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Niche Regulation of Limbal Epithelial Stem Cells: Relationship between Inflammation and Regeneration

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Abstract

Human limbal palisades of Vogt are the ideal site for studying and practicing regenerative medicine due to their accessibility. Nonresolving inflammation in limbal stroma is common manifestation of limbal stem cell (SC) deficiency and presents as a threat to the success of transplanted limbal epithelial SCs. This pathologic process can be overcome by transplantation of cryopreserved human amniotic membrane (AM), which exerts anti-inflammatory, antiscarring and anti-angiogenic action to promote wound healing. To determine how AM might exert anti-inflammation and promote regeneration, we have purified a novel matrix, HC-HA/PTX3, responsible for the efficacy of AM efficacy. HC-HA complex is covalently formed by hyaluronan (HA) and heavy chain 1 (HC1) of inter- α -trypsin inhibitor by the catalytic action of tumor necrosis factor-stimulated gene-6 (TSG-6) and are tightly associated with pentraxin 3 (PTX3) to form HC-HA/PTX3. In vitro reconstitution of the limbal niche can be established by reunion between limbal epithelial progenitors and limbal niche cells on different substrates. In 3-dimensional Matrigel, clonal expansion indicative of SC renewal is correlated with activation of canonical Wnt signaling and suppression of canonical BMP signaling. In contrast, SC quiescence can be achieved in HC-HA/PTX3 by activation of canonical BMP signaling and non-canonical planar cell polarity (PCP) Wnt signaling, but suppression of canonical Wnt signaling. HC-HA/PTX3 is a novel matrix mitigating nonresolving inflammation and restoring SC quiescence in the niche for various applications in regenerative medicine.

Keywords

amniotic membrane; anti-inflammation; anti-angiogenesis; antiscarring; heavy chain; hyaluronan; Inter- α -inhibitor; limbus; stem cell niche; stem cells; umbilical cord

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I. Introduction

Stem cells (SCs) with extensive proliferative potential of giving rise to one or more differentiated cell types are common in early mammalian embryos. By adulthood, such SCs are dispersed and kept in a unique anatomic location (i.e., the niche) of each self-renewing tissue, where they maintain quiescence while performing remarkable and relentless self-renewal to replenish the SC population lost to progeny production and proper fate decision. Among all adult epithelial tissues, the model of the corneal epithelium is most unique in having its SCs located at the basal epithelial layer of the limbus (between the cornea and the conjunctiva) in a special anatomic structure termed “palisades of Vogt, while its transient amplifying cells (TACs), i.e., the immediate progeny of SC, are located in both limbal and corneal basal epithelia (Figure 1).¹ (See reviews.^{2,3}). Because this location is more accessible than other epithelial tissues, the cornea is the prime location where regenerative medicine has long been practiced through transplantation of autologous or allogeneic limbal tissues and ex vivo expanded limbal epithelial cells.

This review appraises the knowledge gathered from the basic and clinical studies of corneal epithelial SCs located at the limbus in the last three decades and recent studies probing into the mechanism by which limbal epithelial SCs are regulated by their niche. By recognizing that inflammatory processes impede regeneration and demonstrating how amniotic membrane (AM) mitigates such inflammation-mediated pathological influence, we have investigated the molecular action mechanism that can be formulated as a new regenerative strategy based on a dual control of inflammation and maintenance of SC quiescence.

A. Tissue Homeostasis Achieved by Limbal Stem Cells

The normal corneal epithelial surface is covered by a nonkeratinized stratified epithelium² expressing cornea-specific markers, such as CK3 and CK12 keratins (Figure 1E). To compensate for a rapid cell loss of the corneal epithelium due to apoptosis and attrition, the homeostasis of the corneal epithelium is ultimately maintained by limbal epithelial SCs (Figure 1). (See reviews.^{2–6}). In the normal uninjured state, limbal SCs are reversibly mitotically quiescent. However, upon wounding, they are activated to proliferate to generate more SCs (to expand the SC pool) and/or to differentiate into TACs (to deplete the SC pool). Hence, the effective preservation of a sufficient and long-lasting pool of limbal SCs is crucial to achieve the homeostasis of the corneal epithelial tissue (Figure 1D). This requires the niche to ensure successful balancing of the conflicting needs between self-renewal (to maintain the SC pool) and progeny production (to deplete the SC pool). Cumulative studies have shown that maintenance of SC quiescence in a number of SCs such as hematopoietic SCs, muscle satellite cells, and skeletal mesenchymal SCs is clinically relevant for enhancing tissue regeneration.⁷

B. Corneal Diseases with Limbal Stem Cell Deficiency

Full regeneration of the entire corneal epithelium is expected when limbal SCs are intact and healthy. Nevertheless, conjunctival epithelial cells migrate onto the corneal surface when limbal SCs are partially⁸ or totally^{9, 10} damaged, leading to a pathologic state termed limbal stem cell deficiency (LSCD). LSCD carries the hallmark of conjunctivalization, i.e., the

corneal surface is covered by an ingrowing conjunctival epithelium containing goblet cells, as first illustrated by impression cytology.¹¹ The process of conjunctivalization is invariably associated with destruction of the basement membrane, emergence of superficial vascularization, and chronic inflammation and scarring.^{8,10, 12–14}

Using impression cytology¹⁵ as a tool to detect conjunctival goblet cells on the corneal surface as a salient feature of conjunctivalization, we¹¹ and others have identified and classified a number of human corneal diseases with LSCD. These diseases can be grossly subdivided into two major categories, i.e., those with primary destructive loss of limbal SCs and those with primary dysfunctional limbal stroma. This information suggests that pathological processes leading to either the loss of the limbal epithelial SC pool or the dysfunctional limbal niche can yield the same phenotype of LSCD. It also points out an important concept, i.e., the limbal niche plays an important role in maintaining the limbal epithelial SC pool. It remains unclear how chronic inflammation may perpetuate, if not trigger, LSCD by perturbing the limbal niche.

C. Corneal Surface Reconstruction by Limbal Stem Cell Transplantation

Patients with LSCD suffer from a severe loss of vision and annoying irritation, and they are poor candidates for conventional corneal transplantation. Hence, new surgical strategies have been devised by transplanting limbal SCs from an autologous or allogeneic source. (See reviews of surgical procedures.^{16–18}) When total LSCD involves only one eye (unilateral), the damaged corneal surface can be effectively reconstructed by conjunctival limbal autograft.¹⁹ Although conjunctival limbal autograft has rather high success rates, the surgical outcome is not satisfactory if transplantation is carried out at the acute stage of chemical burns when inflammation remains “active,”¹⁹ a notion verified in a rabbit model.²⁰ To reduce the potential risk to the patient’s donor eye, the first alternative is to perform limbal SC allograft, in which an allogeneic (not patient’s own) source of limbal SCs are derived from either HLA-matched living donors^{21–23} or non-matched cadavers.^{22,24–26} Because the donor tissue is allogeneic, systemic immunosuppression with cyclosporin A or other agents is necessary but potentially toxic. Even with systemic use of cyclosporin A, the success rate of limbal allografts declines with time.^{27–29}

Severe dry eye, keratinization, chronic inflammation, and uncorrected lid and lid margin abnormalities have all been implicated as factors contributing to the poor prognosis for keratolimbal allografts.³⁰ Meticulous restoration of the ocular surface defense together with a combined immunosuppressive regimen have been shown to further improve the long-term visual outcome of keratolimbal allografts.³¹ In solid organ transplantation, allograft rejection is known as the prime limiting factor against the success of transplantation of allogeneic limbal epithelial stem cells and is exacerbated by “chronic inflammation” derived from the underlying disease process.³² This problem may be circumvented by performing oral mucosal graft as a limbal surrogate³³ or ex vivo expansion of oral mucosal epithelial SCs,³⁴ especially for eyes where transplantation of allogeneic limbal SCs has failed or is not feasible.^{35,36}

To effectively combat the inflammatory process of the recipient bed, another alternative is to perform amniotic membrane transplantation (**AMT**) as an adjunctive therapy to promote the

success of transplanting autologous³⁷ and allogeneic³⁸ limbal SCs. For transplantation of autologous limbal SCs, AMT has allowed the donor site in the fellow eye to be reduced to 60° limbal arc length.³⁹ Recently, Sangwan et al⁴⁰ and others⁴¹ have devised “simple limbal epithelial transplantation (**SLET**)” as an alternative to conjunctival limbal autograft and ex vivo expansion of limbal epithelial SCs. SLET subdivides the limbal biopsy into tissue fragments as a source of regeneration when placed on the limbal deficient cornea that is covered by AM as a graft without⁴⁰ or with⁴¹ another AM as a bandage.

Clinical successes reported from the above studies indicate that AM helps expand residual or transplanted limbal SCs in vivo. It remains unclear whether and how AM might act as “niche” to help the aforementioned “in vivo” expansion of transplanted limbal epithelial SCs. Besides for in vivo expansion, AM has also been used as a substrate for ex vivo expansion of limbal epithelial progenitors as a composite graft for treating human patients with LSCD.^{42,43} It remains unclear whether AM serves as more than a scaffold in the latter applications.

II. Differences between Adult and Fetal Wound Healing

Adult wound healing is heralded by inflammation, which can be subdivided into two major phases that involve cellular infiltration by polymorphonuclear neutrophils (**PMNs**), macrophages, and lymphocytes derived from innate and adaptive immune responses, respectively (Figure 2, left panel). PMNs, first arriving at the scene, have a short life span and will eventually undergo apoptosis. These apoptotic neutrophils are removed by M2 macrophages via phagocytosis, resulting in the restoration and maintenance of anti-inflammatory and immune-tolerogenic milieu.⁴⁴ On the contrary, in pathological states, a wider extent of injury/wound and PMN infiltration, together with a prolonged lifespan of PMNs, results in additional collateral damage by infiltrating PMNs. This may then lead to a significant delay of PMN apoptosis or emergence of PMN necrosis, which exacerbates inflammation and activates M1 macrophages that are ineffective in phagocytic clearance of apoptotic neutrophils.^{45,46} Collectively, these pathological states lead to prolonged inflammation that is the hallmark of a number of diseases.^{47–50}

M1 macrophages are also professed to activate Th1 and Th17 lymphocytes that play a key role in allogeneic rejection and autoimmune dysregulation, respectively (Figure 2, left panel).⁵¹ A lack of transition from M1 to M2 macrophages is a hallmark of non-healing skin wounds.^{45,52} A significant increase of epidermal Langerhans cells (i.e., a special type of macrophages),⁵³ the presence of activated T lymphocytes (CD3+, HLA DR', CD25),⁵⁴ and a high amount of transforming growth factor (**TGF**)β1 during the proliferative phase of wound healing⁵⁵ are characteristic of hypertrophic scars.

In contrast, fetal wound healing is characteristically known as “scarless.”^{56,57} Following injury to the embryo, the inflammatory response (by virtue of a less-than-mature immune system) is less marked and differs in terms of the types and number of inflammatory cells that enter the wound⁵⁸ and diminished production of interleukin (**IL**)-6 and IL-8.^{59,60} In fetal wound healing, there is downregulation of both pro-inflammatory and pro-scarring

responses.^{61,62} One may thus wonder whether the dominant role of inflammatory process in the post-natal state might impede the wishful “regenerative” healing.

III. Molecular Mechanism of Amniotic Membrane’s Therapeutic Actions

Anatomically, the AM is the innermost membrane enwrapping the fetus in the amniotic cavity, and it extends from the fetal membrane, i.e., encompassing both the AM and the chorion, to the placental proper and the umbilical cord (UC), which connects the placenta and the fetus. Histologically, the AM consists of a simple epithelium, a basement membrane, and an avascular stroma. Developmentally, both the AM and the UC share the same cellular origin as the fetus.

The AM’s barrier function is not only “physical” but also “biological.” During pregnancy, the maternal immune system is challenged by the presence of the fetus, which must be tolerated despite being semiallogeneic. One such “biological” barrier function resides at the decidua level, where decidual macrophages contribute to fetal tolerance and are involved in several other processes required for a successful pregnancy. We have long speculated that AM contributes to the fetal immune-tolerance state and the scarless fetal wound healing during pregnancy by delivering anti-inflammatory and antiscarring action and modulating alloreactive immune activation. As a first step to strengthen this hypothesis, it is important to identify the molecule(s) responsible for AM’s anti-inflammatory action. AM’s anti-inflammatory action has been demonstrated in a number of studies, in which transplanted cryopreserved AM induces apoptosis of neutrophils,^{63,64} monocytes, and macrophages⁶⁵; reduces infiltration of neutrophils,^{63,66} macrophages^{67,68} and lymphocytes⁶⁹; and promotes polarization of M2 macrophages.⁷⁰

Our research effort started by first demonstrating that the anti-inflammatory action exerted by cryopreserved AM is retained in the water-soluble AM extract (AME) prepared from cryopreserved AM. Specifically, we have shown that human AME can induce apoptosis of IFN- γ , LPS, and IFN- γ /LPS-activated but not resting macrophages.^{71,72} AME also downregulates expression of M1 macrophage markers such as TNF- α , IL-6, CD86, and MHC II while upregulating M2 macrophage markers such as cytokine IL-10.⁷²

Following research to identify the HC-HA/PTX3 complex as the key component in the cumulus-oocyte complex surrounding the ovulated oocyte to ensure fertilization,^{73,74} our laboratory was the first to report that the biosynthetic pathway used for ovulation also takes place in the AM. In short, we have purified the HC-HA/PTX3 complex from AME by two successive runs of ultracentrifugation in a CsCl gradient in the presence of 4M guanidine HCl.^{75,76} The biosynthetic process of HC-HA/PTX3 by AM epithelial cells and stromal cells involves the following two steps (Figure 3): the first is to form HC-HA complex via tumor necrosis factor-stimulated gene-6 (TSG-6), which is an enzyme that catalyzes the covalent (ester bond) transfer of heavy chains from inter- α -trypsin inhibitor (I α I) to hyaluronan (HA).^{77–80} I α I 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.^{81–86} We have demonstrated that the HC-HA/PTX3 complex purified from AM consists of high molecular weight (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 pro-inflammatory cytokines.^{76,87} Similar to ovulation,^{74,88} the second step is to form the HC-HA/PTX complex by tight association of the HC-HA complex with pentraxin (**PTX3**).

A. Anti-inflammatory Effect of HC-HA/PTX3

As stated above, PMNs are among the first recruited to engulf pathogens and damaged tissues before their eventual apoptosis. In the pathological states, delayed neutrophil apoptosis will lead to chronic inflammation, which is the hallmark of many diseases (Figure 2, left panel).^{49,50} We have reported that water-soluble HC-HA/PTX3, but not HA, significantly promotes apoptosis of freshly isolated neutrophils after activation by fMLP or LPS but sparing resting neutrophils.⁸⁹ Similarly, water-soluble HC-HA/PTX3, but not HA, dose-dependently promotes apoptosis of LPS-activated, interferon (**IFN**)- γ -activated, or IFN- γ /LPS-activated macrophages, but not resting macrophages.^{72,75,89} Hence, the first anti-inflammatory effect of HC-HA/PTX3 is manifested by facilitating rapid apoptosis of activated PMNs (Figure 2, right panel).

Clearance of apoptotic neutrophils by M2 macrophages is essential to resolve inflammation.^{90–92} We have reported that both water-soluble and substrate (plastic)-immobilized HC-HA/PTX3, but not HA, promote phagocytosis of apoptotic neutrophils by resting and LPS-activated macrophages, respectively. Therefore, HC-HA/PTX3 suppresses pro-inflammatory responses of neutrophils and macrophages involved in innate immune responses (Figure 2, right panel). Macrophages, besides undergoing classical M1 activation (e.g., by IFN- γ and/or LPS) to express high levels of pro-inflammatory cytokines (e.g., IL-12, IL-23, and tumor necrosis factor [**TNF**]- α) and activate Th1 and Th17 lymphocytes,⁵¹ can also be polarized toward M2 activation (e.g., by IL-4/IL-13 or immune complex), which express a low level of IL-12 but a high level of anti-inflammatory IL-10, to activate Treg lymphocytes.⁹³ Polarization of M2 macrophages promotes wound healing and resolves inflammation.^{94–96} However, the lack of transition from M1 macrophages to M2 macrophages has been found in non-healing wounds in animals and humans.^{96–98} We have recently reported that immobilized HC-HA/PTX3 promotes polarization of LPS- or IFN- γ /LPS-activated macrophages toward M2 phenotype.^{89,99} These data show that HC-HA/PTX3 can further downregulate the innate immune responses and extends its reach against adaptive immune responses by polarizing M2 macrophages (Figure 2, right panel).

Because HC-HA/PTX3 polarizes M2 macrophages,⁸⁹ and because macrophages are at the crossroad bridging innate immune responses and adaptive immune responses, we speculate that the anti-inflammatory effect of HC-HA/PTX3 in innate immune responses may also be extended to modulate adaptive immune responses. CD4⁺ T cells become activated by contacting with antigen-presenting cells presenting the peptide antigen through MHC II to proliferate rapidly and differentiate into Th1, Th2, Th17, or Treg.^{100–103} Th1 cells secrete IFN- γ and IL-2 to enhance pro-inflammatory responses.^{104,105} These responses can be downregulated by Tregs, which is activated by M2 macrophages.⁹³

In pathological states, activation of Th1 cells is the hallmark of allograft rejection, while activation of Th17 can be found in a number of autoimmune diseases.^{51,105,106,52,106,107} To test the aforementioned hypothesis, we have reported that water-soluble HC-HA/PTX3, but not HA, suppresses activation of CD4⁺ T cells isolated from murine lymph nodes and spleens via ligation with α -CD3/ α -CD28 regarding proliferation and production of Th1 cytokines (IFN- γ , IL-2) and promotes significant expansion of CD25⁺/FOXP3⁺ T cells.⁹⁹ These data indicate that HC-HA/PTX3 also extends its action toward adaptive immune responses by directly suppressing Th1 cells while promoting the expansion of Tregs (Figure 2, right panel).

To demonstrate that the anti-inflammatory actions of HC-HA/PTX3 can downregulate both innate and adaptive immune responses in vivo, we performed corneal allograft transplantation in mice treated with HC-HA/PTX3 and assessed the allograft survival. Our results showed that subconjunctival injection of HC-HA/PTX3 dose-dependently prolonged the corneal allograft survival.⁹⁹ Recently, Coulson-Thomas et al demonstrated that the glycocalyx secreted by umbilical mesenchymal stem cells is capable of suppressing inflammation and endows these cells with the ability to modulate host immune responses.¹⁰⁷ The components found in such glycocalyx are similar to HC-HA/PTX3 purified from AM. Collectively, the above data shed a new light on how this novel matrix, present from ovulation^{74,108} to pregnancy^{75,76,87,89} might contribute to the development of fetal immune tolerance during pregnancy besides immune immaturation of the fetus and those cytokines mentioned above.

B. Antiscarring Effect of HC-HA/PTX3

Although anti-inflammatory effects can indirectly lead to antiscarring effects, experimental evidence also shows that the AM stroma has a direct antiscarring effect. Previously, we have reported that expression of TGF β 1–3 and TGF β RII transcripts (using Northern blot) is downregulated in human corneal fibroblasts and human limbal and conjunctival fibroblasts cultured on the stromal side of cryopreserved amniotic membrane (CAM).^{109,110} This direct antiscarring effect also explains why AM implanted into the corneal stromal pocket reduces myofibroblast differentiation elicited by invading epithelial cells in a rabbit model¹¹¹ and why corneal haze is reduced in excimer laser-induced keratectomy in rabbits.^{63,64,112,113}

We subsequently reported that water-soluble AME induces cell aggregation and prevents expression of α -smooth muscle actin (SMA) by myofibroblasts.¹¹⁴ Human¹¹⁵ and mouse¹¹⁶ keratocytes seeded on the stromal side of cryopreserved AM maintain their normal phenotype without eliciting nuclear translocation of pSmad2/3 even if they were exposed to serum or TGF β 1. Water-soluble HC-HA/PTX3, but not HA, suppresses the TGF- β 1 promoter activity of human corneal fibroblasts.⁷⁵

C. Anti-angiogenic Effect of HC-HA/PTX3

In addition to reduced inflammation and scarring, AM transplanted corneal surfaces also show reduced vascularization.¹¹⁷ This anti-angiogenic action has also been exploited during corneal surface reconstruction in conjunction with transplantation of corneal epithelial stem cells from the limbus.^{118–120} Previously, a soluble AM extract prepared by boiling and

homogenization was shown to prevent angiogenesis in a rat model of corneal neovascularization induced by alkali burn and by suppressing viability and tube formation of cultured human umbilical vein endothelial cells (**HUVEC**).¹²¹ In addition to anti-inflammatory and antiscarring actions, we have also reported that HC-HA/PTX3 suppresses HUVEC viability more significantly than HA and AM stromal extract, and such suppression is not mediated by CD44.¹²² HC-HA/PTX3 also causes HUVEC to become small and rounded with a decrease in spreading and filamentous actin.¹²² Without promoting cell detachment or death, HC-HA/PTX3 dose-dependently inhibited proliferation and was 100 fold more potent than HA.¹²² Migration triggered by VEGF and tube formation were also significantly inhibited by HC-HA/PTX3.¹²²

IV. In Vitro Reconstitution of Limbal Niche

Although the limbal niche is easy to access and plays an important role in regulating limbal epithelial SC function, the progress in understanding the regulatory mechanism considerably lags behind other SC model systems. Anatomically, the limbal stroma is highly vascularized and innervated¹²³ and is a loose connective tissue containing limbal stromal mesenchymal cells. In contrast, the corneal epithelium lies on a prominent Bowman's membrane (a specialized basement membrane), and the underlying corneal stroma is avascular and highly organized into lamellae, which contain neural crest-derived keratocytes. Recent studies have shown that limbal epithelial SCs in this locale lie deep in the stroma beyond the limbal basement membrane to form a crypt-like structure, as suggested by serial histological sections,^{124,125} ultrastructural characterization.¹²⁶

Our laboratory was the first to demonstrate that collagenase rather than dispase can remove those limbal epithelial SCs lying deep into the limbal stroma.¹²⁷ Such collagenase-isolated limbal epithelial SCs exhibit high clonogenicity.^{126–128} More importantly, collagenase digestion also successfully isolates a subset of mesenchymal cells that are located subjacent to limbal basal epithelial progenitors (Figure 1).¹²⁷ These isolated mesenchymal cells, i.e., limbal niche cells (**LNCs**), are closely associated with limbal basal epithelial progenitor cells (**LEPCs**) and are as small as 5 μm in diameter. They heterogeneously express SC markers such as Oct4, Sox2, Nanog, Rex1, Nestin, N-cadherin, SSEA4, and CD34.^{127,129} A close contact with LNCs endows LEPCs with better clonal growth on 3T3 fibroblast feeder layers.¹²⁷

Using collagenase digestion alone¹³⁰ or dispase digestion to remove the limbal epithelial sheet followed by collagenase digestion of the remaining stroma,¹³¹ we have successfully expanded LNCs on coated Matrigel in MESCM (modified embryonic stem cell medium containing basic Fibroblast Growth Factor [**bFGF**] and Leukaemia Inhibitory Factor [**LIF**]) up to 12 passages. During such expansion, accompanied (contaminated) limbal epithelial cells vanish in 1–2 passages. Expanded LNCs possess the plasticity to adopt angiogenesis (pericyte) progenitor cells to help the process of vasculogenesis¹³⁰ and the potential to be differentiated into adipocytes, cartilage cells, and bone cells, i.e., the tri-lineage commonly expressed by mesenchymal stem cells, in the capacity better than bone marrow-derived MSCs.¹³¹ Importantly, such growth potential and plasticity is lost if expanded on plastic and/or serum-containing medium.¹³¹ Using this isolation method, Funderburgh et al

generate “corneal stromal stem cells” as a source of keratocytes to restore corneal stromal clarity. (See review.¹³²) A recent study has shown that these limbal stromal progenitors also possess the potential of being reprogrammed into CK3/CK12-expressing (cornea-like) epithelial cells.¹³³

Upon being reseeded in 3-dimensional (D) Matrigel from coated Matrigel, expanded LNCs form spheres and revert the expression of the aforementioned ESC markers.¹²⁹ Taking advantage of the aforementioned advance of isolation and expansion of LNCs, we have established an in vitro reconstitution of limbal niche by seeding LEPCs over spheres formed by single LNCs¹³⁴ or LNC spheres¹³⁵ in 3D Matrigel or in HC-HA/PTX3 immobilized on plastic (Figure 4).¹³⁶

A. Stem Cell Self-Renewal in 3D Matrigel

Similar to what has been reported in many other SC niches, including bone marrow¹³⁷ and thymus,^{138,139} we have discovered that such reunion between SDF-1-secreting LEPCs and CXCR4-expressing LNCs is mediated through the SDF-1/CXCR4 chemokine axis and is crucial for preventing LEPCs from adopting the corneal fate decision.^{129,130,135} Disruption of such reunion, e.g., by AMD3100, abolishes the ability of LEPCs to form holoclones.¹³⁵ SC quiescence and self-renewal appear to be regulated in two nearby segregated niche compartments through BMP (quiescence) and Wnt (active renewal) signaling pathways, respectively, in skin, gut, intestine, and bone marrow (Figure 4; see reviews^{140–142}). In 3D Matrigel, we noted that the marked clonal growth of LEPCs signifying SC activation is correlated with activation of canonical Wnt signaling.¹³⁴ Additionally, β -catenin was stabilized in the perinuclear cytoplasm in LEPCs and correlated with upregulation of Wnt7A and FZD5, preferentially expressed by LEPCs, which has been correlated with the corneal fate decision controlled by Pax6.¹⁴³ Inactivation of BMP signaling in LNCs is correlated with upregulation of noggin preferentially expressed by LNCs. Indeed, addition of noggin that expectedly downregulated nuclear localization of pSmad1/5/8 in LEPCs led to nuclear localization of β -catenin in larger LEPCs but membrane relocation of β -catenin in smaller LEPCs and significant upregulation of DKK1/2. These results support the notion that SC-NC reunion in 3D Matrigel recapitulates an in vitro limbal niche suggestive of SC self-renewal (Figure 4).

B. Stem Cell Quiescence in HC-HA/PTX3

Compared to 3D Matrigel, which generates fewer and larger aggregates of LNCs, immobilized HC-HA/PTX3 generates more but smaller aggregates revealing significant higher upregulation of ESC markers such as OCT4, SOX2, NANOG and REX1.¹³⁶ Reunion between LEPCs and LNCs in HC-HA/PTX3 also prevents LEPCs from adopting corneal fate decision, as suggested by notable suppression of CK12 more so than 3D Matrigel. When reseeded on 3T3 fibroblast feeders, vivid clonal growth is observed from reunion in 3D Matrigel but nearly nil from reunion in HC-HA/PTX3 (Figure 4). Expression of CEBP δ , a marker of SC quiescence,^{144,145} is significantly higher while expression of CK12¹⁴⁶ is significantly lower on HC-HA/PTX3 than 3D Matrigel.¹³⁶ Expression of CEBP δ is higher both in the cytoplasm and nucleus of LEPCs in 3D Matrigel and HC-HA/PTX3.¹³⁶ However, phosphorylated Bmi-1 (p-Bmi-1), another marker of SC quiescence,¹⁴⁷ is positive

in the cytoplasm of LEPCs in 3D Matrigel, whereas it is positive in the nucleus of LEPCs and LNCs in HC-HA/PTX3 (Figure 1). The percentage of BrdU+ cells in total PCK+ cells is significantly reduced in HC-HA/PTX3 compared to 3D Matrigel at both Day 3 and Day 7 (Fig. 4). These data collectively suggest that LNC aggregates maintained by HC-HA/PTX3, although preventing corneal fate decision (via differentiation), similar to 3D Matrigel, promote higher expression and nuclear localization of Bmi-1 than 3D Matrigel.

The nuclear localization of p-Bmi1 is particularly intriguing because Bmi1, a member of the Polycomb Group (PcG) gene family of proteins that function as chromatin modifiers is a suppressor of the Ink4a locus including p16^{INK4a}.^{148,149,150} Bmi1 has also been found to be necessary for efficient self-renewal cell divisions of adult hematopoietic and neural SCs.^{149,150} In the intestine crypt, Bmi-1 is expressed by SCs situated at the +4 position^{145,151} and its expression marked as a label retaining marker in quiescent SCs.¹⁴¹

The aforementioned outcome is correlated with the suppression of canonical Wnt but activation of non-canonical (PCP) Wnt signaling, as well as BMP signaling in both LEPCs and LNCs (Figure 4).¹³⁶ The activation of BMP signaling in LNCs is pivotal because nuclear translocation of pSmad1/5/8 is prohibited in hLEPCs when reunited with mLNCs of conditionally deleted *Bmpr1a;Acvr1* DCKO mice.¹³⁶ Staining for Bmi-1 is all nuclear in both hLEPCs and mLNCs expanded from WT(+) after reunion, but becomes weakly expressed in the nucleus in LEPCs and mLNCs expanded from *Bmpr1a;Acvr1*^{DCKO}(+). There was no significant difference in CEBP δ staining. Furthermore, ablation of BMP signaling in LEPCs led to upregulation of cell cycle genes, downregulation of Bmi-1, nuclear exclusion of phosphorylated Bmi-1, and marked promotion of the clonal growth of LEPCs. Hence, HC-HA/PTX3 uniquely upregulates BMP signaling in LNCs which leads to BMP signaling in LEPCs to achieve SC quiescence.

V. Looking Forward

Nonresolving inflammation is a common threat not only in a number of degenerative diseases⁹⁸ but also in corneal blindness caused by LSCD. Although SCs hold considerable promise for the treatment of a number of diseases, we need to consider that nonresolving inflammation present in the recipient bed might be a roadblock to the success of transplanted SCs, as disclosed by clinical studies using different types of transplantation of limbal epithelial SCs. Nonetheless, adjunctive transplantation of cryopreserved AM prevents the development of LSCD if it is performed within a critical time window in the acute stage for chemical burns¹⁵² and Stevens-Johnson syndrome/toxic epidermal necrolysis.^{153,154} AM transplantation resurrects the remaining limbal epithelial stem cells in nearly total LSCD³⁹ and augments the success of transplanted limbal epithelial cells in traditionally conjunctival limbal autografts^{37,155} and simple limbal epithelial transplantation.^{40,41}

These clinical results also point out that one important strategy to restore vision in corneal blinding diseases caused by LSCD is through transplantation of cryopreserved AM. We now know that one molecular mechanism explaining AM's anti-inflammatory, antiscarring, and anti-angiogenic actions lies in HC-HA/PTX3 purified from AM.^{75,76,89,99,122} Conventional anti-inflammatory agents, such as glucocorticosteroids, nonsteroidal anti-inflammatory

agents, cyclosporine/tacrolimus, or various humanized antibodies, target a specific action of one particular type of inflammatory/immune cells. However, the anti-inflammatory action of the HC-HA/PTX3 complex stands out as a unique class, as it exerts broad anti-inflammatory actions by targeting at PMNs, macrophages and lymphocytes extending from innate to adaptive immune responses (Figure 2). Hence, we envision that HC-HA/PTX3 might become a new class of therapeutics that can be more effective in modulating inflammation spreading from innate to adaptive immune responses.

It is surprising to find that the same matrix complex, i.e., HC-HA/PTX3, also uniquely maintains the normal limbal NC phenotype to support limbal SC quiescence (Figure 4). Thus, the dual actions of HC-HA/PTX3 in mitigating inflammation and preserving SC quiescence help explain why cryopreserved AM may promote “regenerative” wound healing (Figure 2).¹⁵⁶ Consequently, we envision the HC-HA/PTX3 complex as a novel matrix that can be formulated as a platform technology to launch much other therapeutics to aid regeneration in the future.

Our success in establishing in vitro reconstitution of the limbal niche by reunion of LEPCs and LNCs in different substrates is a first step toward better understanding of how SC-NC interactions might help control quiescence, self-renewal, and fate decision of limbal epithelial SCs (Figure 1). In this respect, our studies have disclosed that self-renewal is governed by the activation of canonical Wnt signaling and suppression of canonical BMP signaling in 3D Matrigel, while quiescence is controlled by the activation of canonical BMP signaling and non-canonical Wnt (PCP) signaling but suppression of canonical Wnt signaling in HC-HA/PTX3 (Figure 4). Such an in vitro reconstitution of the limbal niche can also be used to investigate how nonresolving inflammation might threaten the well-being of the limbal niche resulting in LSCD. Further studies are necessary to characterize how extracellular HC-HA/PTX3 might exert these actions regarding the receptor binding and the elicited signaling pathways. These studies may also unravel a new engineering strategy of a surgical graft containing limbal epithelial SCs with their native NCs for correcting LSCD.

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Figure 1. Schematic Representation of Human Limbal SC Niche

The human corneal SC niche is located at the limbus (A) in an microenvironment termed palisades of Vogt (B, bar = 200 μ m), where the limbal basal epithelial cells are undulated into folds (C) and invaginated into the limbal stroma as crypts (not shown). A subset of mesenchymal cells (MC) subjacent to the limbal basal epithelial SCs functions as niche cells (C). The close interaction between NCs modulates functions of limbal epithelial SCs such as quiescence, self-renewal and differentiation (D), of which each process is highlighted by the expression of certain markers, of which the tissue location is based on the limbal but not corneal region (E). TAC: transient amplifying cells, PMC: post-mitotic cells, TDC: terminally differentiated cells.

Figure 2. Nonresolving inflammation is correlated to progression from innate to adaptive immune responses

The wound healing process follows four major steps of coagulation, inflammation, granulation tissue formation involving re-epithelialization and angiogenesis, and tissue remodeling. The step of inflammation involves activation of both innate and adaptive immune responses. Under normal circumstance, the innate immune response is activated by apoptosis of infiltrating PMNs and by phagocytosis of apoptotic PMNs by M2 macrophages without activation of adaptive immune response. Under pathological states leading to non-healing chronic wounds or ulcers (left panel), prolonged PMN infiltration delays their apoptosis and delayed phagocytic clearance of apoptotic PMNs by M1 macrophages activates Th1 or Th17 lymphocytes of the adaptive immune response. In contrast, this pathological state can be reversed by HC-HA/PTX3 purified from AM (right panel), which facilitates PMN apoptosis, polarizes M2 macrophages, and suppresses lymphocyte activation (taken from ¹⁵⁷).

Figure 3. Formation of HC-HA/PTX3

In the first stage of biosynthesis, IaI composed of two heavy chains (HC1 and HC2) covalently linked to bikunin via a chondroitin sulfate is obtained from the blood secreted from the liver in ovulation, but is synthesized *de novo* by amniotic epithelial cells and stromal cells. Both HC1 and HC2 from IaI are covalently transferred to HMW HA to form HC-HA complex in the ovary but only HC1 is transferred in the AM via the catalytic action of TSG-6. In the second stage of biosynthesis, PTX3 octamers are tightly associated with the HC-HA complex via binding with HCs. Taken from ¹⁵⁷.

Figure 4. *In Vitro* Reconstitution of Limbal Niche on 3D Matrigel or HC-HA/PTX3

Limbal niche can be reconstituted by reunion between LEPCs and LNCs to generate sphere growth in either 3D Matrigel or HC-HA/PTX3. Switching on or off of Wnt and BMP signaling pathways controls SC quiescence and activation, respectively, in several SC niches (taken from¹⁴¹). Similarly, SC renewal as evidenced by clonal expansion and high BrdU labeling is mediated by activation of Wnt (on) and suppression of BMP (off) signaling in the niche reconstituted in 3D Matrigel. In contrast, SC quiescence as evidenced by the lack of clonal growth and low BrdU labeling is mediated by activation of BMP and PCP (both on) signaling and suppression of Wnt signaling (off) in the niche reconstituted in HC-HA/PTX3.