Scientific Background Information on BrightMEM Allograft

Significance of Research Question/Purpose:

Limbal stem cell deficiency (LSCD) is a devastating disease that accounts for an estimated 15-20% of corneal blindness worldwide.[1] This disease results from loss or dysfunction of LSCs, a population of pluripotent cells that continuously regenerates the transparent epithelium of the cornea throughout life.[2–4] LSCs are found on the ocular surface in the limbus, where they divide and differentiate into transient amplifying cells (TAC), which then migrate centripetally across the cornea and further differentiate into mature corneal epithelium.[5] Loss of these cells from chemical burns, autoimmune diseases, various congenital disorders, ocular surgeries, and other surface insults can result in partial or total LSCD and associated vision loss.[2]

Without a healthy population of LSCs to regenerate the corneal epithelium, LSCD patients are at risk for recurrent erosions, persistent epithelial defects (PED), corneal conjunctivalization, corneal scarring, and corneal melting. Standard corneal transplants are ineffective. Instead, transplantation of donor limbal tissue containing donor LSCs (keratolimbal allografts or KLAL) is often necessary. However, because systemic immunosuppression with prednisone, mycophenolate, and tacrolimus is required to prevent rejection of the highly antigenic limbal grafts, KLAL is almost always reserved for only the most severe cases.[6-8] For many patients, the risks associated with immunosuppression outweigh the benefits of treatment. Artificial corneas made from plastic polymers such as the Boston keratoprosthesis (KPro) have also been used to treat LSCD because their ability to remain clear in the absence of LSCs and healthy corneal epithelium;[9] however, the significant rates of glaucoma, corneal melt, implant extrusion, infectious keratitis, and endophthalmitis associated with KPros have also limited their use in mild to moderate disease.[10] As such, current treatment options for LSCD remain limited.

Persistent epithelial defects (PED) are a well-known complication of LSCD that results from the failure of the corneal epithelium to regenerate an injury within 10-14 days.[11] PEDs can also occur due to neurotrophic keratopathy, infectious keratitis, chronic exposure keratopathy, or other etiologies where proliferation, migration, and adhesion of the corneal epithelium are impaired. Both LSCD and PEDs are challenging disorders of the corneal epithelial regeneration where therapies to promote durable corneal re-epithelialization remain lacking.

The limbal niche is the microenvironment surrounding the LSCs and limbal TACs that is critical for maintaining their survival and proliferative potential under physiologic conditions.[12,13] The limbal niche is extremely complex and includes the limbal basement membrane, the limbal stroma, the microstructures of the limbus (such as the limbal crypts), as well as the limbal nerve plexus, microvascular, melanocytes, dendritic cells, and mesenchymal stem cells.[14-19] Extracellular signals from this microenvironment are critical to the normal function and maintenance of pluripotent stem cells.[20,21]

Without the support of a niche-like environment, LSCs are often dysfunctional or have limited survival over time. This is evidenced by the fact that, in contrast to KLAL, where limbal tissue is transplanted with LSCs, transplantation of cultured LSCs alone often results in poor long-term survival of the transplanted LSCs. Even in the absence of rejection, transplanted cultured LSCs have been reported to have limited survival beyond 9 months.[22,23]

Similar evidence is also seen in patients with congenital aniridia. In congenital aniridia, limbal niche dysfunction is believed to contribute in part to the later development of LSCD. Due to a PAX6 mutation, patients with congenital aniridia have malformation of their limbus.[24] Consequently, although aniridics do not have LSCD early in life, without a normal limbal niche, these patients gradually develop attrition of their LSCs over time and typically present with LSCD in the second or third decades of life.[24] As such, the limbal niche is critical for long-term LSC and limbal TAC survival and function.

At the same time, extracellular signals from the limbal niche can be powerful drivers of LSC, TAC, and even corneal epithelial proliferation. In fact, treatments that leverage components of the limbal niche to stimulate epithelial cell proliferation on the corneal surface have been explored extensively. Mesenchymal stromal cells are an important component of the limbal niche. Topical application of mesenchymal stromal cells and mesenchymal stromal cell-derived exosomes have been shown to stimulate corneal epithelial regeneration in both LSCD and PEDs.[25,26] By activating cell signaling pathways normally stimulated by the extracellular matrix and cells of the limbal niche, limbal and epithelial cell proliferation can be stimulated even outside of the native limbus.

Isolated components of the limbal niche thus holds significant promise as potential therapies for ocular surface diseases. Descemet's membrane (DM), an acellular, naturally occurring basement membrane found on the posterior surface of the cornea, is a promising substitute for limbal basement membrane. DM is routinely isolated and transplanted intraocularly for treatment of diseases such as Fuchs' dystrophy and corneal bullous keratopathy. However, its application on the ocular surface has not been explored.[27] DM is optically clear and highly resistant to collagenase digestion.[28,29] This is evidenced by the fact that Descemetoceles can remain intact for months in the setting of a sterile corneal melt. Taken together, this makes DM attractive as a potential long-term corneal on-lay and substrate for supporting corneal epithelial regeneration.

The anterior fetal banded layer of DM shares key compositional similarities with limbal basement membrane, which is a major component of the limbal niche. These similarities include limbus-specific extracellular matrix proteins such as collagen IV that is restricted to the α1 and α2 subtypes, vitronectin, and BM40/SPARC.[30-32] Of these, vitronectin and BM40/SPARC are known to promote proliferation of LSCs and induced pluripotent stem cells (iPSC) in culture.[33,34]

Because of this, DM is a promising biological membrane for establishing a pro-proliferative substrate on the corneal surface in patients with LSCD and PEDs.

	Epithelial BM		DM
	Central	Limbus	Stromal face
Type IV collagen chains	α3-α6	α1,α2,α5,α6	α1,α2
Laminin chains	α1,α3-α5,β1,β3,γ1-3	α2-α5,β1-3,γ1-3	none
Nidogen-1/entactin-1	+	+	-
Nidogen-2/entactin-2	+	+	-
Perlecan	+	+	-
Netrin-4/β-netrin	-	-	-
Matrilin-4	+	+	-
Tenascin-C	-	+	-
Fibrillin-1	-	+	-
Type VIII collagen			+
Type XII collagen	+ (both forms)	+ (short form)	-
Type XVIII collagen	+	+	-
Thrombospondin-1	+	-	-
SPARC/BM-40/osteonectin	-*	+*	±
Fibronectin	+	+	+
Vitronectin	-	+	+
Endostatin	+	+	
Versican	-	+	

±weak staining in some cases.

**Sclotzer-Shrehardt, et al. (Exp Eye Res 2007) reported (-) in the central epithelial BM, and (++) in the limbal BM. However, Kabasova, et al. (Invest Ophthalmol Vis Sci 2008) reported weak staining in some cases for epithelial BM, limbal BM, and DM.*

Preliminary Data:

Preliminary in vitro data from our lab suggests that DM does indeed have some niche-like properties. Data from our labs shows that DM supports proliferation of LSCs in vitro. LSCs cultured on DM strongly maintain their stem cell phenotype (stemness) as well as their proliferative potential. LSCs cultured for 1 week on DM showed similar expression of putative LSC biomarkers p63α (t-test, p=0.34) and ABCG2 (t-test, p=0.29) and similar co-localized expression of p63α/ABCG2 (t-test, p=0.20) than cells grown on human amniotic membrane (HAM). This suggests that DM, as a substrate, is comparable to HAM at preserving the stemness of LSCs (Fig 1). Currently, HAM is the most widely used substrate for culturing and transplanting cultivated LSCs.[27] HAM is also widely used as a corneal on-lay alone in the treatment of ocular surface diseases such a persistent epithelial defects (PED) and LSCD.

On in-cell western, LSCs cultured for 1 week on DM showed comparable expression of putative LSC biomarkers: p63α (t-test, p=0.35), ABCG2 (t-test,

p=0.17), and ABCB5 (t-test, *p*=0.36) when compared to HAM (Fig 2). And on BrdU pulse-chase assays, LSC cultured on DM showed similar proliferative rates to HAM (t-test, *p*=0.52) (Fig 3). This suggests that DM is comparable to HAM in maintaining both the stemness and proliferative potential of LSCs.

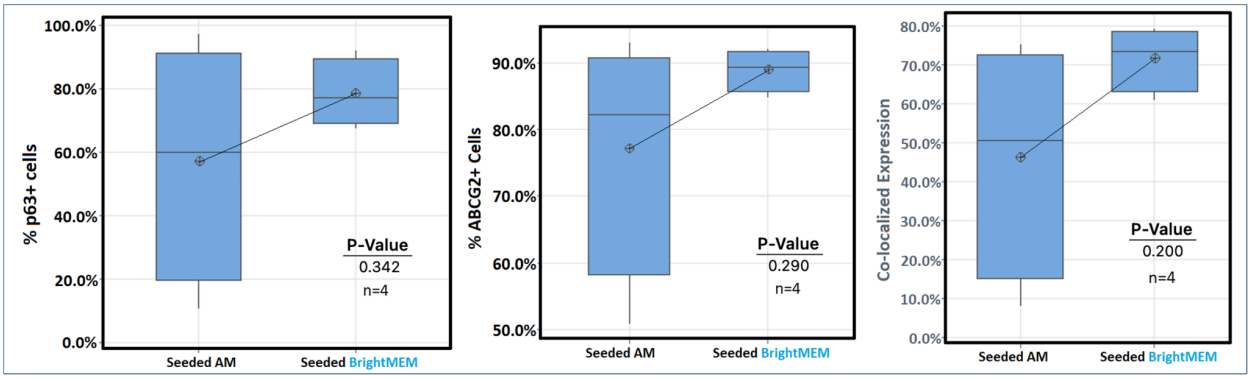

Figure 1. Limbal stem cells(LSC) isolated from donor tissue were seeded onto amniotic membrane (AM) and BrightMEM and cultured in serum-free media to confluence (10-14 days). LSC biomarker expression in cells cultured on BrightMEM was non-inferior and less variable then in cells cultured on AM.

Fig 2. Expression of LSC Biomarkers (In-cell Western)

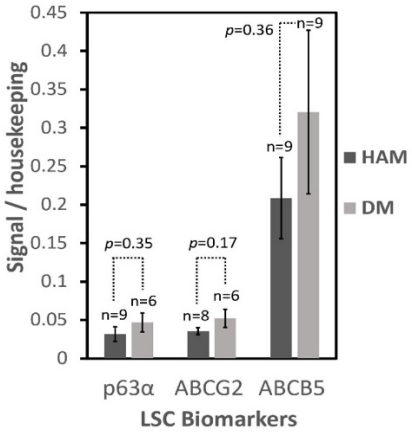

Fig 2. Donor LSPC showed equal expression of LSC biomarkers (p63α, ABCG2, ABCB5) when cultured on DM versus HAM based on in-cell Western. (μ ± SEM).

Fig 3. BrdU Assay

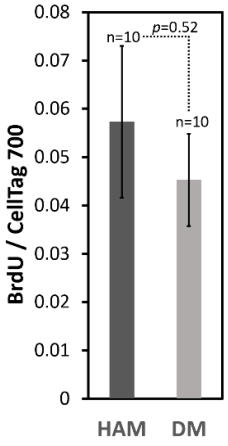

Fig 3. BrdU assay showed similar proliferative rates in LSPC cultured on DM vs HAM (μ ± SEM).

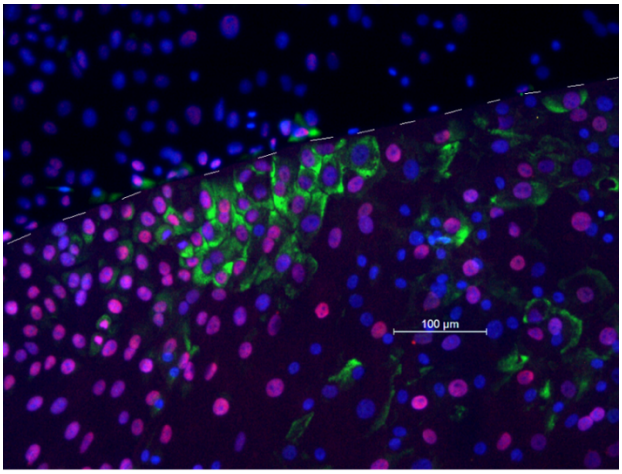

Fig 4. Outgrowth culture of LSPCs on DM growing onto tissue culture plastic (TCP). Cells on DM (below the white arrows) show greater expression of p63α (red) and ABCG2 (green) compared to cells growing off DM on TCP.

Similarly, in outgrowth cultures of LSCs growing from DM onto adjacent tissue culture plastic (TCP), there was loss of ABCG2 and p63α expression in cells that grew off DM after 2 weeks, while cells that remained supported on DM had greater retention of ABCG2 and p63α expression (Fig 4). This supports the niche-like function of DM as a substrate.

LSCs cultured on DM also demonstrated normal stratification and differentiation into mature corneal epithelium (K12+) when airlifted, suggesting that DM can support normal stratified corneal epithelium as well as LSCs. Furthermore, in stratified epithelium cultured on DM, expression of ABCG2 and p63α was preserved in the basal cell layer in contact with DM. This suggests that DM, as a substrate, can help preserve a reservoir of highly proliferative, LSC-like cells in the basal epithelium (Fig. 5).

Fig 5. Immunohistochemistry of Cultured LPSC on DM under Airlifted Conditions

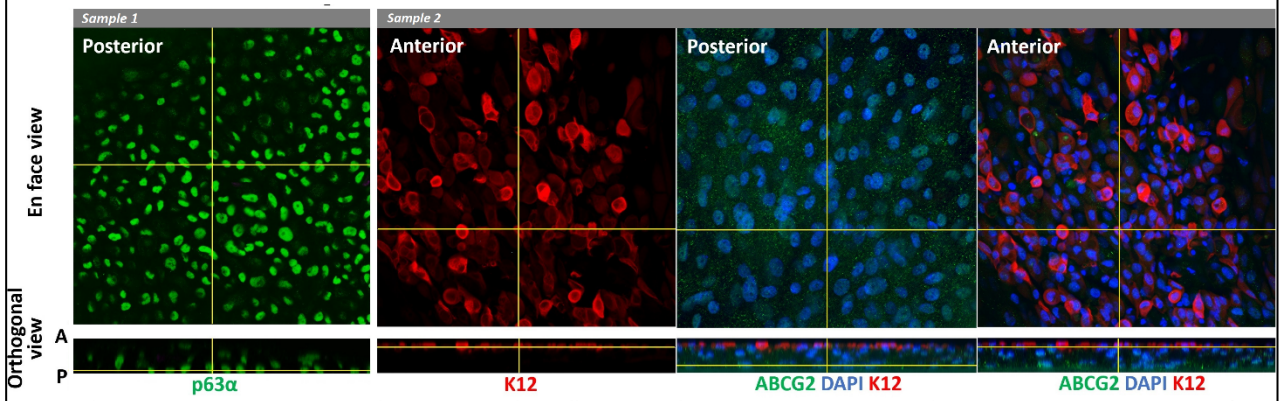

Fig 5. Confocal microscopy demonstrating stratification and superficial differentiation of LSPC cultured on DM under airlifted conditions. LSC marker expression (p63α, ABCG2) was greatest in the basal cells (posterior) adjacent to DM. Superficial cells (anterior) showed corneal epithelial differentiation (K12+). A: anterior. P: posterior.

In degradation studies, DM is also shown to be far more resistant to collagenase digestion than HAM. When submerged in serial dilutions of collagenase A (0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, and 5 mg/ml), DM persisted for at least 24 hours at all concentrations, while HAM was completely degraded within 8 hours. Though HAM is effective in promoting corneal re-epithelialization, HAM is known to rapidly degrade within weeks on the ocular surface, limiting the durability of any therapeutic effect. Our preliminary data suggests that DM is far more durable and has the potential to be a long-term substrate on the ocular surface.

In early clinical cases, DM has been shown to adhere well and re-epithelialize in all cases (Fig. 6). DM has been shown to persist out to at least 13 months. In addition, early evidence suggests that DM may help prevent or delay recurrence of LSCD and corneal neovascularization in partial LSCD (Fig. 7).

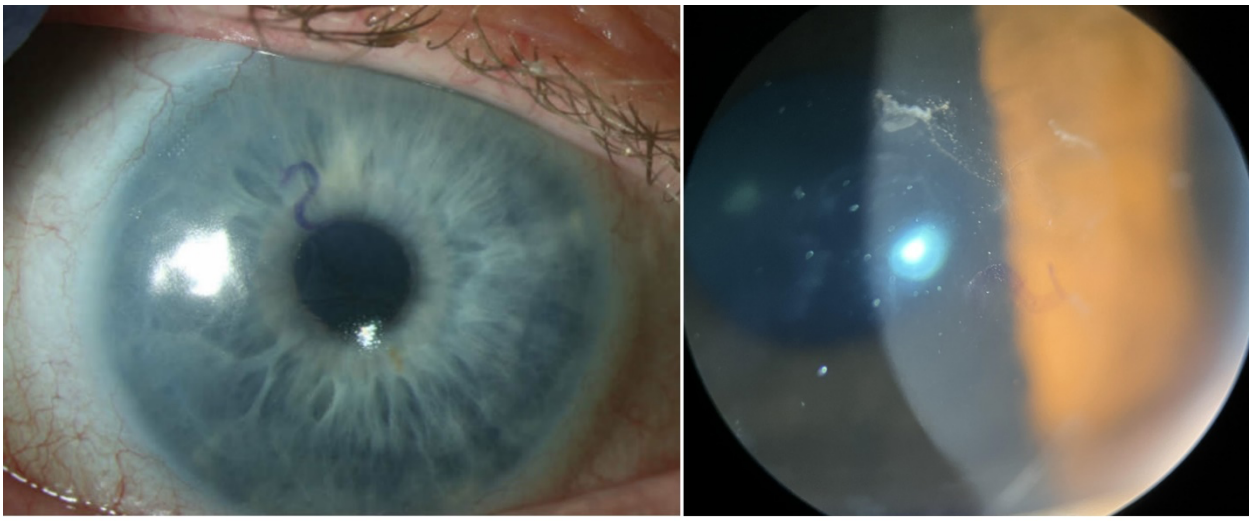

Fig 6. POD#1 and POM#2 after DM corneal on-lay in two different patients. The backwards S-stamp orientation marker is seen at slit lamp confirming the presence of the graft under the epithelium.

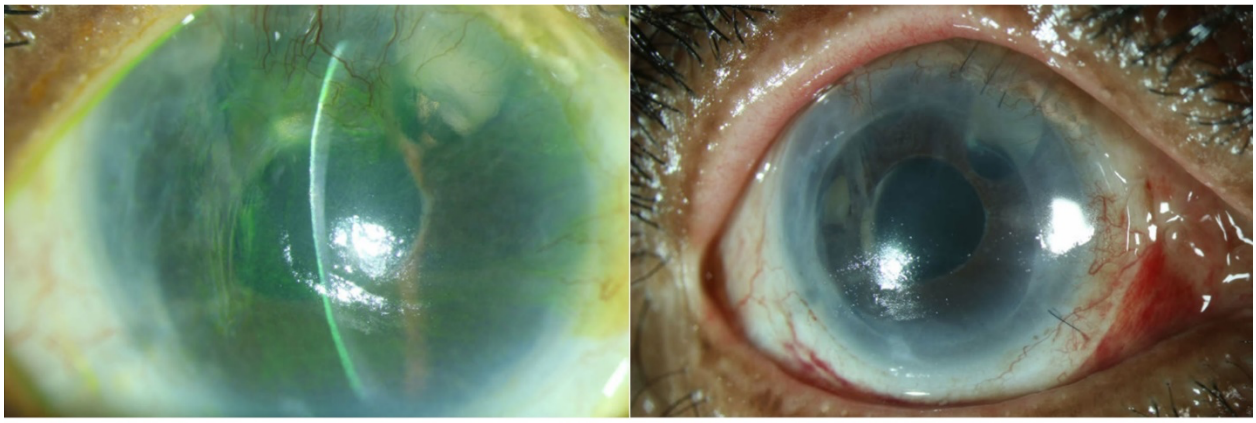

Fig 7. Pre-op and POM#3 after DM corneal onlay + DSAEK for treatment of corneal endothelial decompensation and partial superior LSCD (2/2 trabeculectomy with MMC). Post-operatively, there was regression of the pre-operative superficial corneal neovascularization. Note: a bandage lens is present in the POM#3 image.

Summary:

Limbal niche components are potent drivers of LSC, TAC, and corneal epithelial proliferation and may have a therapeutic benefit in management of corneal epithelial disease. DM is naturally occurring basement membrane that is biochemically similar to limbal basement membrane, which is a critical component of the limbal niche. DM is routinely isolated for intraocular

transplantation by eye banks, optically clear, and resistant to collagenase digestion, making it an ideal long-term niche substitute on the ocular surface. Compared to HAM, which is widely used as a temporary corneal graft for treatment of ocular surface disease (HAM degrades after 1-2 weeks on the ocular surface), DM is relatively stable on the ocular surface and resistant to degradation. Preliminary data from our lab suggests that DM can provide a stable substrate on the corneal surface to support the proliferative potential of the basal epithelium. This suggests that DM may be an ideal corneal allograft for long-term support of LSCs and corneal epithelium on the corneal surface. Early clinical cases have demonstrated good adhesion and re-epithelialization of DM on the ocular surface. However, prospective clinical data is necessary for establishing the safety and efficacy of DM corneal allografts in patients with corneal epithelial disorders.

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