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Niche Regulation of Limbal Epithelial Stem Cells: *HC-HA/PTX3 as Surrogate Matrix Niche*

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Abstract

Homeostasis of the corneal epithelium is ultimately maintained by stem cells that reside in a specialized microenvironment within the corneal limbus termed palisades of Vogt. This limbal niche nourishes, protects, and regulates quiescence, self-renewal, and fate decision of limbal epithelial stem/progenitor cells (LEPCs) toward corneal epithelial differentiation. This review focuses on our current understanding of the mechanism by which limbal (stromal) niche cells (LNCs) regulate the aforementioned functions of LEPCs. Based on our discovery and characterization of a unique extracellular matrix termed HC-HA/PTX3 (Heavy chain (HC1)-hyaluronan (HA)/pentraxin 3 (PTX3) complex, “-” denotes covalent linkage; “/” denotes non-covalent binding) in the birth tissue, i.e., amniotic membrane and umbilical cord, we put forth a new paradigm that HC-HA/PTX3 serves as a surrogate matrix niche by maintaining the *in vivo* nuclear Pax6+ neural crest progenitor phenotype to support quiescence and self-renewal but prevent corneal fate decision of LEPCs. This new paradigm helps explain how limbal stem cell deficiency (LSCD) develops in aniridia due to Pax6-haplotype deficiency and further explains why transplantation of HC-HA/PTX3-containing amniotic membrane prevents LSCD in acute chemical burns and Stevens Johnson syndrome, augments the success of autologous LEPCs transplantation in patients suffering from partial or total LSCD, and assists *ex vivo* expansion (engineering) of a graft containing LEPCs. We thus envisage that this new paradigm based on regenerative matrix

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HC-HA/PTX3 as a surrogate niche can set a new standard for regenerative medicine in and beyond ophthalmology.

Keywords

amniotic membrane; HC-HA/PTX3; limbus; limbal stem cell deficiency; niche; stem cell

1. Limbal Niche

The corneal epithelium is composed of non-keratinized, stratified squamous epithelial cells held together by tight junctions to protect the cornea from external insult and ensure corneal transparency and clear vision. To maintain corneal epithelial homeostasis and ultimately vision, the corneal epithelium self-renews throughout life from a population of stem cells in the limbal niche (Ang et al., 2004; Cheng et al., 2016; Schermer et al., 1986). Anatomically and clinically, the native limbal niche can be recognized by the pigmented “palisades of Vogt”, which has a vascularized and innervated underlying stroma (Lavker et al., 2004) (Fig. 1A). Palisades are more pronounced in the superior and inferior limbus (Goldberg and Bron, 1982; Townsend, 1991), presumably due to a greater need of limbal epithelial stem cells (LEPCs) to withstand shear stress forces from blinking and progressive attrition of corneal epithelial cells. Other possible explanation suggests the role of eyelids in limbal niche protection from UV radiations may also explain the higher density of LSC in the vertical meridian in humans which is not found in nocturnal mammals such as mice (Grieve et al., 2015). Within this locale, LEPCs reside in the basal epithelial layer (Schermer et al., 1986) between palisade epithelial ridges that extend deep into the stroma beyond the limbal basement membrane to form limbal epithelial crypts (Chen et al., 2011), (Dua et al., 2005), (Kulkarni et al., 2010) (Fig. 1B and 1C). Several studies have found an age-related decline in the number of palisades of Vogt and limbal crypts in humans (Zheng and Xu, 2008), (Notara et al., 2013). This may explain why the basal cell density in the limbal epithelium decreases with age (Niederer et al., 2007), (Tseng, 1989); (Pellegrini et al., 1999), and the epithelial thickness of the limbus and the peripheral cornea gradually deteriorates over time (Yang et al., 2014).

Compared to corneal epithelial cells, LEPCs are smaller in size with a higher nucleus-to-cytoplasm ratio (Shortt et al., 2007), (Pellegrini et al., 1999), (Romano et al., 2003), have a higher proliferative capacity (Cotsarelis et al., 1989; Ebato et al.; Kruse et al.; Lindberg et al.; Pellegrini et al., 1999), and are slow-cycling as demonstrated by their ability to retain DNA labels such as tritiated thymidine ($^3\text{H-TdR}$) over long periods of time (Cotsarelis et al., 1989). Under a steady state, the majority of LEPCs are “mitotically” quiescent and resting in the G_0 phase, with only 4–5% of the LEPC population actively undergoing mitosis (Sagga et al., 2018), (Cotsarelis, Cheng et al. 1989). However, upon wounding of the corneal epithelium, LEPCs are activated to divide asymmetrically to produce two daughter cells: a stem cell, which remains in the limbal niche to replenish the stem cell pool for “self-renewal”, and a transient amplifying cell (TAC) that moves suprabasally in the limbus and centripetally into the cornea for “corneal fate decision” (Tseng, 1989); (Cotsarelis et al., 1989), (Di Girolamo et al., 2015). After a limited number of cell divisions, TAC

differentiates into non-proliferative post-mitotic cells (PMCs), which lose contact with the basement membrane and migrate suprabasally as “terminally differentiated cells” (TDCs) (Pellegrini et al., 1999; Tseng, 1989) (Fig. 1D).

While a marker specific to LEPCs has yet to be confirmed, LEPCs can be distinguished from their progeny by a panel of markers including transporters such as ABCB5 and ATP binding cassette (ABCG2) (Ksander et al., 2014),(Watanabe et al., 2004); transcription factors such as Sox9 (Bath et al., 2013), C/EBP6 (Barbaro et al., 2007), Bmi-1 (Umemoto et al., 2006), Pax6 (Ramaesh and Dhillon, 2003), and p63 α and its Np63 α isoform (Di Iorio et al., 2005; Shortt et al., 2007), cell adhesion molecules and receptors including Frizzled 7 (Mei et al., 2014), N-cadherin (Hayashi et al., 2007), and Notch-1 (Yoshida et al., 2006); and cytokeratins (CKs) such as CK15 (Yoshida et al., 2006), CK14 (Dua et al., 2005);(Ksander et al., 2014), CK3 (Schermer et al., 1986), and CK12 (Liu et al., 1994). Fig. 1 illustrates how these putative markers have been used to denote quiescence, self-renewal, and fate decision from LEPCs to TAC, PMC and TDC (Fig. 1E).

2. Neurovascular Support of Limbal Niche

Among all stem cell niches in the body, nerve fibers frequently coincide and coexist with blood vessels to form a neurovascular network. Taking the epidermis as an example, the upper bulge of the hair follicle, which harbors epidermal stem cells, is extensively innervated by cutaneous fibers (Larson et al., 2010). Whole mount immunostaining of the skin shows perfect alignment of blood vessels and nerves within a neurovascular niche (Larrivee et al., 2009). Sonic Hedgehog signaling pathway that mediates the sensory nerve influence on the epidermal stem cell niche in the upper bulge.(Larson et al., 2010) The unique expression of Gli1 by epidermal stem cells at the upper bulge depends on sensory nerves derived from dorsal root ganglion neurons expressing Sonic Hedgehog to wrap around it.(Larson et al., 2010) Surgical denervation selectively deprives the Gli1 expression resulting in a reduced number of bulge stem cells. A similar pattern of nerve-derived Sonic Hedgehog signaling is observed in controlling the taste bud density on the tongue,(Castillo et al., 2014) and renewal of touch dome stem cells in a perineural microenvironment in the epidermis. (Xiao et al., 2015)

Palisades of Vogt within the cornea limbus are extensively innervated (Lavker et al., 2004) and vascularized (Muller et al., 2003), suggesting that the neurovascular support is critical in maintaining limbal niche integrity. Sensory denervation of the ophthalmic branch of the trigeminal nerve leads to a 2 to 4-fold loss of limbal stem cells (Ueno et al., 2012). Similarly, capsaicin denervation impairs stem cell progeny egress from the hair follicle, resulting in delayed healing of denervated skin (Martinez-Martinez et al., 2012);(Gho et al., 2016). It is no wonder why insults to the limbal neurovascular network can lead to chronic inflammation as well as corneal pathologies including epithelial keratitis, epithelial defect, and stromal ulceration. For example, disruption of the first branch of the trigeminal nerve can cause neurotrophic keratitis, which overlaps with severe dry eye disease due to diminished blinking and tearing reflexes (Mead et al., 2020). Furthermore, the severity of chemical burns is graded by the degree of limbal ischemia (Dua et al., 2001). Nerve growth factor with its receptors TrkA and p75^{NTR} are constitutively expressed in basal limbus and cornea.

(Touhami et al., 2002),(Lambiase et al., 1998) and had shown to promote limbal stem cells phenotype and its proliferative capacity and clonal forming efficiency.(Kolli et al., 2019)Additional studies are needed to understand how the limbal niche is supported by its neurovascular network.

3. Limbal Niche Cells

In addition to its neurovascular network, the native limbal niche comprises extracellular matrix components and a subset of neighboring cells including stem cell progeny and subjacent mesenchymal cells (Fuchs et al., 2004). For the extracellular matrix, it has been shown that Laminin N-211, -213, -221, Vitronectin, Versican, BM40/SPARC, and Tenascin-C are more specific to the limbus than the cornea (Echevarria et al., 2011), (Schlotzer-Schrehardt et al., 2007);(Mei et al., 2012). Furthermore, a specialized hyaluronan (HA) matrix that differs from the rest of the cornea has been found in the murine limbal niche, and disruption of this matrix within the niche compromises corneal epithelial regeneration (Gesteira et al., 2017). The mesenchymal cells in the limbal niche include intraepithelial melanocytes, subepithelial mesenchymal cells, and migrating immune cells. Melanocytes, which are relatively concentrated in the limbal epithelial crypts, have been shown to maintain the undifferentiated state of epithelial progenitors during *in vitro* co-culture (Dziasko et al., 2015).

Our cumulative studies over the last decade have allowed us to conclude the following three salient features of a subset of mesenchymal cells that lie closely subjacent to limbal basal epithelial cells termed limbal niche cells (LNCs) in the limbal stroma (Table 1A):

The *first* salient feature is the physical close contact between LEPCs and LNCs in the vicinity of the limbal basement membrane. This feature explains why (1) the conventional isolation method utilizing dispase, which digests the basement membrane, has failed to isolate LNCs in the past, and (2) isolation the entire limbal stroma via trypsin may have included other mesenchymal cells deep into the stroma. In contrast, the isolation method based on collagenase, which digests interstitial collagens, but not the basement membrane, retains the close physical contact between LEPCs and LNCs so as to enable successful isolation of LNCs lying in the limbal epithelial crypt deep into the stroma (Chen et al., 2011). Such collagenase-isolated clusters obtained directly from an intact limbal tissue contain both Pancytokeratin (PCK) + / Vimentin - LEPCs that exhibit high clonogenicity and a subset of PCK - / Vimentin + mesenchymal cells termed LNCs (Chen et al., 2011). When freshly isolated, these LNCs are characterized as small as 10 μ m in diameter and heterogeneously express embryonic stem cell (ESC) markers such as Oct4, Sox2, Nanog, Rex1, Nestin, N-cadherin, SSEA4 and CD34 (Chen et al., 2011), angiogenesis progenitor (pericyte) markers such as Platelet-derived growth factor receptor beta (PDGFR- β), VEGF receptor 2 (FLK-1), CD31, von Willebrand Factor (vWF), and α -SMA (Li et al., 2012a),(Li et al., 2012b), and neural crest progenitor markers such as Pax6, Sox2, Nestin, p75^{NTR}, and Musashi-1 (Chen et al., 2019). Similar collagenase isolation method has been adopted by others to confirm the expression of ESC markers and derivation of neural crest progenitors after expansion (Basu et al., 2014; Chen et al., 2014b; Tomasello et al., 2016). Although the expressions of these and other markers has also been reported by collagenase digestion of

the limbal stroma following manual scraping or dispase digestion of the overlying epithelial cells (Branch et al., 2012; Bray et al., 2014; Katikireddy et al., 2014), such methods may have unintentionally left some progenitor cells in the fraction retained by the filter due to tight association with the basement membrane matrix (Chen et al., 2014a). Table 1A summarizes markers identified for freshly isolated LNCs by us upon isolation and compared to those by others based on expansion after isolation using different enzymatic digestion methods from the human limbal stroma. LNC may also express neural cell crest markers such as HNK1, SOX9 and SOX10 when expanded in subsequent cultures. (Ghoubay-Benallaoua et al., 2017) Further studies are needed to determine the relationship between LNCs subjacent to the LEPCs and those lying deep in the stroma.

The *second* salient feature is the stringent *ex vivo* environment to maintain the *in vivo* LNC phenotype. We achieve this goal by culturing freshly-isolated LNCs on coated two-dimensional (2D) Matrigel in serum-free modified embryonic stem cell medium (MESCM) containing basic fibroblast growth factors (bFGF) and leukemia inhibitory factor (LIF) for up to 10 passages (Li et al., 2012a),(Li et al., 2012b). A number of studies have shown that corneal epithelial cells grown on relatively soft substrates are able to retain limbal stem cell markers, whereas cells cultured on stiff substrates are prone to differentiate (Foster et al., 2014; Gouveia et al., 2019; Jones et al., 2012; Moers et al., 2013). Others have also noted that increased matrix rigidity threatens mesenchymal stem cells and epithelial stem cell lineages (Abdeen et al., 2014; Engler et al., 2006; Wang and Chen, 2013). Although the expression of ESC markers and angiogenic progenitor markers by LNCs is gradually lost during serial passage on 2D Matrigel, such a loss is transient and can be regained when reseeded back on three-dimensional (3D) Matrigel (Li et al., 2012a; Xie et al., 2012). Besides matrix rigidity, we also noted that the aforementioned plasticity, i.e., reversibility by changing from 2D to 3D Matrigel, is irreversibly lost if LNCs are expanded in serum-containing medium that is traditionally used for mesenchymal stem cells expansion (Li et al., 2012b).

The *third* salient feature is that LNCs with an *in vivo* phenotype of nuclear Pax6+ neural crest progenitors have multipotent potential to differentiate into the classical tri-lineages of bone, cartilage and adipose tissue similar to mesenchymal stem cells (Li et al., 2012b), pericytes and angiogenic progenitors to promote angiogenesis (vasculogenesis),(Li et al., 2012a), and neurons, oligodendrocytes, and astrocytes (Chen et al., 2019) (Table 2). Others have also discovered similar multipotent potential in mesenchymal cells isolated from the human limbal stroma (Table 2). We discovered that nuclear Pax6+ staining is uniquely found in freshly-isolated LNCs but not corneal stromal cells (Chen et al., 2019). The hallmark of nuclear Pax6+ staining to denote the neural crest progenitor phenotype is causally correlated with neural crest marker expression, neutrosphere formation, and neuroglial differentiation (Chen et al., 2019). This finding is consistent with the notion that the limbal stroma cells are derived from migrating Pax-expressing neural crest during embryonic development (Baulmann et al., 2002). In fact, human cornea and limbal stromal cells have been reported to express neural crest genes such as Pax6 (Basu et al., 2014), ABCG2 (Du et al., 2005), Nestin (Chen et al., 2011), and Sox2 (Chen et al., 2011). Sox2-expressing neural crest progenitor cells have been found in the dermal papilla to support dermal follicular niche (Driskell et al., 2009). It is tempting to speculate that the multipotent potential of LNCs

summarized in Table 2 supports their regenerative potential to restore the neurovascular network during wound healing. This speculation is supported by the finding that nestin-driven GFP cells that co-express with CD31 and vWF can grow into vessels from the bulge region of hair follicles during wounds healing (Amoh et al., 2004) and can subsequently be isolated and differentiated into neurons, glia, keratinocytes, smooth muscle, and melanocytes *in vitro* (Amoh et al., 2004). Although corneal epithelial differentiation by that limbal stromal neural crest progenitors has been suggested (Dravida et al., 2005), (Katikireddy et al., 2014), it has been challenged by the recent data from Ghouby-Benallaoua et al 2017. (Ghouby-Benallaoua et al., 2017)

4. Regulatory Mechanism of LEPCs by LNCs

The *in vivo* close physical contact between LNCs and LEPCs can be recapitulated by an *in vitro* reunion assay by seeding single isolated LNC and LEPCs, which contain LEPC, at a 1:1 ratio on 3D Matrigel (Li et al., 2012a). A number of studies have shown that chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12) is critical for the recruitment and retention of CXCR4+ bone marrow stem/progenitor cells to the neo-angiogenic niches to promote revascularization during wound healing (Petit et al., 2007; Petit et al., 2002; Ratajczak et al., 2004). We discovered that *in vitro* reunion of LNCs and LEPCs is also mediated by this chemokine axis (Xie et al., 2011). Specifically, we found that the SDF-1/CXCR4 axis is predominantly expressed in the human limbus but not the cornea and that SDF-1 is preferentially expressed by limbal epithelial cells and CXCR4 is expressed by subjacent stromal mesenchymal cells (Xie et al., 2011). Such reunion prevents fate decision of LEPCs, i.e., differentiation into corneal epithelial cells, upregulates p63 α expression, and maintains holoclone growth signifying self-renewal of LEPCs (Fig. 2A). Disruption of such reunion by AMD3100, a small molecular inhibitor or a blocking antibody to CXCR4, at the time of seeding of both LNCs and LEPCs, but not later, promotes corneal fate decision and reduces holoclone growth of LEPCs (Xie et al., 2011). Furthermore, reunion between LEPCs with cells other than LNCs, such as remaining human limbal stromal cells, human mesenchymal stem cells, human corneal stromal cells, or human umbilical endothelial cells, leads to adoption of corneal epithelial fate (Xie et al., 2012); (Li et al., 2012a) (Fig. 2A). These findings collectively provide molecular mechanism whereby LNCs forms a close physical contact with LEPCs to maintain stem cell self-renewal and prevent corneal epithelial fate decision (Xie et al., 2011, 2012).

Self-renewal and quiescence of stem cells are regulated by two segregated niche compartments through Wnt signaling pathways and bone morphogenetic protein (BMP) signaling pathways, respectively (Greco and Guo, 2010). We thus deployed the aforementioned *in vitro* reunion assay to see if these two signaling pathways also operate between LNCs and LEPCs. In 3D Matrigel, we noted marked clonal growth of LEPCs, signifying stem cell activation is correlated with activation of canonical Wnt signaling (Han et al., 2014). The involvement of Wnt signaling has been noted in the corneal fate decision controlled by Pax6 in human corneal epithelium where β -catenin is found in the perinuclear cytoplasm of LEPCs and correlated with upregulation of Wnt7A and FZD5 (Ouyang et al., 2014). Inactivation of BMP signaling in LNCs by noggin leads to downregulation of nuclear pSmad1/5/8 in LEPCs and nuclear β -catenin in larger LEPCs but membrane relocation of β -

catenin in smaller LEPCs and significant upregulation of DKK1/2 (Han et al., 2014), suggesting the upregulation of DKK1 and DKK2 might suppress Wnt signaling in LEPCs adjacent to LNCs. These results collectively support the notion that the *in vitro* reunion of LNCs and LEPCs in 3D Matrigel recapitulates an *in vitro* limbal niche suggestive of stem cell self-renewal (Fig. 2B).

5. HC-HA/PTX3 from Amniotic Membrane as a Surrogate Matrix Niche

Since the reintroduction of amniotic membrane (AM) transplantation (AMT) in ophthalmology by Kim and Tseng in 1995 (Kim and Tseng, 1995), there has been a surge of interest in using this surgical procedure for ocular surface reconstruction (Fernandes et al., 2005; Liu et al., 2010; Sangwan et al., 2007; Sangwan and Tseng, 2001; Tseng, 2001), presumably due to the advent of cryopreservation, which devitalizes living cells to minimize immune reaction while preserving the remaining matrix integrity for long-term storage (Kruse et al., 2000). In 2001, the U.S. Food and Drug Administration (FDA) designated cryopreserved AM (Amniograft®; Bio-Tissue, Inc., Miami, FL) as a “361 human cell and tissue-based product (HCT/P)” for ocular wound repair and healing through exertion of anti-scarring and anti-inflammatory actions. Clinical use of cryopreserved AM is now considered the standard of care for many ophthalmic indications, is reimbursed by Centers for Medicare and Medicaid Services (CMS), and has over a 20-year safety record. Not only AM is useful for ocular surface reconstruction but also an efficient substrate for growing LSC with no 3T3 feeders as demonstrated by cultured cell transplantation (Sangwan et al., 2011), (Tsai et al., 2000), (Pauklin et al., 2010), (Borderie et al., 2019). Human AM epithelial cells can serve as feeder layers for promoting ex-vivo expansion of limbal epithelial progenitor cell. (Chen et al., 2007)

Our cumulative research effort over a period of more than a decade under the research support from the National Institute of Health has identified and characterized one key matrix component responsible for the aforementioned therapeutic actions of AM. We first demonstrated that the biological activity of cryopreserved AM is retained in a water-soluble AM extract. More specifically, we noted that this AM extract induces an anti-inflammatory effect by promoting apoptosis of activated macrophages (He et al., 2008; Li et al., 2006) and upregulating anti-inflammatory cytokines (i.e. IL-10) while downregulating pro-inflammatory markers (i.e. TNF- α , IL-6, CD86) (He et al., 2008). AM extract also exhibits an anti-scarring action by preventing myofibroblast expression of α -smooth muscle actin (α -SMA) (Li et al., 2008). We then utilized successive ultracentrifugation in a CsCl gradient in the presence of 4M guanidine HCl to isolate a unique HA-containing matrix from AM extract, termed HC-HA/PTX3, which consists of high molecular weight HA covalently linked with heavy chain 1 (HC1) from inter- α -trypsin inhibitor (IaI) through the catalytic action of TNF α -stimulated gene 6 (TSG-6) and is further complexed with pentraxin 3 (PTX3) (He et al., 2009; Zhang et al.) (Fig. 3). The biosynthetic process of HC-HA/PTX3 involves the following two steps: the first is to form HCHA complex via tumor necrosis factor-stimulated gene-6 (TSG-6), which is an enzyme that catalyzes the covalent (ester bond) transfer of HCs from IaI to HA. (Rugg et al., 2005); (Fulop et al., 2003); (Jessen and Odum, 2003); (Sanggaard et al., 2006) IaI contains two HCs (i.e., HC1 and HC2) and a light chain termed bikunin jointed a chondroitin sulfate chain and is present in the blood after

being secreted by the liver.(Enghild et al., 1989);(Odum, 1990); (Salier et al., 1996); (Mizushima et al., 1998);(Blom et al., 1999);(Zhuo et al., 2004) We demonstrated that the HC-HA/PTX3 complex purified from AM consists of HMW HA (>3000 kDa) covalently linked with HC1 and tightly bound PTX3, but not HC2, bikunin, and TSG-6. Unlike the cumulus-oocyte complex, the source of IaI is endogenously produced by AM epithelial cells and stromal cells but not derived from the liver, and the expression of TSG-6 and PTX3 is constitutive (i.e., without relying on proinflammatory cytokines).(Blom et al., 1999),(Zhang et al., 2014) Similar to ovulation (Salustri et al., 2004);the second step is to form the HC-HA/PTX3 complex by tight association of the HC-HA complex with PTX3. Recent studies further disclose that HC-HA/PTX3 is a unique matrix component abundantly present in the birth tissue, i.e., not only in AM but also in umbilical cord (Tan et al., 2014),(Cooke et al., 2014).

5.1. HC-HA/PTX3 Exerts Anti-Inflammatory and Anti-Scarring Effects

HC-HA/PTX3 exerts potent anti-inflammatory and anti-scarring actions. Similar to AM extract, HC-HA/PTX3 dose-dependently promotes apoptosis of activated but not resting macrophages (He et al., 2009; He et al., 2013) and suppresses activation of Th1 and Th17 lymphocytes (He et al., 2009), thus capable of modulating inflammation triggered by both innate and adaptive immune responses. Consequently, subconjunctival injection of HC-HA/PTX3 dose-dependently prolongs corneal allograft survival (He et al., 2014) and suppresses inflammation and scarring of both conjunctiva and lacrimal tissue thus preserving lacrimal tear production and conjunctival goblet cells in a murine model of graft-versus-host disease following allogeneic bone marrow transplantation (Ogawa, 2017). HC-HA/PTX3 also suppresses the TGF- β 1 promoter activity of human corneal fibroblasts (He et al., 2009) and reverts human corneal fibroblasts and myofibroblasts to keratocytes by inhibiting canonical TGF- β signaling and activating BMP signaling (Zhu et al., 2020). Moreover, both AM extract and HC-HA/PTX3 suppress viability and tube formation of cultured HUVEC (Shay et al., 2011) but promote growth when HUVEC are cocultured with LNCs (unpublished observation), collectively suggesting that HC-HA/PTX3 suppresses abnormal angiogenesis mediated by endothelial cells alone but promotes vasculogenesis when endothelial cells are associated with pericytes.

5.2. HC-HA/PTX3 Preserves Quiescence of LEPCs through LNCs

The majority of LEPCs are “mitotically” quiescent and resting in the G₀ phase under a steady state (Cotsarelis et al., 1989; Sagga et al., 2018). The G₀ phase of quiescence stem cells has been assigned as a strategy for stem cells in rapid response to injury-induced signals (Rodgers et al 2014). Due to the complexity of factors in AM, purified HC-HA/PTX3 matrix helps delineate the potential mechanism explaining how AM might modulate interactions between LNCs and LEPCs.(Chen et al., 2015) Therefore, HC-HA/PTX3 may someday be deployed as a surrogate matrix niche in lieu of AM to support ex vivo expansion of LEPCs. Taking advantage of the aforementioned *in vitro* reunion assay, we have investigated whether stem cell quiescence can be maintained by HC-HA/PTX3 to replace 3D Matrigel. Others have reported that LEPCs use integrins α 3 β 1 and α 6 β 4 to anchor to their basement membrane niche (Polisetti et al., 2016), presumably for self-renewal. Our results showed that HC-HA/PTX3 promotes expression of ESC markers by LNCs more so

than 3D Matrigel (Chen et al., 2015). Reunion of LNCs and LEPCs on HC-HA/PTX3 results in sphere growth that exhibits similar suppression of fate decision, i.e., preventing from differentiation into corneal epithelial cells but negligible clonal growth of LEPCs, which exhibit upregulation of quiescence markers including nuclear translocation of phosphorylated Bmi-1 (Chen et al., 2015). Furthermore, the above finding is correlated with the suppression of canonical Wnt but activation of noncanonical Wnt (PCP) signaling and BMP signaling in both LEPCs and LNCs. The activation of BMP signaling in LNCs is crucial because ablation of BMP signaling results in upregulation of cell cycle genes, downregulation of Bmi-1, nuclear exclusion of phosphorylated Bmi-1, and marked promotion of LEPC clonal growth (Chen et al., 2015). Hence, HC-HA/PTX3 uniquely preserves quiescence of LEPCs through the upregulation of BMP and non-canonical Wnt signaling in LNCs and subsequent BMP signaling in LEPCs (Fig. 2B). Therefore, the limbal niche resembles other stem cell niches such as dermal papillae, epidermal bulges, intestinal crypts, and bone marrow, where both Wnt and BMP signaling have been found to play an imperative role in two adjacent compartments that regulate stem cell self-renewal and quiescence, respectively (reviewed in (Li and Xie, 2005),(Greco and Guo, 2010; Li and Clevers, 2010). Along with the gradual loss of the expression of ESC markers and angiogenic progenitor markers (Li et al., 2012a; Xie et al., 2012), the nuclear Pax6⁺ neural crest progenitor status also gradually declines in LNCs following serial passage (Chen et al., 2019). Such a gradual loss can be reverted by seeding the late-passaged LNCs back to HC-HA/PTX3, but not 3D Matrigel, suggesting that HC-HA/PTX3 is capable of rejuvenating differentiated (aged) LNCs to restore their *in vivo* phenotype to support quiescence and self-renewal of LEPCs (Chen et al., 2020, manuscript submitted). Our recent data showed that activation of BMP signaling in LNCs proceeds after cell aggregation of LNCs mediated by SDF-1/CXCR4 signaling (Chen et al 2020 manuscript submitted), a finding that is also noted in human corneal fibroblasts when seeded on HC-HA/PTX3 (Zhu et al., 2020). We speculate that this signaling is triggered by the binding between HC-HA/PTX3 and CD44, a well-known receptor for HA. If proven, it suggests that limbal stem cell niche uses different matrix ligand and receptor to control stem cell quiescence and self-renewal, respectively.

6. Clinical Evidence Supporting Amniotic Membrane as Surrogate Niche

Therefore, HC-HA/PTX3 as a single matrix can orchestrate a myriad of actions collectively translated to promote regenerative healing (reviewed in (Tseng, 2016; Tseng et al., 2016)). This notion has been demonstrated in the clinical efficacy of cryopreserved AM in promoting corneal nerve regeneration in patients suffering from moderate to severe dry eye (John et al., 2017) and chronic neuropathic pain (Morkin and Hamrah, 2018). We thus envisage that HC-HA/PTX3 is a unique matrix that can serve as a surrogate limbal niche. This notion is also supported by the finding that disruption of a specific HA matrix within the limbal niche leads to compromised corneal epithelial regeneration and more inflammatory response in knockout mice of HA synthetases or TSG-6 (Gesteira et al., 2017), which is involved in biosynthesis of HC-HA/PTX3 (Fig. 3). If LEPCs can be analogized as “seeds” to planted in the garden, the regenerative properties of HC-HA/PTX3 in supporting LNCs can be viewed as a key nutrient “fertilizer” that enriches the “top soil”

(limbal niche) given the close interaction between LNCs and LEPCs (Fig. 4). This new paradigm is supported by the following clinical evidence:

6.1. The Role of Pax6 in Pathogenesis of LSCD

Limbal stem cell deficiency (LSCD) is the pathological process leading to corneal blindness where the corneal tissues manifest chronic inflammation, scarring, vascularization, and conjunctivalization (see reviews in (Ahmad, 2012; Fernandez-Buenaga et al., 2018; Kim and Mian, 2017)). Besides direct destructive insults to reduce the LEPC pool by chemical/thermal burns, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), or contact lens wear, LSCD can also ensue from a dysfunctional limbal stromal niche. To understand a variety of etiologies of LSCD, readers might like to see the reviews and references cited therein (Hatch and Dana, 2009; Rossen et al., 2016; Vazirani et al., 2018); (Deng et al., 2019) Among diverse causes that may lead to a dysfunctional stromal niche, one prototypic disease is aniridia caused by haploinsufficiency mutations in the Pax6 gene (Cvekl and Callaerts, 2017). For the corneal epithelium, Pax6 plays an important role in maintaining epithelial proliferation (Collinson et al., 2004);(Hsueh et al., 2013), wound healing (Doran, 2008), and corneal fate decision (Ouyang et al., 2014). Nonetheless, Pax6 also plays a key role in maintaining the *in vivo* Pax6⁺ neural crest progenitor phenotype in LNCs that is crucial for supporting self-renewal and preventing fate decision (i.e., terminal differentiation) of LEPCs (Chen et al., 2019). Thus, the finding that HC-HA/PTX3 can help rejuvenate “aged” LNCs to regain the nuclear Pax6⁺ neural crest progenitor status may one day shed insight not only into the pathophysiology of LSCD caused by aniridia but also into the therapeutic potential of using HC-HA/PTX3-containing birth tissue (AM or umbilical cord) to restore and strengthen the supporting role of LNCs.

6.2. Amniotic Membrane Transplantation for Preventing and Treating LSCD

The idea that HC-HA/PTX3-containing birth tissue (AM or umbilical cord) can be used to restore and strengthen the supporting role of LNCs was first demonstrated when AM transplantation (AMT) was re-introduced in ophthalmology in 1995 (Kim and Tseng, 1995) in a rabbit model of LSCD induced by chemical and surgical insults. To a great surprise, that study disclosed that AMT prevented LSCD by restoring the normal corneal epithelial phenotype. Subsequently, this clinical effectiveness was demonstrated in human patients with “partial” LSCD, i.e., the cornea without total loss of LEPCs (Tseng et al., 1998), (Anderson et al., 2001);(Gomes et al., 2003);(Ivekovic et al., 2005); (Kheirkhah et al., 2008a);(Sharma et al., 2018)). Because AMT can prevent LSCD, especially if implemented within two weeks of acute chemical or thermal burns (Kheirkhah et al., 2008c; Meller et al., 2000; Prabhasawat et al., 2007), self-retained cryopreserved AM (e.g., PROKERA®; BioTissue, Inc., Miami, Florida, USA) was developed in 2004 to facilitate early intervention because its placement on the eye surface can readily be executed in-office or bed side. This sutureless approach prevents LSCD and subsequent cicatricial complications in managing acute chemical or thermal burns (Chirapapaisan et al., 2018; Kheirkhah et al., 2008c; Shupe and Cheng, 2017) as well as acute SJS/TENS (Kolomeyer et al., 2013; Shamma et al., 2010; Tomlins et al., 2013).

For treating one eye with the total loss of LEPCs in the setting of unilateral “total” LSCD, Kenyon and Tseng (Kenyon, 1989) first reported transplantation of 240° of conjunctival-limbal tissue from the healthy contralateral eye in a surgical procedure termed conjunctival limbal autograft (CLAU). Because of the concern that the donor eye might develop LSCD in the long run based on the experimental model (Chen and Tseng, 1990, 1991), others began to reduce the size of the harvested CLAU graft and noted that the clinical benefit in treating unilateral total LSCD vanishes when the size of CLAU is reduced to less than 90° (Moldovan et al., 1999; Rao et al., 1999). Because the experimental evidence shows that chronic inflammation within the limbal niche contributes to the CLAU failure (Tsai and Tseng, 1995), we and others have incorporated AMT with CLAU by taking the advantage of AM’s anti-inflammatory and anti-scarring actions (Ivekovic et al., 2005; Kheirkhah et al., 2008b; Meallet et al., 2003; Santos et al., 2005). Indeed, the addition of AMT has successfully reduced the size of CLAU to 60° for unilateral total LSCD (Kheirkhah et al., 2008d). Sangwan et al. (Sangwan et al., 2012) introduced simple limbal epithelial transplantation (SLET) where a small limbal graft is harvested from the healthy contralateral eye, cut into small pieces, and fixed with fibrin glue on an AM graft that covers the cornea for unilateral partial or total LSCD. The combined use of single (Sangwan et al., 2012) or double (Amescua et al., 2014) layers of AM in SLET has successfully reduced the size of limbal stem cell removal from the fellow eye down to less than 30° as testified by an overall success rate of 80% in a total of 378 cases during a long-term follow up (Jackson et al., 2020). Therefore, the new paradigm that AM can be used as a surrogate niche is also supported by the above clinical evidence showing successful *in vivo* expansion of LEPCs.

6.3. Ex Vivo Expansion (Tissue Engineering) of LEPCs

When treating patients with bilateral total LSCD, one would have to resort to transplantation of allogeneic LEPCs in a procedure termed keratolimbal allograft (Kim and Tseng, 1995). However, one key hurdle impeding the success of this procedure is allograft rejection, which is combated with long-term use of systemic immunosuppressive therapies (Liang et al., 2009). One way to circumvent this difficulty is by transplanting LEPCs following *ex vivo* expansion, a concept first introduced by Pellegrini et al. in 1997 using a culturing technique based on the use of mitomycin C-treated murine 3T3 fibroblast feeder layers (Pellegrini et al., 1997). Although *ex vivo* expansion was suggested to potentially reduce the risk of allograft rejection as antigen-presenting cells do not survive during culture (Shortt et al., 2011), this notion has not been demonstrated because systemic immunosuppression is still required according to long-term follow-up studies of cultured allogeneic limbal stem cells transplantation. (Shortt et al., 2014), (Borderie et al., 2019), (Pauklin et al., 2010). *Ex vivo* expansion of LEPCs has since been explored by many as a new therapeutic approach to address LSCD. They vary in methods of epithelial isolation from limbal biopsy, the use and preparation of AM as the substrate, the expansion medium, the use of airlifting to promote epithelial stratification, and the use of additional feeder layers such as mitomycin C-arrested murine 3T3 fibroblasts, mesenchymal stem cells, or human stromal cells (reviewed in (Tseng et al., 2010)). Now that we understand the importance of LNCs in supporting LEPC function, it is important to re-examine these variables by asking the question whether a surrogate niche has been restored and maintained to preserve the function of LEPCs during *ex vivo* expansion. If the said paradigm that HC-HA/PTX3 serves as a surrogate matrix

niche holds true, one may like to build additional process controls to ensure HC-HA/PTX3 is preserved and matrix rigidity not jeopardizing the phenotype and function of LNCs during preparation or further manipulation of AM as the substrate (Borrelli et al., 2013),(Le and Deng, 2019). Furthermore, one may also like to pay attention to the preservation of the *in vivo* phenotype of nuclear Pax6+ neural crest progenitors in LNCs that can be threatened by the use of serum-containing media and serial passages.

7. Conclusion

In summary, the new paradigm that HC-HA/PTX3 or HC-HA/PTX3-containing birth tissue can serve as a surrogate matrix niche surely explains why AM plays a pivotal role in managing LSCD during *in vivo* or *ex vivo* expansion of LEPCs. Taking the metaphor of planting seeds in the garden (Figure 4), the anti-inflammatory and anti-scarring actions exerted by HC-HA/PTX3 act as “weeding” measures to fend untoward external insults to the niche, i.e., “soil”. The supportive action of HC-HA/PTX3 to LNCs, which in turn maintain stem cell quiescence and self-renewal, can be viewed as “fertilizer” that enriches the niche to become “top soil”. In conjunction with its anti-inflammatory and anti-scarring actions, HC-HA/PTX3 can promote regenerative healing (Tseng, 2016; Tseng et al., 2016). That is why such action paradigm has been successfully applied beyond ophthalmology as evidenced by the use of cryopreserved umbilical cord graft in promoting healing in complex diabetic foot ulcers (Caputo et al., 2016; Ghoubay-Benallaoua et al., 2017; Marston, 2019), radionecrotic wounds (Fernandez, 2019), and severe spina bifida (Papanna et al., 2016), preventing cicatricial complications in orthopedic reconstruction (DeMill, 2014) and expediting functional recovery of continence following robot-assisted radical prostatectomy (Ahmed et al., 2019). We thus envisage that the new paradigm based on HC-HA/PTX3 as a novel regenerative matrix to serve as a surrogate niche can set a new standard for regenerative medicine in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Limbal niche nourishes, protects, and regulates quiescence, self-renewal, and differentiation decision of limbal epithelial stem cells (LESCs)
- HC-HA/PTX3 found in amniotic membrane and umbilical cord may serve as a surrogate matrix niche to support quiescence and self-renewal but prevent corneal fate decision of LESCs
- This may explain why amniotic membrane plays a pivotal role in managing limbal stem cell deficiency during in vivo or ex vivo expansion of LESCs
- HC-HA/PTX3 as a surrogate niche can set a new standard for regenerative medicine in ophthalmology and beyond

Figure 1: Schematic Representation of Human Limbal Niche.

The human limbal niche (A) is located in a microenvironment termed palisades of Vogt (B, bar = 200 mm), where SCs are within the limbal basal epithelial cells undulated into folds (C) and invaginated into the limbal stroma as crypts (not shown). A subset of mesenchymal cells immediately subjacent to LEPCs functions as LNCs (C). The close interaction between LNCs modulates functions of LEPCs such as quiescence, self-renewal and fate decision (D), of which each process is highlighted by the preferential expression of certain markers specific to the limbus (E). TAC: transient amplifying cells, PMC: post-mitotic cells, TDC: terminally differentiated cells. (Image Taken from (Tseng et al 2016) with modification.)

Figure 2. *In Vitro* Reunion of LNCs and LEPCs.

Reunion between LNCs and LEPCs in 3D Matrigel prevents corneal fate decision as illustrated by expression of cytokeratin 12 (CK12) by LEPCs as the corneal fate marker (A). Expression of CK12 is abolished when LNCs cultured in MESCM (Modified embryonic stem cells medium) are compared to reunion with RSC (remaining stromal cells after LNCs have been removed), bone marrow mesenchymal stem cells, human corneal fibroblast (HCF) or human umbilical endothelial cells (HUVEC) cultured in Endothelial Growth Media (EGM2), or LNC cultured in DF (DMEM/F12 with 10% FBS). In contrast, the expression of p63 α as a marker for LEPCs is promoted (A). (Data taken from Li et al 2012a, Li et al 2012b) The reunion in 3D Matrigel promotes stem cell self-renewal by activating Wnt signaling but suppressing BMP signaling. In contrast, the reunion in HC-HA/PTX3 promotes stem cell quiescence by suppressing Wnt signaling but activating BMP signaling (B) LNC: Limbal Niche Cells; SC: Stem Cells (Quiescence or Self-Renewal); TAC:

Transient Amplified Cells; TDC: Terminal Differentiated Cells. (Data taken from (Han et al 2014; Chen et al 2015))

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1 Formation of HC-HA

2 Formation of HC-HA/PTX3

Figure 3. Biosynthesis of HC-HA/PTX3.

HC-HA/PTX3 is synthesized by the following two steps: (1) In the first step, TNF-stimulated gene 6 protein (TSG-6) covalently binds heavy chain 1 (HC1) of inter- α -trypsin inhibitor (IaI) and transfers it to high molecular weight hyaluronan (HA), at which time HC1 becomes conjugated and TSG-6 released. (2) In the second step, PTX3, an octamer, is tightly associated with the HC-HA complex via binding with HCs. (Image Taken from (Tighe et al 2020) with modification.)

Figure 4. HC-HA/PTX3 as a Surrogate Matrix Niche.

HC-HA/PTX3 exerts anti-inflammatory and anti-scarring actions, of which both act as “weeding” measures to fend off two untoward threats, i.e., chronic inflammation and scarring. HC-HA/PTX3 also acts as a “fertilizer” that provides essential nutrients to developing “seeds” (stem cells) and improves the quality of the “top soil” (limbal niche) through its supporting action to LNCs to maintain SC quiescence. Collectively, this myriad of actions collectively promote regenerative healing.

Table 1.**Salient Features and Phenotypic Characterization of Limbal Niche Cells**

A. Salient Features	
<ul style="list-style-type: none"> • Close Physical Contact with Limbal Epithelial Stem Cells • Stringent <i>ex vivo</i> Environment to Maintain the <i>in vivo</i> Phenotype • Multipotent Potentials to Differentiate into Neuroglial, Angiogenesis Progenitors, Tri- (Bone, Fat, Cartilage) lineage 	
B. Phenotypic Characterization	
Embryonic Stem Cell Markers	Oct4, Sox2, Nanog, Rex1, SSEA4, CD34, ABCG2 (Chen et al 2011)
Angiogenesis Progenitor Markers	PDGFR- β , CD31, α -SMA, FLK-1 (Li et al 2012a)
Neural Crest Progenitor Markers	Pax6, Sox2, Nestin (Chen et al 2019),(Chen et al 2011)
Mesenchymal Stem Cells Markers	CD73, CD90, CD105 (Li et al 2012)

Table 2.

Multipotent Potential of Mesenchymal Cells from Human Limbal Stroma

MULTIPOTENT POTENTIAL	
Angiogenic Potential	References
Pericyte	(Li et al., 2012a) *
Vascular Endothelial Tube Formation	(Li et al., 2012a) *
Neuroglial Potential	
Neuron	(Dravida et al., 2005) (Chen et al., 2019) *
Astrocytes	(Chen et al., 2019) *
Oligodendrocytes	(Dravida et al., 2005) *
Neurospheres	(Ushida et al 2004) (Chen et al., 2019) * (Chen et al 2014)
Trilineage Potential	
Adipocytes	(Li et al 2012) * (Branch et al 2012) (Dravida et al., 2005) (Liang et al 2014) (Tomasello et al., 2016)
Osteocytes	(Li et al 2012) * (Dravida et al., 2005) (Liang et al 2014) (Tomasello et al., 2016)
Chondrocytes	(Li et al 2012) * (Dravida et al., 2005) (Liang et al 2014) (Tomasello et al., 2016)
Other Lineage Potentials	
Corneal Epithelia	(Dravida et al., 2005) (Gallagher et al., 2014)
Keratocytes	(Du et al., 2007) (Branch et al., 2012) (Basu et al., 2014)
Cardiac Myocytes	(Dravida et al., 2005)
Hepatocytes	(Dravida et al., 2005)
Pancreatic Islet cells	(Dravida et al., 2005)

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