Primary Mechanisms of Thymosin β4 Repair Activity in Dry Eye Disorders and Other Tissue Injuries

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Dry eye disorders are becoming more common due to many causes, including an aging population, increased pollution, and postrefractive surgery. Current treatments include artificial tears; gels; lubricants; tear duct plugs; and anti-inflammatory agents such as steroids, doxycycline, and cyclosporine. For more severe forms of the disease, serum tears and scleral contact lenses are employed. Despite these therapies, successful resolution of the problem is limited because none of these treatments fully addresses the underlying causes of dry eye to promote ocular surface repair. Thymosin β4 (Tß4), a small, naturally occurring protein, promotes complete and faster corneal healing than saline alone or prescription agents (doxycycline and cyclosporine) in various animal models of eye injury. In human trials, it improves both the signs and symptoms of moderate to severe dry eye with effects lasting beyond the treatment period. This review will cover the multiple activities of Tß4 on cell migration, inflammation, apoptosis, cytoprotection, and gene expression with a focus on mechanisms of cell migration, including laminin-332 synthesis and degradation, that account for this paradigm-shifting potential new treatment for dry eye disorders. We will also speculate on additional mechanisms that might promote eye repair based on data from other tissue injury models. Such studies provide the rationale for use of Tß4 in other types of eye disorders beyond dry eye. Finally, we will identify the gaps in our knowledge and propose future research avenues.

Keywords: laminin-332, thymosin beta 4, dry eye, migration, cell–cell adhesion

Overview of Corneal Epithelial Wounds

The cornea is avascular, transparent, and rigid and consists of three layers (epithelial, stromal, and endothelial). In order to optimally refract light and ensure clear vision, the cornea must maintain optical transparency. The corneal epithelium serves as a barrier and also contributes to the maintenance of corneal transparency. Rapid resurfacing the injured area is essential to prevent loss of both normal corneal function and vision. Dry eye is becoming more common due to many causes or predisposing factors, including an aging population, increased pollution, postrefractive surgery (LASIK), female sex, smoking, extreme hot or cold weather, low relative humidity, use of video screen display, contact lens wear, and certain medical conditions (rheumatoid arthritis, lupus, diabetes, graft-versus-host disease, blepharitis).

Following wounding, corneal epithelial healing initiates with the migration of the epithelial cells from the damaged edge of the wound. The cells migrate to cover the denuded area and then proliferate and differentiate to restore the normal epithelial cytoarchitecture. In most instances, corneal epithelial defects caused by simple injury are rapidly resurfaced. However, in individuals with certain clinical conditions, such as diabetic keratopathy, corneal epithelial defects persist and do not respond to conventional treatment regimens because of delayed epithelial wound healing. Complete and rapid corneal re-epithelialization after injury protects the cornea from infectious agents and prevents epithelial fragility that can lead to recurrent erosions. After the corneal epithelium is damaged, the remaining epithelial cells migrate as an intact sheet over the denuded surface via both the interaction of the cells with the underlying substrate and with each other. This migration is mediated by adhesion via substrate fibronectin and laminin-332, integrin receptors, and other unknown factors. In addition, laminin-332 is produced by the cells at the leading edge of the migrating sheet and promotes migration via multiple mechanisms, including haptotaxis, chemotactic activity of a protease-derived fragment of laminin-332, and increased cell–cell and cell–matrix interactions.

Thymosin β4 Activities

Thymosin β4 is a ubiquitous, 43-amino acid acidic polypeptide with a molecular weight of 4.9 kDa. It is highly conserved across species, and is found in all tissues and cell types except red blood cells. Thymosin β4 is a multifunctional protein that promotes cell migration, stem cell recruitment and differentiation, protease production, and the expression of various regulatory genes, such as laminin-332, fibronectin, zyxin, VEGF, matrix metalloproteases, hepatocyte growth factor, and anti-oxidative enzymes (Table 1). It inhibits inflammation, microbial growth, scar formation (by reducing the level of myofibroblasts), and apoptosis, and protects cells from cytotoxic damage, including glutamate neuronal toxicity.

Thymosin β4 binds to G-actin, blocks actin polymerization, and...
is coreleased with factor XIIIa by platelets, suggesting its importance in wound healing.18

Thymosin β4 promotes full thickness dermal wound repair in normal, steroid-treated, and diabetic animals.25–28 Following dermal injury, high levels of Tβ4 are naturally present (15 µg/mL) in the wound fluid.29 Thymosin β4 is also active for repair and regeneration in the eye, heart, brain, peripheral nervous system, and spinal cord and promotes angiogenesis in some tissues, but not when added topically to the wounded eye surface.18 A number of active sites on Tβ4 have been identified for some of these activities.30,31 Amino acid fragments 1-4 is anti-inflammatory, 1-15 is anti-apoptotic and cytoprotective, and 17-23 is active for cell migration, actin binding, dermal wound healing, angiogenesis, and hair growth. Surprisingly little is known about the potential receptors. Purinergic signaling pathways have been reported, but given the number of activities and active sites, one would expect several receptors.32 Much is also unknown about the role of Tβ4 in the nucleus. Upon incubation with cells, it is rapidly (30 minutes) transported to the nucleus where it may function as a transcription factor.21,33

**Tβ4 in Basic and Clinical Eye Studies**

**Preclinical Studies.** In various animal eye injury models, Tβ4 is efficacious for ocular repair, including heptanol debridement, alkali injury, ethanol exposure, secondhand cigarette smoke exposure, and ultraviolet light.34–39 In a controlled adverse environment that results in severe dry eye, it also promoted murine corneal epithelial healing with a controlled adverse environment that results in severe dry eye phase 2 trial, patients showed statistically significant decrease in corneal staining over that eye, it also promoted murine corneal epithelial healing with a controlled adverse environment that results in severe dry eye phase 2 severe dry eye phase 2 trial with 72 patients, statistically significant improvements in both the signs (reduced wound size) and symptoms (ocular discomfort).42 Finally, in a controlled adverse environment phase 2 severe dry eye phase 2 trial with 72 patients, statistically significant improvements in both the signs and symptoms of dry eye were found, and these improvements were maintained during the 28-day follow up with no Tβ4 treatment.44 These studies demonstrate the safety and rapid repair activity of Tβ4.

Given the clinical applications for Tβ4 in ocular repair and its advanced current clinical development, it is important to define as much as possible, the mechanisms involved in ocular repair and identify future studies needed to fill in the gaps in our knowledge on the activity of this molecule. In most tissues, a decrease in inflammation is credited with being a major therapeutic advance. Six patients, with nonhealing neurotrophic keratitis wounds that had not healed for at least 6 weeks, completely healed by the end of the 28-day study and reported a reduction in eye discomfort.42 In a moderate dry eye phase 2 trial, patients showed statistically significant improvements in both signs (reduced wound size) and symptoms (ocular discomfort).43 Finally, in a controlled adverse environment phase 2 severe dry eye phase 2 trial with 72 patients, statistically significant improvements in both the signs and symptoms of dry eye were found, and these improvements were maintained during the 28-day follow up with no Tβ4 treatment.44 These studies demonstrate the safety and rapid repair activity of Tβ4.

**Mechanism of Action of Tβ4 in Dry Eye: Migration**

Cell migration is a complex process. Thymosin β4 promotes chemotactic and haptotactic cell migration.18,41,46,49-53 Such
epithelial sheet migration is critical to the healing process. Cells must change from a stationary adherent mode to a migrating nonadherent mode to effect repair. A better understanding of the processes by which Tß4 promotes cell migration could lead to improved treatments for dry eye disorders. Given the published data on the role of Tß4 in stem cell migration and differentiation in other tissues, we also speculate that it may promote limbal or other types of stem cell migration in the eye as well. Based on our evaluation of the current data in the eye field and in other tissues, we present new insights on at least five possible ways in which Tß4 regulates such motility (Fig. 3).

**Actin Binding.** Thymosin ß4 binds to actin and regulates actin polymerization which is important for cells to attach and detach and extend cellular protrusions during migration. Thymosin ß4 has been localized at the leading edge of the lamellipodia and defined as a key molecule that localizes actin monomers to this region for neuronal cell motility. The role of actin polymerization in cell migration is well documented for many cell types, including the corneal epithelium. There is a connection between integrins (the matrix receptors), adhesion molecules (laminin-332 and fibronectin), and actin. Proteases destroy these connections resulting in loss of adhesion and then adhesion reforms only to be lost again and the cycle continues, allowing cell migration.

**Proteases.** Thymosin ß4 promotes matrix metalloproteinase activity that is necessary for epithelial cell migration. Inhibitors of these enzymes reduce the migration of various cell types, including corneal epithelial cells. Such enzymes also degrade and release matrix molecules, which may be chemotactic or haptotactic migration factors as well. It would be important to test specific protease inhibitors in vivo in animal models and define the degraded matrix fragments that are active for migration.

**Laminin-332.** Thymosin ß4 promotes the synthesis of fibronectin and laminin-332 (Fig. 4) both of which are migration factors for many cell types. Laminin-332 is a migration factor for human corneal epithelial cells, where it is active as both a chemoattractant and haptotactic factor. As mentioned above, laminin-332 mRNA and protein are expressed at the leading edge of the migrating tongue in wounded epidermis. It is not known exactly how Tß4 induces laminin-332, but it has been shown that Tß4 stabilizes HIF1, which is a transcription factor that binds to the promoter of the ß3 chain of laminin-332. Laminin-332 is deposited between the clot and the migrating epidermis, and such deposition in the healing dermis is critical for cell migration.

### Table 2. Completed Ocular Clinical Trials and Findings

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<tr>
<th>Neurotrophic phase 2 trial</th>
<th>Significant healing in patients with neurotrophic keratitis who had lesions that had not healed in 6 weeks to several years before treatment, completed 2009 (physician sponsored), 28-day trial</th>
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<td>No drug-related or serious side effects observed in six treated patients</td>
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<th>Moderate dry eye phase 2 trial</th>
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<td>No drug-related or serious adverse events observed in 72 patients</td>
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<td>Statistically significant sign (size of lesion) and symptom (patient comfort) improvements in patients</td>
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<th>Severe dry eye phase 2a trial</th>
<th>Significant sign and symptom improvements in 28-day trial, completed 2013 (physician sponsored)</th>
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<td>No drug-related or serious adverse events observed in nine patients</td>
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<td>Statistically significant increase in healing (59% reduction of corneal staining) and symptom improvements (35%)</td>
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migration and basement membrane matrix organization. Additional studies are needed to support the production and deposition of laminin-332 in the Tβ4-induced migrating corneal epithelium. One could also argue that laminin-332 is an adhesion molecule and cells must adhere in order to migrate so this additional function is likely a component of the migration activity of laminin-332.

**Laminin-332 Degradation.** Proteases degrade laminin-332 and release a potent migration-promoting fragment. In particular, the laminin-332 α2 chain is processed to a smaller size that functions to promote epithelial sheet migration over the dermal wound bed. This proteolytic processing is required to switch from stationary to migratory activity whereby the cells detach from the matrix and then reattach as they migrate.

**Cell-Cell and Cell-Matrix Adhesion.** Laminin-332 functions to anchor the cells to each other and to the substratum. Laminin-332 maintains the cell–cell and cell–matrix interactions that allow for the sheet of epithelium to be stabilized and to migrate intact over the wounded area. Some processing is involved in the laminin-332 to form the tight cell–cell interactions. One could also possibly argue here that the antiapoptotic activity of Tβ4 mediated by a reduction in the oxidative enzymes prevents cell loss and thus maintains the intact epithelial sheet. Such antiapoptotic activity indirectly supports sheet migration.

While these five activities of Tβ4 support its role in migration and provide mechanistic pathways, much needs to be further investigated. Many of these studies have not been performed in eye-derived cells or eye tissue.

**Additional Important Activities for Tβ4-Mediated Dry Eye Repair**

**Stem Cell Recruitment.** Stem cell recruitment/migration and differentiation by Tβ4 has been found to be an important component of both heart and neural repair, as well as hair growth and tooth development. Thymosin β4 has also been shown to promote mesenchymal stem cell proliferation and may also have such activity in the eye for the regeneration of the epithelium. The corneal epithelium is thought to be regenerated from stem cells in the limbus which are activated with injury. Some studies have shown successful stem cell transplants for ocular repair, but these studies are at an early stage. The concerns with stem cell transplantation generally include the limited number of cells requiring expansion in vitro, teratoma formation, and immune reaction, lifelong immunosuppression. The role of stem cells in Tβ4-mediated ocular repair has not yet been investigated, but it can be assumed that these cells play an important role. Related studies have been performed in the heart where endogenous stem cells are known to improve heart function after injury, and Tβ4 has shown improvement in stem cell recruitment and cardiac repair. Exogenously added stem cells have shown improvement over no treatment in the heart as well. Interestingly, when Tβ4 treatment versus stem cell transplantation are
compared in heart repair, both have similar efficacy suggesting that Tβ4 may be able to replace stem cell therapy in certain tissues.86 It has also been shown that stem cells overexpressing Tβ4 further repair the injured over that observed with stem cells alone.81 Finally, one group has shown that silencing Tβ4 in stem cells and then transplanting them into the heart reduces their efficacy for cardiac repair. These studies demonstrate that short-term cardioprotection in the heart is mediated in part by endogenous stem-cell derived Tβ4.80 Thus, Tβ4 alone would potentially be an effective heart therapy and would avoid the concerns with using stem cells for tissue repair stated above. The important questions remain in the eye on whether Tβ4 recruits stem cells for repair, and if this process is a major driver in corneal healing. The mechanisms of the stem cell recruitment beyond increased migration are not known. Increased stem cells in response Tβ4 are also observed in nervous system repair, and there is increased hair growth in the skin suggesting that the tissue regeneration activity of Tβ4 may be mediated through a common mechanism but too little is known yet about that process.

**Anti-Inflammation.** Inflammation is reduced by Tβ4 in the eye and in other tissues after various types of injuries, and the pathways of this activity are being defined at the molecular level.19,36,39,45,82 This reduction in inflammation in Tβ4-mediated repair in dry eye is important and permits the specific migration and activities mediated by Tβ4 to effect repair. For example, Tβ4 reduces inflammatory cytokines and chemokines in many tissues, including the eye, and decreases inflammatory cell infiltration, upregulates antioxidative enzymes, and decreases reactive oxygen species. Thymosin β4 inhibits TNF-α-induced nuclear factor kappa B activation and blocks Ral/Gap65 translocation and the sensitizing effects of its intracellular binding partners PINCH-1 and integrin-linked kinase.19

**Additional Ocular Disorders That May Improve With Tβ4**

Based on the defined mechanism of action of Tβ4 in the eye, it is likely that it will promote repair in variety of other eye disorders involving corneal injury, including postrefractive surgery, blepharitis, graft-versus-host disease, limbal stem cell deficiency, diabetic keratitis, and eye surgery. Below are some examples of such eye disorders that may benefit from treatment with Tβ4.

**Postrefractive Surgery Corneal Wound Healing.** Corneal wound healing plays a major role in the visual outcome after LASIK, photorefractive keratectomy (PRK), and other commonly performed refractive surgeries.85 We hypothesize that Tβ4 treatment will accelerate and improve corneal epithelial healing after refractive surgery, thereby reducing ocular morbidity, such as pain, inflammation, and corneal haze. Promoting corneal epithelial cell migration and repair will lead to improved epithelial flap healing in LASIK and faster re-epithelialization in PRK laser-treated eyes. Reduced healing time will allow patients to return to their activities sooner and should lead to superior visual outcomes.

**Blepharitis.** Blepharitis is an inflammatory condition where the meibomian glands in the lids fail to function properly, resulting in inflamed, irritated, and itchy eyelids and abnormal tear film.84 This can cause dry eye and even some scarring in the eyelid that can result in injury to the corneal surface. Recent findings demonstrated that Tβ4 is the sixth highest expressed gene in human meibomian gland disease samples.85 The authors concluded that keratinization plays an important role in meibomian gland disease. We hypothesize that Tβ4 would be an excellent candidate drug for this problem as it reduces inflammation and scarring and promotes corneal repair with less discomfort.15

**Limbal Stem Cell Deficiency.** The corneal epithelium is continuously and naturally replaced by limbal stem cells.54,55 When the stem cells are reduced, the ocular surface becomes unstable. There are various causes of the loss of limbal stem cells, including trauma (chemical injury, contact lens wear), multiple surgeries, and genetic and autoimmune disorders. When the cornea cannot be repopulated, patients suffer from pain due to the corneal erosions and may also have reduced vision from scarring. Thymosin β4 can reduce inflammation and scarring and recruit stem cells from adjacent tissues to effect repair. All three layers of the cornea contain stem cells so it is possible that stem cells can be recruited from nearby locations.55

**Diabetic Keratitis.** Diabetes can have a deleterious effect on the eye resulting in pain and reduced vision.86 Chronic hyperglycemia weakens the cell-matrix hemidesmosomal attachments between the corneal epithelium and its underlying basement membrane, making the patients much more susceptible to both incidental corneal abrasions and recurrent corneal erosions. Thymosin β4 promotes laminin-332 synthesis that maintains cell-matrix contacts and also healing through increased corneal epithelial migration.47 Thus, Tβ4 would be expected to prevent or reduce diabetic eye injuries and possibly aid in the healing of injuries due to hyperglycemia or other causes in diabetics.

**CONCLUSIONS AND QUESTIONS FOR THE FUTURE**

Although significant progress has been made in understanding how the cornea heals, corneal wounds pose significant challenges to the ophthalmologist because current treatment regimens are limited. Clinicians provide the patient with an environment conducive to healing and rely on the eye’s innate reparative ability. The development of new therapies that selectively regulate specific steps in ocular surface healing has lagged far behind. Agents, such as corticosteroids, lubricating ointments, artificial tears, and amniotic membranes, fail to adequately address clinical needs. Additionally, there are many potentially severe complications from corticosteroids, such as corneal ulceration and perforation, cataract formation, steroid-induced glaucoma, and increased risk of infection.80 Defining Tβ4-modulated pathways that regulate corneal healing will facilitate translation of basic scientific findings into safe and effective therapeutic regimens for the treatment of ocular surface disorders. In turn, the tissue repair and anti-inflammatory capabilities of Tβ4 in the cornea are clinically relevant in a wide array of eye conditions.

Many questions still remain that could lead to a better understanding of the therapeutic potential of Tβ4. What we know about the receptors is limited and needs to be expanded. It may be possible that short sequences or even mimetics may be able to be used to optimize the tissue responses. Given the multiple activities and defined active sites, there are multiple receptors yet to be identified. Thymosin β4 is in tears at a level ranging from 0.5 to 0.7 μg/mL, which is twice the amount found in saliva.89 What is its function in tears and does the level present tell us anything important for the clinical application? One important thought is that chronic use of Tβ4 might be safe. No long-term safety studies have been done with Tβ4 treatment in the eye for more than a month, and these should be done to determine if there are any adverse effects due to chronic usage. This is important since in certain chronic conditions there may be a need for longer and/or continuous usage. In the clinic, 0.1% Tβ4 eye drops are used 2 to 6 times per day which translates to 1.0 mg/mL that exceeds the
physiological tear level. Eye platelet rich plasma (E-PRP) has shown efficacy in corneal wound healing.\(^9\) It contains many factors one of which is likely T\(\beta 4\) which is in serum and is released by platelets. The amount of T\(\beta 4\) in E-PRP is unknown.

Laminin-332 is pivotal for corneal epithelial migration both as a cell–cell and cell–matrix adhesion factor and both the intact molecule and at least one fragment are chemotactic/haptotactic factors. It is important that a full understanding of how laminin-332 and its receptors regulate repair. Testing of the different types of proteases that might be required to disrupt the cell–cell and cell–matrix interactions as well as the generation of active laminin-332 fragments is needed. A full analysis of all the proteases and their inhibitors should be done as it is not clear which proteases are present, active, and affect cell migration directly or via laminin-332 in the eye. Based on inhibitor studies, it is clear that proteases are needed for migration, but in vivo studies have not been done. It is important to understand which are the active proteases in corneal repair and whether this information can be used to improve the therapeutic approaches.

In summary, there is an increasing need for better treatments for dry eye and other ocular disorders. Thymosin \(\beta 4\) represents a new class of bioactive molecules that affect repair by multiple mechanisms. Understanding these mechanisms can lead to better patient care.

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