or Thermal Burns	• Aniridia / Iris Coloboma
• Stevens 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

Ophthalmology 102:1476, 1995; *AJO* 120:368, 1995
BJO 80:911, 1996; *Cornea* 7:31, 1998

AM: Fetal Strategy for Ocular Surface Reconstruction



Niche Features: *Learned from AM*

- *Anti-scarring*: suppress Smad-mediated TGF-β signaling.

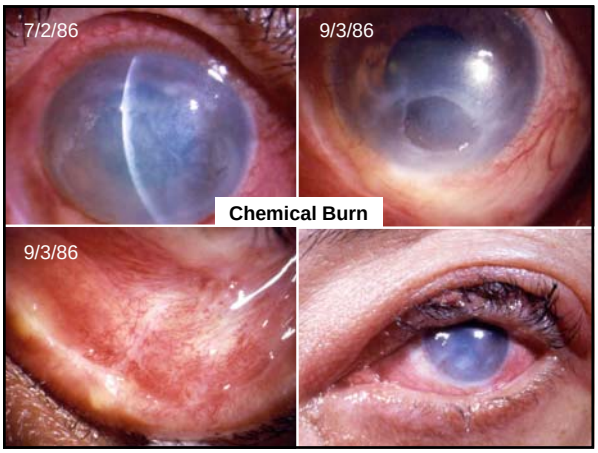
  Tseng et al, 1999; Lee et al, 2000; Choi et al, 2001; Espana et al, 2004; Kawakita et al all, 2005

- *Anti-inflammatory*: facilitate apoptosis of PMN, lymphocyte, macrophage; downregulate IL-1; NF-κB signaling

  Park et al, 2000; Wang et al, 2001; Heiligenhaus et al, 2001; Solomon et al, 2001; Solomon et al, 2005; Li et al, 2006

Two Major Modes of AMT

As a **Permanent Graft**	As a **Biological Bandage**
➢ AM does **not dissolve** but is integrated into the host tissue (cornea or conjunctiva) ➢ One or multiple layers as a filler for stromal defect of any shape (the orientation of membrane matters only for the top layer with the sticky side down) ➢ Suture (10-0 nylon) interrupted (bulbar) and 8-0 Vicryl (fornix) ➢ **Fibrin Glue** without sutures	➢ AM is either **dissolves or removed** after healing ➢ Time of AM dissolution coincides with healing ➢ If AM dissolves in one week, suspect "**exposure**" problem ➢ Suture (10-0 nylon) purse-string running perilimbal sclera ➢ **Prokera™** without sutures ➢ IOP by Tonopen and fluorescein staining without removing **Prokera™**

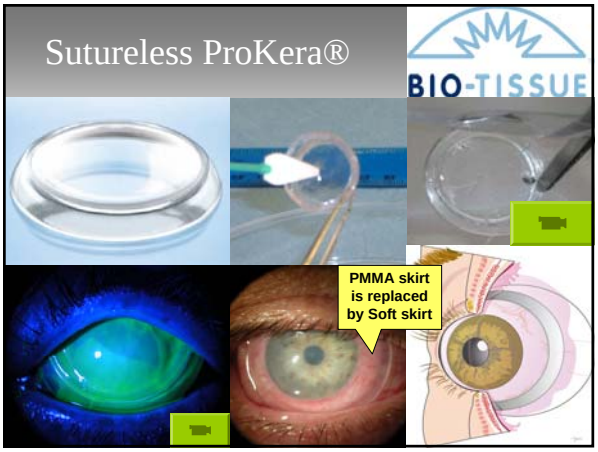


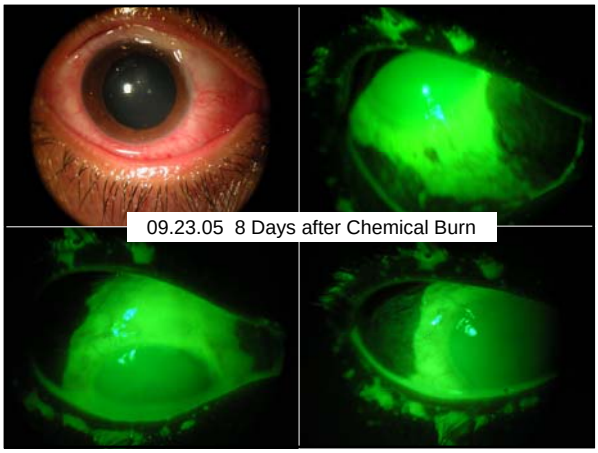
AM Strategies for LSCD

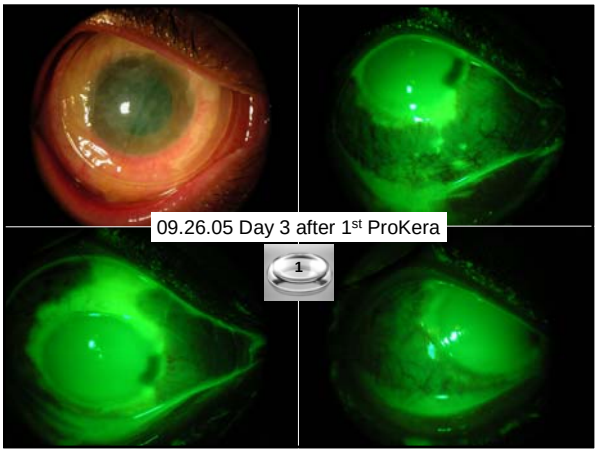
❶ For **acute** burn, rapid application of AM as a temporary biological bandage can **prevent LSCD by resurrecting remaining limbal SCs**.

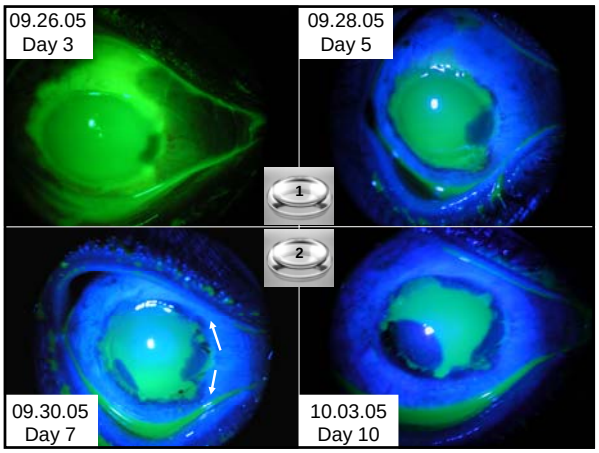
❷ For **chronic** burn with "**nearly total LSCD**", AM as a permanent graft can **promote remaining limbal SC expansion**.

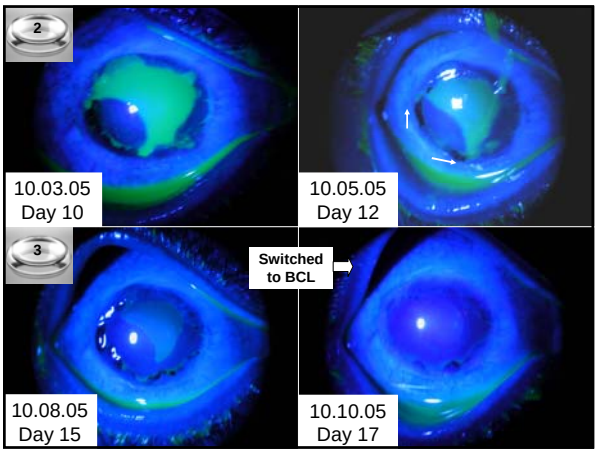
❸ For **chronic** burn with **"total LSCD",** AM as a permanent graft can **promote *in vivo* expansion** of transplanted autologous and allogeneic limbal SCs.

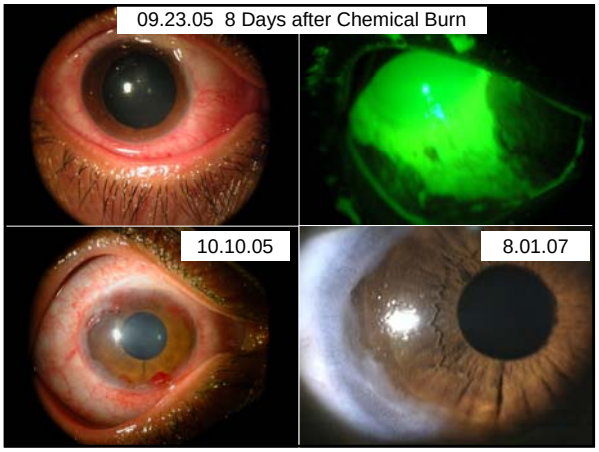


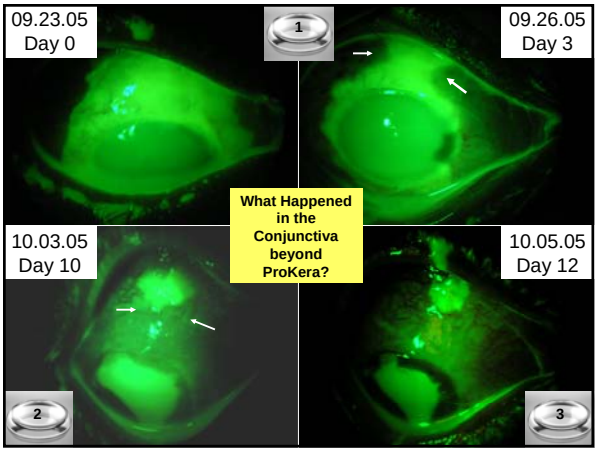


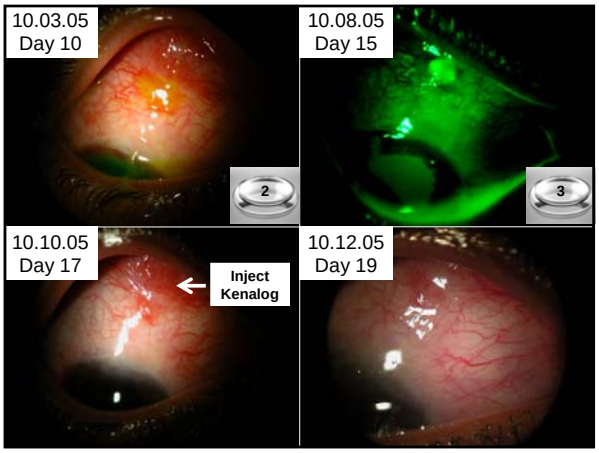










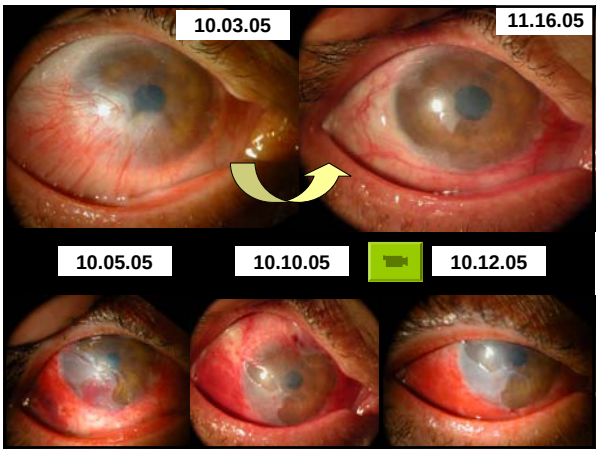


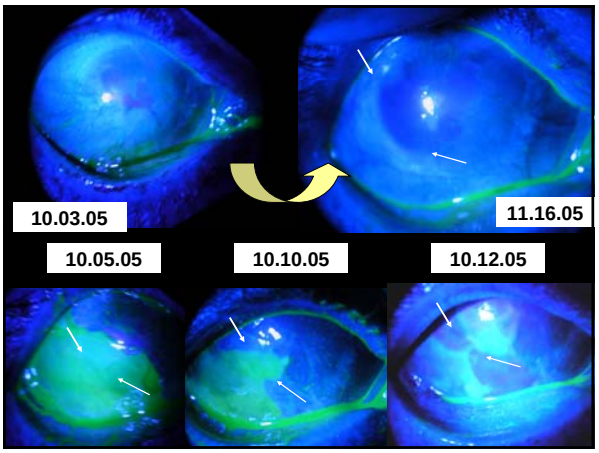
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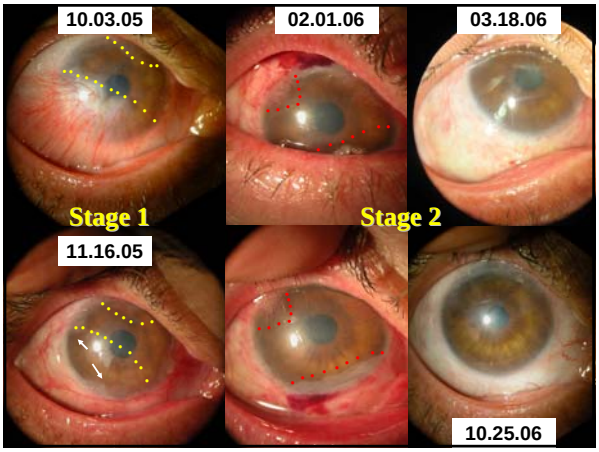
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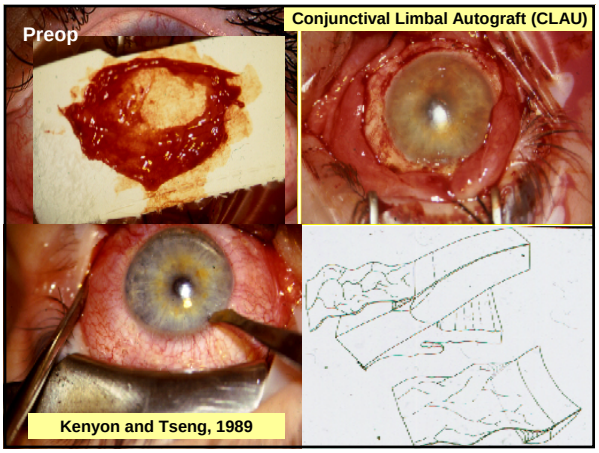


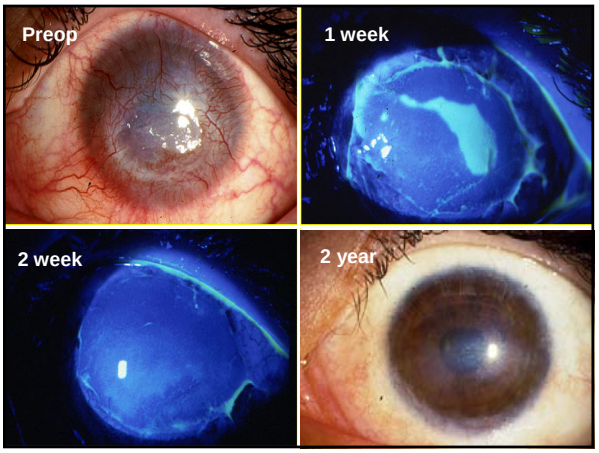
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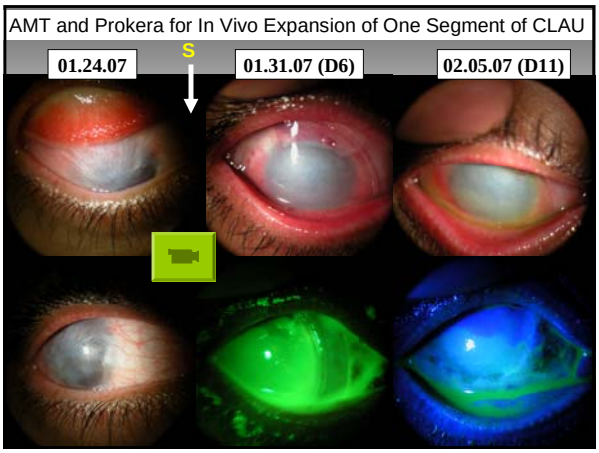
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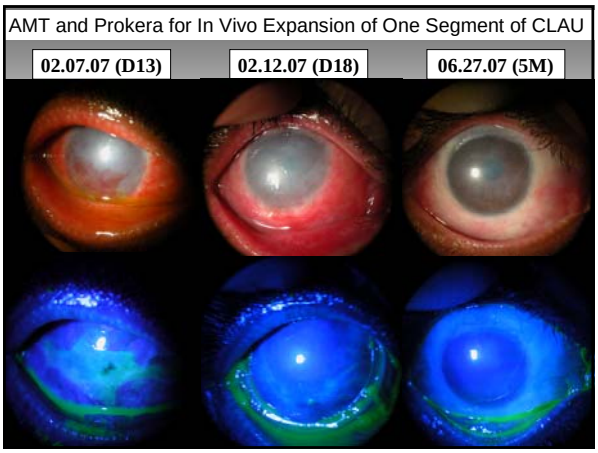




AMT and Prokera for In Vivo Expansion of One Segment of CLAU

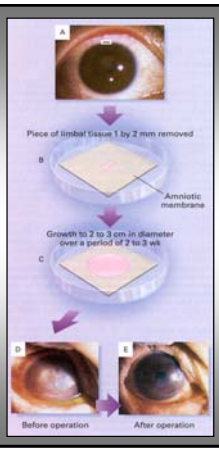


AMT and Prokera for In Vivo Expansion of One Segment of CLAU

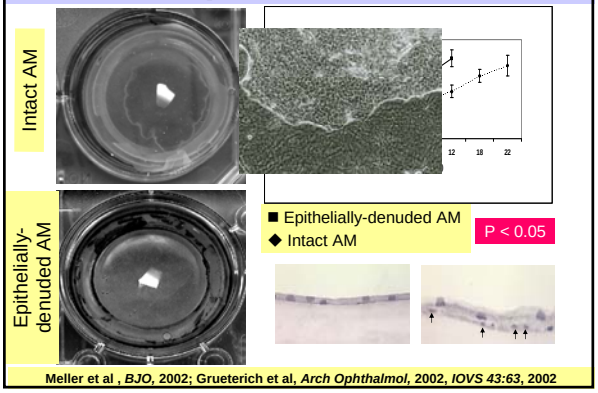


AM as a Surrogate Niche for *Ex Vivo* Expansion of Limbal Epithelial Stem Cells

- Encouraging **human** results
  - *Autologous* — Pellegrini et al, 1997; Tsai et al, 2000; Schwab et al, 2000
  - *Allogeneic* — Koizumi et al, 2001
- Encouraging **rabbit** results
  - *Short-term* — Koizumi et al, 2000
  - *Long-term* — Ti et al, 2002, *IOVS*

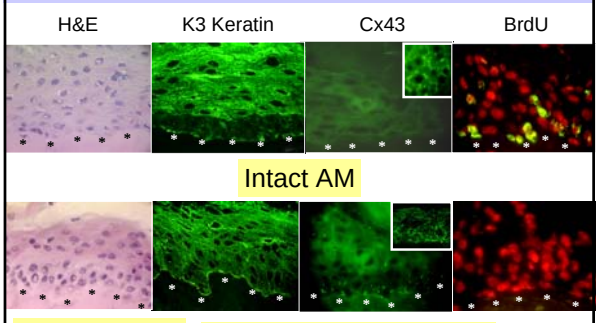


Epithelial Outgrowth of Human Limbal Explants on Amniotic Membrane

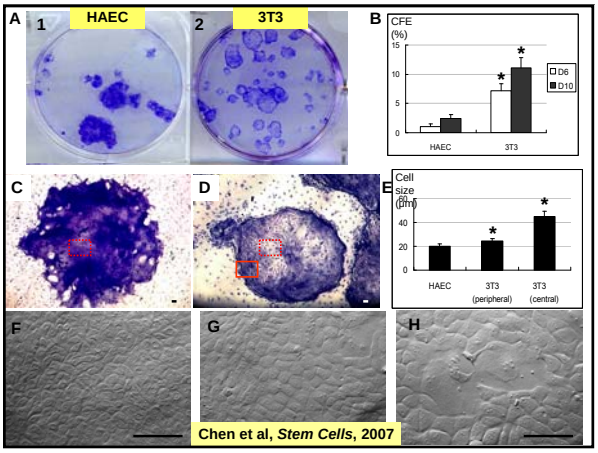


Meller et al , *BJO*, 2002; Grueterich et al, *Arch Ophthalmol*, 2002, *IOVS 43:63*, 2002

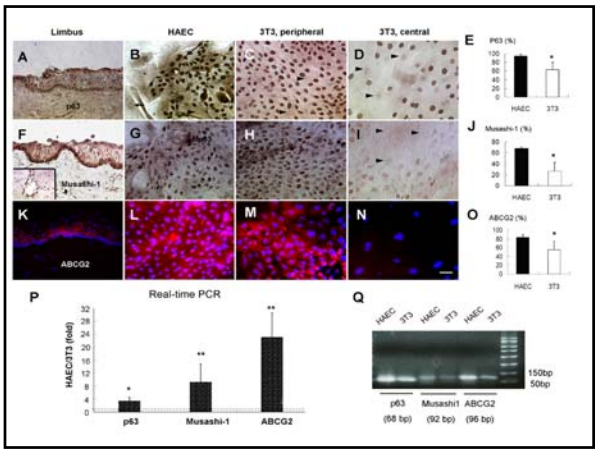
Intact AM Preserves Limbal Epithelial Phenotype While Epithelially-denuded AM Promotes Corneal Epithelial Phenotype after Xenotransplantaion to Nude Mice

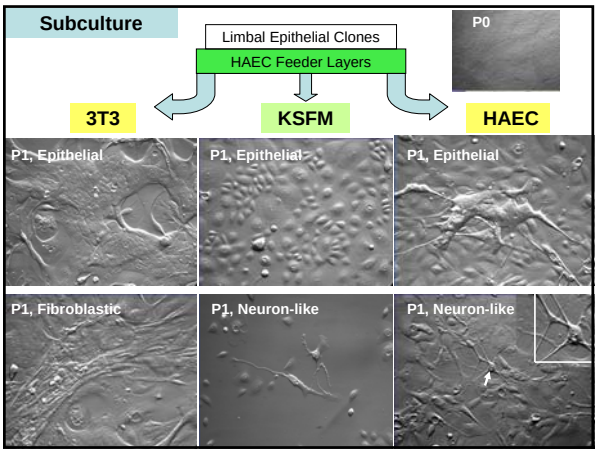


Grueterich et al, *IOVS 43:63*, 2002

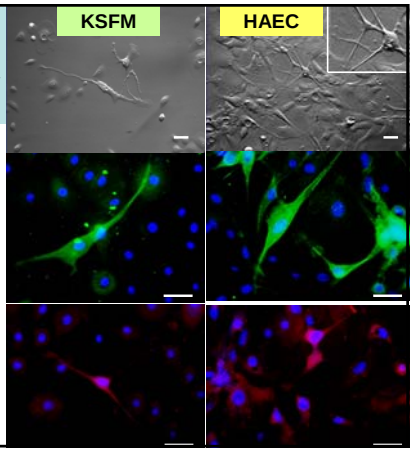


Chen et al, *Stem Cells*, 2007



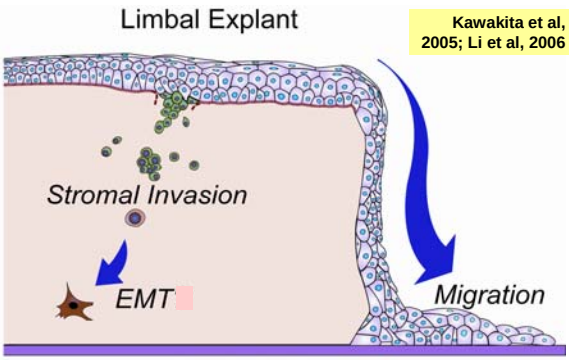


HAEC-supported Clones Exhibited Neuronal Plasticity upon Subculturing

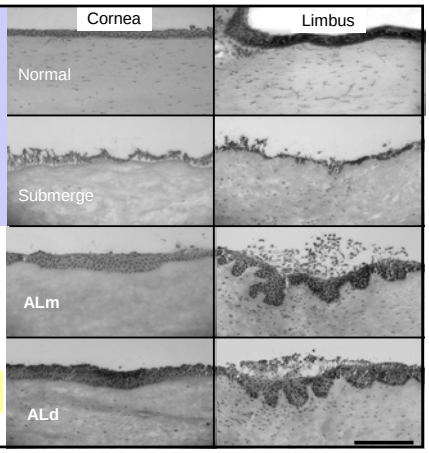


Chen et al, *Stem Cells*, 2007

Intrastromal Invasion by Human Limbal Basal Progenitors

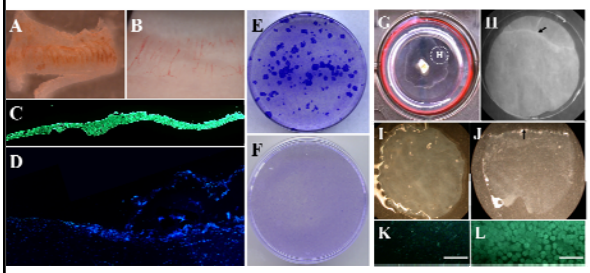


Airlfit Increased **Stratification** in both Corneal and Limbal Epithelium, but Induced **Intrastromal Invasion *only*** in Limbal Basal Epithelial Cells



Kawakita et al, *Am J Pathol* 167:381, 2005

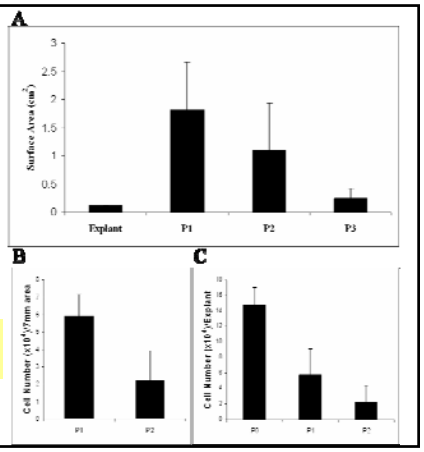
Human Limbal Explants Cultured on iAM to Generate Epithelial Outgrowth

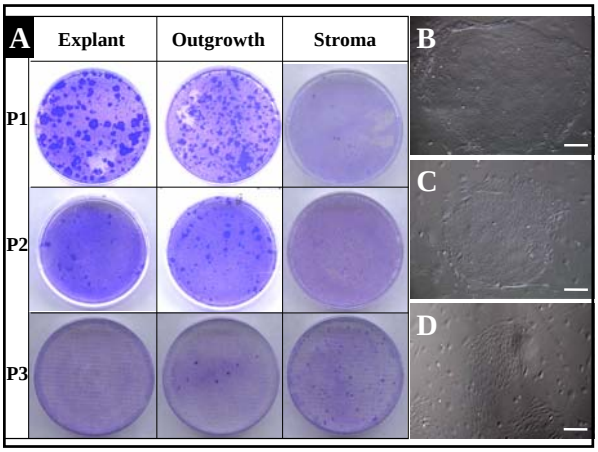


Li et al, *Invest Ophthalmol Vis Sci*, 48:605, 2007

Progressive Decline of Epithelial Outgrowth from Human Limbal Explants

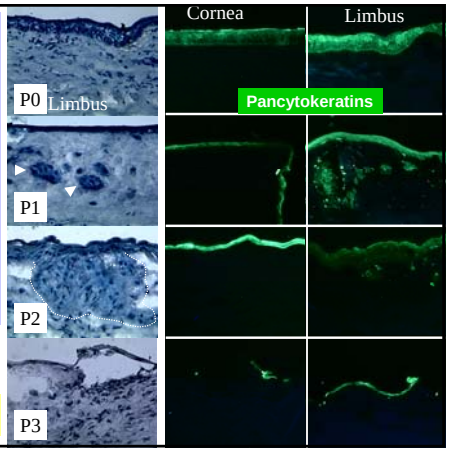
Li et al, *Invest Ophthalmol Vis Sci*, 48:605, 2007





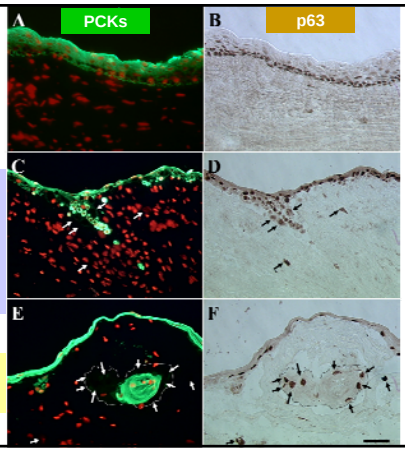
Intrastromal Invasion of Limbal Basal Epithelial Cells Occurs in **Human** Limbal Explants **Cultured on AM** for **15 days** with **repetitive passages**

Li et al, *IOVS*, 48:605, 2007



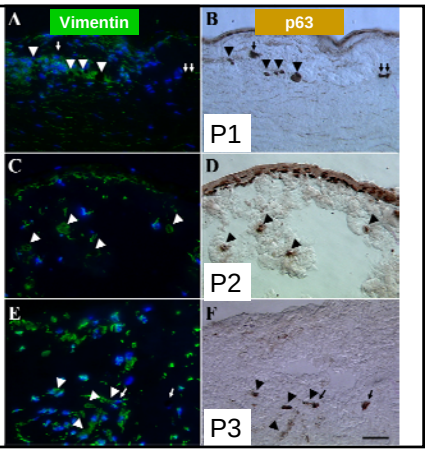
Invading Cells Were
p63(+)PCK(-)
p63(+)PCK(+)
p63(-)PCK(+)

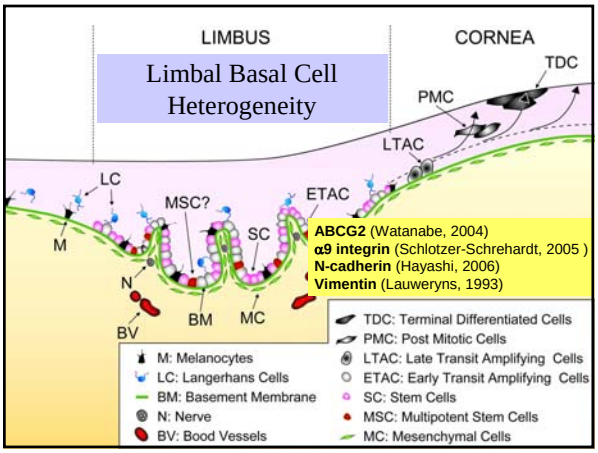
Li et al, *Invest Ophthalmol Vis Sci*, 48:605, 2007

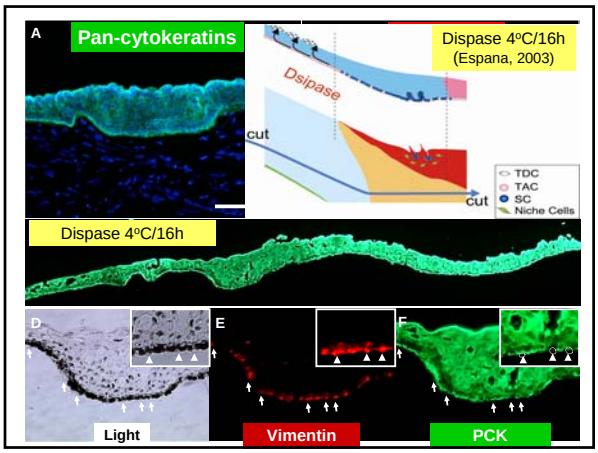


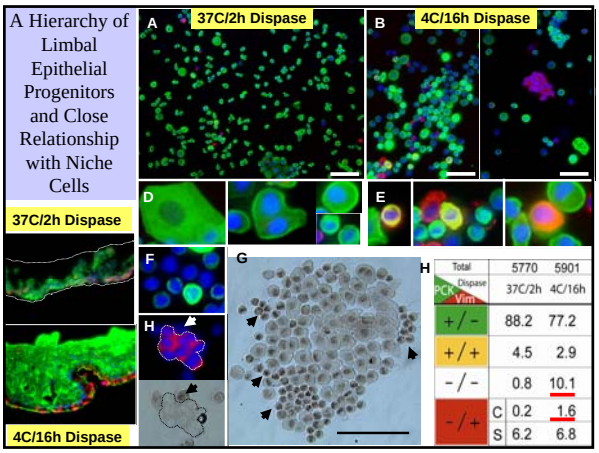
Invading Cells Were
p63(+)Vim(+)
▲
p63(+)Vim(-)
↑

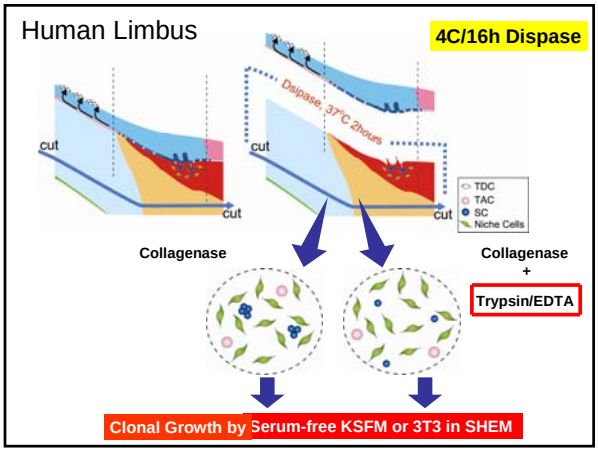
Li et al, *Invest Ophthalmol Vis Sci*, 48:605, 2007

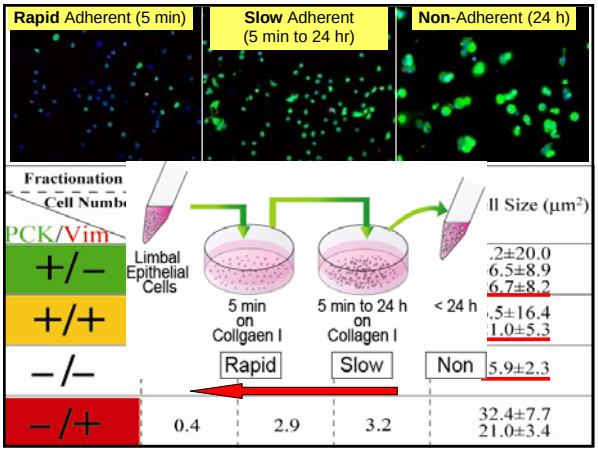


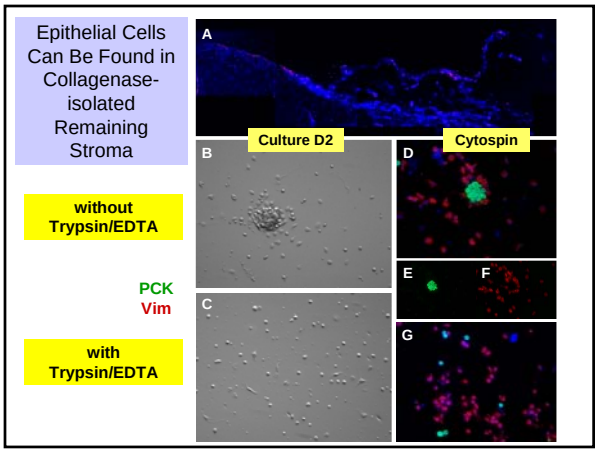


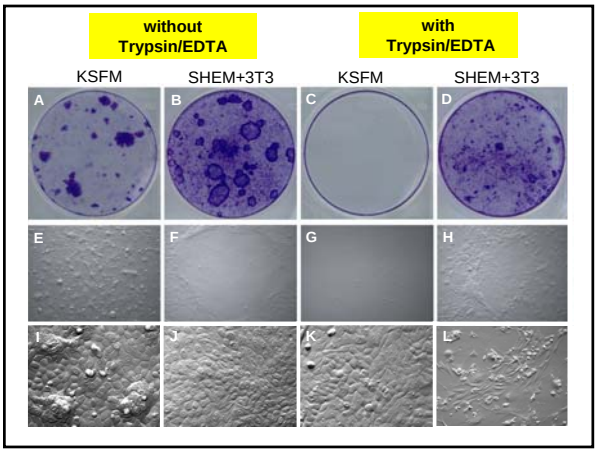

A
Pan-cytokeratins
Dispase 4ºC/16h
(Espana, 2003)
Dsipase
cut
cut
TDC
TAC
SC
Niche Cells
Dispase 4ºC/16h
D
E
F
Light
Vimentin
PCK

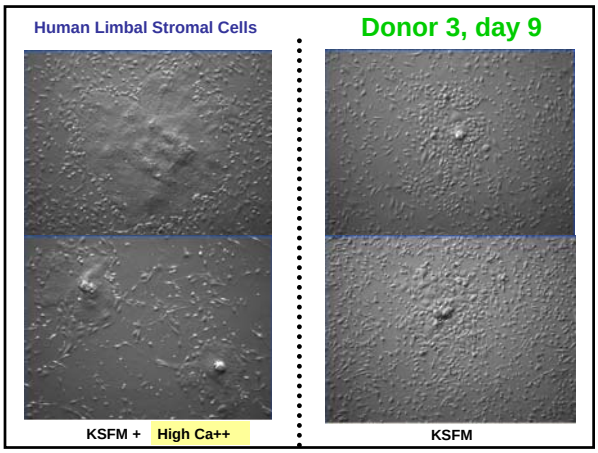

A Hierarchy of Limbal Epithelial Progenitors and Close Relationship with Niche Cells
A
37C/2h Dispase
B
4C/16h Dispase
D
E
37C/2h Dispase
F
G
H
H
4C/16h Dispase
Total 5770 5901
Dispase 37C/2h 4C/16h
PCK Vim
+/- 88.2 77.2
+/+ 4.5 2.9
-/- 0.8 10.1
-/+ C 0.2 1.6
S 6.2 6.8


Human Limbus
4C/16h Dispase
Dispase, 37°C 2hours
cut
cut
cut
cut
TDC
TAC
SC
Niche Cells
Collagenase
Collagenase
+
Trypsin/EDTA
Clonal Growth by Serum-free KSFM or 3T3 in SHEM


Rapid Adherent (5 min)
Slow Adherent
(5 min to 24 hr)
Non-Adherent (24 h)
Fractionation
Cell Numb
ll Size (μm²)
PCK/Vim
+/–
+/+
–/–
–/+
Limbal Epithelial Cells
5 min on Collgaen I
5 min to 24 h on Collagen I
< 24 h
Rapid
Slow
Non
.2±20.0
6.5±8.9
6.7±8.2
.5±16.4
1.0±5.3
5.9±2.3
0.4
2.9
3.2
32.4±7.7
21.0±3.4


Epithelial Cells Can Be Found in Collagenase-isolated Remaining Stroma
without Trypsin/EDTA
PCK
Vim
with Trypsin/EDTA
A
Culture D2
Cytospin
B
C
D
E
F
G


without Trypsin/EDTA
with Trypsin/EDTA
KSFM
SHEM+3T3
KSFM
SHEM+3T3
A
B
C
D
E
F
G
H
I
J
K
L



Conclusion

- **AM** matrix can restore stem cell "**niche**" by suppressing inflammation and scarring, and by promoting ***in vivo*** and ***ex vivo*** expansion of limbal SCs.
- **Caution** should be exercised in the use of enzymatic digestion during isolation and subsequent cultivation of limbal SCs and niche cells.
- **PCK-/Vim-/p63+** putative SCs with smallest size, poor differentiation, and highest clonal growth can partially be enriched by differential dispase digestion and rapid adhesion.
- There is **close physical interaction** between **PCK-/Vim-/p63+ SC** and **PCK-/Vim+/p63- niche cell**.
- **New strategies** to encourage interaction between SC and native or surrogate (e.g., HAECs) niche cell might prevent EMT, ensure SC self renewal/expansion, and preserve plasticity.