• Review Article •

Research progress on animal models of corneal epithelial-stromal injury

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Abstract

● A corneal epithelial-stromal defect is recognized as a major contributor to corneal scarring. Given the rising prevalence of blindness caused by corneal scarring, increasing attention has been focused on corneal epithelial-stromal defects. Currently, the etiology and pathogenesis of these defects remain inadequately understood, necessitating further investigation through experimental research. Various modeling methods exist both domestically and internationally, each with distinct adaptive conditions, advantages, and disadvantages. This review primarily aims to summarize the techniques used to establish optimal animal models of corneal epithelial-stromal injury, including mechanical modeling, chemical alkali burns, post-refractive surgery infections, and genetic engineering. The intention is to provide valuable insights for studying the mechanisms underlying corneal epithelial-stromal injury and the development of corresponding therapeutic interventions.

● **KEYWORDS:** cornea; corneal epithelial-stromal defect; corneal epithelial-stromal injury; corneal scarring; animal models

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INTRODUCTION

The cornea, a transparent non-vascular connective tissue, serves crucial roles in both refraction and mechanical barrier functions. Its intricate anatomical structure and cellular components enable us to perceive a clear visual field[1]. Positioned in the outermost layer of the eyeball, the cornea is susceptible to various exogeneous or endogenous pathogenic factors, including infections, surgeries, immune-mediated reactions, and traumas. Consequently, these factors can compromise the cornea's integrity, leading to the development of corneal ulcers. Corneal ulcers manifest as surface defects in the corneal epithelium, often accompanied by necrosis and thinning of the corneal stroma. In severe cases, such defects can result in corneal clouding, dissolution, and even perforation, causing visual impairment and blindness and significantly impacting patients' quality of life, as well as their physical and mental well-being[2].

The corneal stroma, comprising approximately 90% of the corneal thickness, plays a pivotal role in maintaining corneal transparency and refractive function by forming the majority of the corneal framework. However, when trauma, infection, or other factors result in partial or complete penetrating damage to the corneal stroma, fibroblasts and multinucleated leukocytes initiate fibrin clot formation within 7d. Subsequently, these clots undergo continuous refinement and remodeling of extracellular matrix components, ultimately leading to the irreversible loss of corneal transparency through scar formation. Corneal scarring stands as a major cause of visual impairment and vision loss worldwide[3]. Currently, corneal scarring can be addressed through corneal transplantation or the development of corneal stromal equivalents. However, the efficacy of these approaches requires further investigation due to challenges such as the limited availability of corneal donors, immune rejection, scarcity of suitable animal models, and the need for validation in human subjects[4].

The utilization of an appropriate *in vivo* animal model of corneal injury allows for a deeper comprehension of the reparative mechanisms involved in corneal epithelial-stromal damage, thereby facilitating the development of improved treatment modalities for individuals suffering from corneal scarring-induced blindness. Presently, numerous techniques

have been employed both domestically and internationally to establish animal models of corneal stromal defects. These techniques encompass methods such as mechanical resection, filter paper cauterization, microbial inoculation, and gene modeling. However, a standardized approach for establishing such models is yet to be universally adopted.

ANIMAL SPECIES FOR *IN VIVO* STUDIES

Corneal defects have been extensively investigated in several animal models, including rabbits, mice, rats, zebrafish, pigs, and chickens. Among these models, rabbits are particularly well-suited for experimental studies due to their corneal structure, which closely resembles that of humans, and the ease with which local surgical procedures can be performed, observations can be made, and experimental specimens can be obtained. Esteves *et al*[5] have comprehensively documented the sustained and extensive use of rabbits as a reliable model for studying various human diseases, such as syphilis, tuberculosis, norovirus, and ocular herpesvirus infections. This model has contributed significantly to our understanding of the cellular and molecular mechanisms underlying these diseases, as well as facilitating investigations into antibody structure and diversification mechanisms and advancing drug development and testing. Nonetheless, there are certain drawbacks associated with using rabbits, including high costs, limited availability of polyclonal antibodies targeting specific proteins of interest, and constraints on access to genetically diverse strains and transgenic animals.

Mice are the preferred experimental animals for many corneal studies due to the availability of genetically modified resources and their relatively low cost. The advent of genetically engineered mice in the 20th century ushered in the era of the mouse model. Currently, the most commonly utilized mouse strains are BALB/c and C57BL6, and differences among strains can directly impact corneal wound healing[6]. Pham *et al*[7] have conducted corneal epithelial-stromal injury using a corneal rust ring remover. They have found that the BALB/c and CFW mouse with the higher density of corneal nerve plexus show faster wound healing than C57BL/6. On the other hand, zebrafish, as described by Puzzolo *et al*[8], lack the Bowman layer, post-elastic layer, and nerve fibers, and have a thinner extracellular matrix compared to the human cornea. Therefore, they are not suitable for corneal defect models. Regardless of the chosen animal model, variations in anatomical structure and function exist. Therefore, the selection of an appropriate animal model should be based on the specific experimental purpose and objective conditions. Prior to conducting experiments, animals should undergo adaptive breeding, and their eyes should be carefully examined. Compliance with animal ethical regulations and policy guidelines is imperative.

OPERATION METHODS

Mechanical Wound Models

Lamellar keratectomy wounds In the 1960s, Barraquer[9] introduced the concept of keratomileusis, which involved altering corneal curvature through lamellar surgery to correct refractive errors. Various methods have been employed for corneal lamellar cutting in keratomileusis, including the use of blades, micro-electric lamellar knives, or automatic gear rotation. Rittie *et al*[10] have provided a detailed description of the lamellar keratotomy procedure. In brief, a ring drill with a diameter of 1.5–2.5 mm is gently rotated into the corneal stroma at the central cornea. Subsequently, forceps are utilized to separate the epithelial layer, basement membrane, and thin anterior mesenchyme from the remaining mesenchyme, successfully establishing a mouse model of corneal scarring. This model is employed to test the potential of drugs in reducing corneal fibrosis.

Shirzaei Sani *et al*[11] have employed a 3-mm biopsy punch to create a hole at the central cornea of the right eye of male New Zealand white rabbits aged 8 to 12wk, followed by lamellar keratotomy at approximately 50% depth using a scalpel. This approach aims to evaluate the biocompatibility and biointegration of engineered GelCORE hydrogel. However, it is worth noting that in mice, there is a risk of penetrating the cornea and achieving an incorrect cut diameter due to the thinness of the cornea. Moreover, manual separation-induced matrix fluctuations can result in optical distortion and irregular astigmatism.

Photorefractive keratectomy wounds To minimize the variability in corneal injury, researchers have the option to utilize an excimer laser for keratectomy. Refractive keratectomy, specifically photorefractive keratectomy (PRK), involves the removal of corneal epithelium using mechanical or chemical methods, followed by the use of an excimer laser to precisely ablate the corneal stroma. This approach results in minimal collateral damage to surrounding tissues. Park and Kim[12] have conducted a comparison between the corneal wound healing processes of PRK using the VISXSTAR 193 nm excimer laser and laser-assisted *in situ* keratomileusis (LASIK) employing the MicroTech microkeratome in rabbit eyes. They have observed inward growth of corneal epithelium and regenerating stroma between the flap and stromal bed at the wound edge in both procedures. However, the number of regenerating stromal tissue and keratinocytes within the wound area is found to be smaller in the LASIK group compared to the PRK group. The loss of dehydrated and contracted flap or corneal epithelium in PRK can lead to exposure of the corneal stroma and activation of corneal cells, potentially leading to excessive wound healing in the annular region. This phenomenon may be associated with the occurrence of significant corneal haze following clinical PRK surgery.

Femtosecond-assisted LASIK (FS-LASIK) utilizes the nonlinear optical detonation principle of the femtosecond laser to create a separation between the corneal flap and the stromal bed. Subsequently, the excimer laser is employed to perform the refractive cut on the stromal bed. FS-LASIK offers several advantages, including the generation of a more predictable corneal flap, reduced ocular aberration, improved uncorrected visual acuity, and minimal intraocular pressure (IOP) fluctuations.

Koulikovska *et al*[13] have employed a novel FS-LASIK model to implant an acellular bioengineered porcine construct (BPC) into the corneal stroma for a duration of 8wk. This approach results in transparent corneas with rapid healing and devoid of inflammation. Postoperative administration of steroids is unnecessary. The procedure minimizes interference with the epithelium and endothelium and preserves the shape and curvature of the cornea without sutures, thereby reducing the risk of stimulating positive wound healing and minimizing the likelihood of rejection. Consequently, this approach enables the production of truly individualized valves, contributing to optimal clinical outcomes. It should be noted, however, that a drawback of this modeling approach is the inability to utilize laser-ablated tissue for subsequent RNA or biochemical analysis.

Incisional wounds Sumioka *et al*[14] have utilized a surgical blade to create a full-thickness penetrating incision injury, measuring 1.5 mm in length, in the central cornea of both wild-type C57BL/6 (WT) mice and KO mice of C57BL/6 background. Their findings reveal that the absence of tenascin C inhibits the effect of transforming growth factor β1 (TGFβ1) in accelerating fibroblast expression and myofibroblast production, ultimately impairing the primary healing of the corneal stromal injury. Li *et al*[15] have employed a scalpel to generate a triangular corneal defect measuring 2×2×2 mm^3. They have promptly filled the area with trabecular tissue, sutured the laceration, and debrided the defect using an oblique suture. After a period of 3mo, all rabbits exhibit favorable anterior chamber formation, smooth corneal curvature, and healing of the fibrous scar. To prevent chronic iris incarceration into the corneal incision, it is recommended to administer atropine to dilate the pupils before conducting the experiment. This model is characterized by its simplicity, ease of operation, low cost, and lack of specialized equipment requirements. Consequently, it is commonly employed to investigate the mechanisms and roles of corneal defect replacement repair. However, it is essential to note that the extent of the damage may vary, and the manipulation technique should be carefully regulated.

Chemical Wound Models With the progress of economic development and modern industrialization, corneal chemical injuries are increasingly prevalent. These injuries can be attributed to approximately 10 categories of chemicals, primarily categorized as either acids or alkalis. When eye tissue meets alkaline substances, rapid dissolution of intraocular fat and protein occurs, leading to deep infiltration. Additionally, leukocytes accumulate and release various proteases and free radicals, causing the dissolution of corneal collagen and the subsequent development of corneal ulcers[16]. On the other hand, acidic substances can induce the solidification of tissue proteins, forming scab membranes that prevent further penetration of the acid into deeper layers. Nevertheless, high concentrations of acid can still permeate the deep matrix, resulting in similarly severe consequences as alkali burns.

Alkali injury Corneal neovascularization and scarring are significant characteristics of alkali burns[17]. Animal models of corneal alkali burns are valuable for studying the underlying regulatory mechanisms, evaluating the effectiveness of drugs, and exploring prevention and treatment strategies. Currently, rats, mice, Japanese big-eared rabbits, and New Zealand rabbits are commonly used as experimental subjects[18-20]. The shape of the filter paper used for the burn can vary, including ring, circular, or triangular shapes. The burn area can encompass the central cornea, limbus, or the entire cornea. For instance, Koivusalo *et al*[13] have accurately placed NaOH-soaked filter paper on the central cornea for 40s, followed by immediate rinsing with BPC and epithelial scraping. Subsequently, a 5-mm trephine is used to excise the corneal stroma, and the stromal buttons are removed. This study demonstrates the concept of implanting limbal stem cells and stromal repair cells into an adherent scaffold of corneal regenerative tissue without the need for sutures.

Acid injury In comparison to alkali burns, corneal acid burns have a lower incidence of ulceration and reduced secretion of inflammatory mediators and proteases. He *et al*[21] have established a model of low-concentration acid burn by immersing a single-layer filter paper with a diameter of 2 mm in 25% sulfuric acid for 10s and then evenly applying it to the center of the cornea of the right eye for 60s. The results indicate that the scars and opacities left by dexamethasone are more pronounced in the early stage of repair compared to the acute and late stages of repair.

Hydrofluoric acid (HF), as a highly corrosive liquid, induces severe pain upon eye contact and rapidly forms white pseudomembranous turbidity, causing extensive dose-related damage to the superficial and deep structures of the eye[22]. The severity of progressive corrosion of the eye by HF is determined by a combination of pH value and the toxic effect of free fluoride ions. While mild burns are reversible, severe burns can lead to the formation of corneal stromal scars, vascularization, edema, calcification zone formation[23]. Altan

and Ogurtan[24] have successfully established an HF burn model in rabbits by applying 0.05 mL of 2% HF drops into the eyes for 60s under general anesthesia. The healing of corneal opacity and conjunctival injury is evaluated clinically based on corneal opacity, IOP, conjunctivitis, conjunctival status, and corneal erosion area, as well as pathologically by assessing stromal thickness, inflammatory cell infiltration, and corneal angiogenesis.

Silver nitrate injury In the study of corneal neovascularization and corneal opacity induced by injury, as well as the behavioral evaluation of ocular hyperalgesia, topical application of silver nitrate cauterization of the cornea has been employed. A modified procedure for silver nitrate cauterization involves using a smear or filter paper coated with a mixture of 75% silver nitrate and 25% potassium nitrate to cauterize the central cornea for a duration of 2–20s, resulting in the formation of gray-white discrete lesions. Subsequently, the residues are rinsed with normal saline, and the local application of eye ointments or antibiotic eye drops is performed[25-26].

Vesicant injury Vesicants, also known as blister agents, are cytotoxic alkylating agents that can cause tissue damage and poisoning when they enter the body through the skin or respiratory tract. Common types of vesicants include nitrogen mustard (NM), sulfur mustard (SM), and Lewisite (LEW). The functional recovery from vesicant damage is determined by the exposure dose and the total area of exposure[27].

In the study of the ocular toxicity effects of NM, NM is often used as a substitute for SM in acute corneal injury models. The animals are exposed to 1% NM for 5min, and corneal stromal injury and ulceration are evaluated using slit-lamp imaging 28d after exposure[28]. Experimental models have been established in rabbits, pigs, and rodents to investigate the potential mechanisms of SM-induced skin and lung injury[29-30]. SM, a highly toxic agent, is first used in World War I and has long-lasting effects on veterans and survivors due to its delayed complications[31]. In the experimental setup, the animals' eyes are protected with a shield, and their heads are fixed with protective glasses while being exposed to SM vapor at concentrations of 400 μg/L (390–420 μg/L) for short (5min) or long (7min) durations. SM exposure leads to maximum epidermal degradation on day 3 after exposure in both groups, and the inflammatory response results in corneal ulceration and opacity. SM also induces an increase in the number of stromal blood vessels and activates inflammatory indicators such as cyclooxygenase-2 (COX-2), Matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8). Corneal thickness, cloudiness, and epithelial degradation are affected by the duration of exposure[32].

In studies involving LEW, rabbits' eyes are exposed to LEW vapor at a concentration of 0.2 mg/L for 2.5 or 7.5min using a vapor exposure system. This allows for the quantification of blood vessels and inflammatory cells in the entire stromal region of the cornea, providing valuable endpoints to assess LEW-induced corneal injury at different exposure durations. These models contribute to the identification and screening of therapies for LEW-induced corneal injury[33].

Acrolein is a highly reactive unsaturated aldehyde that can be found in various substances, including smoke, automobile exhaust, and fungicides. Gupta *et al*[34] have conducted a study to optimize the effect of continuous exposure to acrolein on eye injuries in living rabbits. They investigated both local and vapor exposure modes and evaluated the resulting eye injuries. In the vapor exposure method, an 8-mm diameter filter paper disc impregnated with acrolein (30 μL) is placed in the center of a circular clear acrylic chamber. The acrylic chamber is then inverted and placed on the rabbit's eye for either 1 or 3min. On the other hand, in the local exposure method, an acrolein-impregnated filter paper disc (30 μL) is placed directly in the center of the cornea for either 1 or 5min. All procedures for both exposure methods are conducted in a chemical hood to ensure safety. The results of the study show that the vapor-exposed group exhibits more corneal fibrosis, while the locally exposed group has more corneal neovascularization. These findings indicate that different exposure methods can lead to distinct patterns of eye injury in response to acrolein exposure.

The establishment of a vesicle-induced corneal injury model can aid in detecting relevant molecular markers, developing treatment methods to minimize toxicity, promoting tissue repair processes, and optimizing the effectiveness of drug therapy.

It is crucial to strictly control the concentration and duration of substance application to accurately gauge the level of cautery. This is necessary to prevent complications such as corneal perforation or excessive diffusion of the substance towards the conjunctiva and eyelids, which can lead to conjunctival burns and eyelid deformities.

Infectious Keratitis After Laser Vision Correction

Postoperative infectious keratitis represents a challenging clinical entity that often leading to significant diagnostic and therapeutic distress. Infectious keratitis, which can be caused by bacteria, viruses, fungi, and echinococcosis, is the primary source of this condition[35].

Since the initial clinical use of femtosecond lasers for LASIK flap creation in 2001, these lasers have progressively become the preferred technology worldwide. The representative procedure, femtosecond laser small incision stromal lens extraction (SMILE), has demonstrated superior repeatability, safety, precision, and versatility compared to traditional stromal refractive surgery. Although the incidence of postoperative complications is low, certain issues such as dry eye, diffuse

laminar keratitis, pressure-induced stromal keratitis, interface fluid syndrome, interface debris, and infectious keratitis can arise due to its invasive nature[36]. Among these complications, postoperative infectious keratitis is one of the most severe, characterized by rapid progression and the potential to severely impair vision or even lead to blindness if left untreated.

Incidence and pathogen The incidence of keratitis following refractive surgery varies considerably across different data sources. Schallhorn *et al*[37] have conducted a review of suspected microbial keratitis cases after laser refractive surgery from 2008 to 2015, reporting an incidence of 0.013% for post-PRK and 0.0046% for post-LASIK microbial keratitis. Soleimani *et al*[38] have found an overall incidence of 0.02% for keratitis post-PRK. Liu *et al*[39] have retrospectively summarized the clinical treatments of patients who undergo SMILE in local hospital from 2019 to 2021, only 5 patients develops infectious corneal infiltration. Ivarsen *et al*[40] have retrospectively analyzed 1800 eyes treated with SMILE and found that five patients returned for treatment due to ocular irritation, experiencing slight interface infiltration once or more. Liu *et al*[41] have analyzed 306 cases of infectious keratitis occurring after refractive surgery. These cases include 68 cases of Staphylococcus aureus, 23 cases of Coagulase-negative Staphylococcus (CNS), 62 cases of Mycobacterium and 14 case of *Aspergillus*. Currently, only around 17 cases of infectious keratitis post-SMILE have been reported internationally[39-40,42-48]. Among these cases, ten are bacterial infections (including *Staphylococcus aureus*, *Streptococcus pneumoniae*, hemolytic Streptococcus, *Mycobacterium tuberculosis*, and bacterial keratitis without culture confirmation), two are unilateral fungal infections[45,48], and one is caused by unilateral herpes simplex virus[47]. Except for one case of non-tuberculous mycobacterial infection[44], all cases exhibit clinical signs of corneal infection within 7d after the surgery. Most patients receive antimicrobial agents for interface flushing, and seven patients undergo photoactivated chromophore for infectious keratitis-corneal collagen cross-linking (PACK-CXL) and drug therapy concurrently[43,46-47], including one patient who receives penetrating keratoplasty (PKP) treatment for corneal perforation due to aspergillosis[48]. *Staphylococci* and *Streptococci* are the most commonly detected microorganisms within the first 2wk after the operation, while atypical mycobacteria and fungi are more prevalent from 2wk to 3mo postoperatively.

Post-LASIK infectious keratitis *Staphylococcus aureus* is the primary culprit responsible for bacterial infections following LASIK surgery. To establish a rabbit model of methicillin-resistant *Staphylococcus aureus* (MRSA) keratitis post-LASIK, a corneal flap is created using a microknife, and a MRSA keratitis isolate [5 mL, 500 colony-forming units (CFU)] is inoculated beneath the flap. This model is employed to compare the effectiveness of moxifloxacin and gatifloxacin in preventing, treating, and managing MRSA keratitis[49].

In addition to *Staphylococcus aureus*, *Streptococcus species* are the second most common cause of non-opportunistic bacterial infections after LASIK. *Streptococcus pneumoniae* is responsible for approximately 35% of infectious keratitis cases. Donnenfeld *et al*[50] have conducted a study using 12 New Zealand white rabbits, which are randomly divided into three groups: topical 0.3% gatifloxacin, 0.3% ciprofloxacin, and control groups. In this study, 0.1 mL of *Streptococcus pneumoniae* is inoculated at the interface to examine the effects of the respective treatments.

Post-SMILE infectious keratitis Currently, the literature primarily consists of case reports on post-SMILE infections, and there is a lack of information regarding the establishment of animal models for post-SMILE infection[51]. SMILE surgery offers several advantages, such as a small incision, avoidance of corneal flap creation, minimal corneal damage, favorable biomechanical properties, and clinical safety. The one-step lenticular creation and extraction technique eliminates the need for previous excimer laser ablation, thus preventing exposure to the corneal stromal bed. However, the space left by the corneal cap can create a conducive environment for microbial growth. Additionally, antibiotics may have difficulty penetrating necrotic tissue and may be challenging to remove, making infection control difficult and potentially leading to severe visual impairment. Consequently, the establishment of a reproducible animal model for SMILE postoperative infection holds significant importance. Such a model would allow for the study of infection characteristics and pathological processes, providing a foundation for diagnosis and treatment. It is important to note that there are some drawbacks to establishing this model, including the need for expensive equipment, the relative complexity of the procedure, and the high skill requirements for the research staff involved.

Genetically Engineered Animal Models

Gene knockout mice Genetic modification is a technique that involves the integration of foreign genes into a specific location on the target cell genome through homologous recombination. This process allows for the modification of a gene on the chromosome. Knockout technology, developed in the late 1980s, relies on locus integration and *in vitro* culture of mouse embryonic stem cells (ESCs). The first knockout animal model was established in 1988, marking the beginning of knockout animal model development[52]. Currently, knockout mouse models are widely utilized in fundamental ophthalmology research to elucidate the role of specific genes in eye development and the maintenance of normal visual function.

Osteopontin (OPN) is a phosphorylated glycoprotein that is secreted and belongs to the small integrin-binding ligand N-linked glycoprotein family. It is widely distributed in human tissues and plays a role in inducing fibroblast proliferation, neovascularization, and regulation of scar formation. The loss of OPN impairs the proliferation, osteogenic differentiation, mineralization, and angiogenic potential of mesenchymal stem/stromal cells[53]. Dai *et al*[51] have demonstrated that OPN induces the expression of VEGF through the PI3K/AKT and extracellular regulated protein kinases (ERK) signaling pathways, thereby promoting angiogenesis. In ocular fibroblasts of both WT and OPN-null (KO) mice, the absence of OPN attenuates TGFβ1 signaling (Smad3 and p38) as well as VEGF expression. This leads to a reduction in injury-induced neovascularization in the corneal stroma of mice[54].

Miyazaki *et al*[52] have established corneal injury repair models using corneal epithelial debridement, full-thickness penetrating incision injury, and alkali burn in both OPN-KO and OPN-WT mice. In WT mice, the injured stroma is covered by granulation tissue by day 7 and becomes fibrotic after 21d. However, in the KO group, there is minimal extracellular matrix deposition for 14d, and the wounds remain unhealed until 21d later. KO mice show lower levels of myofibroblasts and reduced expression of TGFβ1. Furthermore, the higher incidence of ulceration and perforation in the alkali-burned eyes of KO mice can be attributed to impaired adhesion and migration of fibroblasts.

Tenascin is a family of oligomeric glycoproteins that exhibit a distinctive hexameric structure, comprising five isomers known as tenascin R, X, Y, and W. Tenascin-C, similar to OPN, serves as a ligand for A9 integrin and contributes to traumatic corneal fibrosis and neovascularization. In comparison to WT mice, Tenascin C-KO mice experience significantly prolonged repair time for corneal stromal incisions. Moreover, there is a notable decrease in the number of myofibroblasts, the infiltration capacity of macrophages, and the expression levels of collagen Ia1, fibronectin, and TGF-β1 in Tenascin C-KO mice. It is suggested that Tenascin C may regulate the expression of fibrotic genes in ocular tissues and the repair process of stage I corneal stroma[14].

Decorin is a proteoglycan that regulates keratinocyte-collagen matrix assembly and corneal wound healing. Gupta *et al*[53] have demonstrated that injured decorin null corneas had poorly organized collagen fibrils compared to WT mice using a standard injury technique.

V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), a member of the MYC proto-oncogene family, plays a role in various cellular processes, including cell proliferation, differentiation, and tumorigenesis. In neuroblastoma, MYCN amplification is associated with a poor prognosis. Furthermore, MYCN gene expression is dynamically observed in EpCAM cancer stem cells of hepatocellular carcinoma, serving as a biomarker for tumor stemness and plasticity[55]. Current research on MYCN in the eye primarily focuses on retinal and lens development.

To study the role of MYCN in the cornea, researchers have induced a chemical mutation in the MYCN gene using the mutagen ethylnitrosourea (ENU), resulting in mice with corneal opacity. MYCN-KO mice were generated by crossing MYCNtm1psk/J ($MYCN^{flox/+}$) mice with EllaCre transgenic mice expressing the Cre enzyme, following the principles of the Cre-loxP system. Approximately 68.4% of MYCN-KO mice exhibit corneal opacity, and some mice are born with open eyelids and microphthalmia. The findings demonstrate that the epithelial thickness of MYCN-KO mice is variably altered. This mechanism may be associated with increased expression levels of integrin β1, integrin β4, and CyclinD1 in the corneal epithelium of these mice.

S100A4 protein, a multifunctional calcium-binding protein, belongs to the S100 family. It plays a role not only in tumor formation and metastasis but also in the progression of inflammation and the promotion of organ fibrosis pathways[55]. In a study conducted by Wang *et al*[56], alkali burn models are established in rabbits by placing a 10-mm diameter Whatman II filter paper soaked in 1 mol/L NaOH on the eyes for 30s. To investigate the effects of S100A4 depletion, a transfection solution consisting of an equal amount of S100A4 siRNA expression plasmid and liposome Lipofectamine 2000 is injected into the empty vector group and the S100A4 siRNA group, respectively. Real-time quantitative assays show that the mRNA expressions of S100A4, VEGF, and tumor necrosis factor (TNF)-α are down-regulated in the S100A4 siRNA group compared to the empty vector group. This suggests that depletion of the S100A4 gene can inhibit corneal neovascularization induced by alkali burn. The inhibition of corneal wound healing by S100A4 is attributed to the activation of the PI3K/Akt/mTOR signaling pathway[57].

Integrins are a prominent family of cell surface glycoproteins that facilitate cell adhesion to the extracellular matrix. Integrin αvβ6 is primarily expressed in epithelial cells and is normally expressed at low levels in healthy tissues. However, its expression is up-regulated during wound healing and in cancer. Its main function is to activate TGF-β1 through thrombospondin-1 (TSP-1)[58]. Under pathological conditions involving TGFβ1-mediated repair, excessive collagen deposition and fibrosis can occur, impairing organ function and potentially leading to organ failure[59].

Studies using $\beta6^{-/-}$ mice have revealed slowed wound healing and reduced production of laminin. These mice also exhibit persistent stromal edema due to the absence of Descemet's membrane and corneal endothelium. Additionally, they

experience prolonged wound gaping due to impaired myofibroblast formation after total penetrating corneal injury (1.5 mm in length). Similarly, Thbs1$^{-/-}$ mice lacking TSP-1 show impaired corneal wound healing, characterized by delayed closure and persistent inflammation.

Wu *et al*[58] have conducted a study using C57BL/6-Itgb6 KO (β6$^{-/-}$) mice and created an incisional wound to investigate the role of αvβ6 integrin in wound healing. Their findings suggest that αvβ6 integrin may play a role in initiating rapid fibrosis during the early stages of wound healing, while TSP-1 acts as an amplification factor in this process.

Although knockout mice have been valuable tools in studying corneal injury, there are certain limitations associated with their use. First, the depletion of essential genes can lead to animal death, limiting the ability to study their specific functions. Second, the relationship between specific genes and complex phenotypes is not always one-to-one, necessitating the reintroduction of foreign genes or proteins to clarify causality. Third, the loss of knockout genes can trigger compensatory processes, leading to secondary changes in gene expression. At last, gene ablation does not fully mimic the disease processes caused by specific base mutations. These factors should be taken into consideration when interpreting the results obtained from knockout mouse models.

Transgenic mice Transgenic technology is a powerful tool that allows for the introduction of artificially isolated or modified genes into an organism's genome, resulting in predictable and directed genetic changes. Various techniques are employed in generating transgenic animals, including nuclear microinjection, sperm-mediated gene transfer, nuclear transfer, and retroviral methods. Unlike gene knockout approaches, transgenic technology enables site-directed mutagenesis of the target gene, connection with tissue-specific expression promoters, and simultaneous observation of gene expression function, regulation, and phenotypic effects from both temporal and spatial perspectives.

In the study by Khandaker *et al*[60], the researchers have constructed and modified the mouse genotype by introducing multiple copies of a modified bacterial artificial chromosome (BAC), directly inserting the enhanced green fluorescent protein (EGFP) reporter gene upstream of the COL3A1 coding sequence, and incorporating the DNA into the mouse genome through nuclear microinjection. Through screening and breeding, they have successfully established a transgenic mouse line called Tg (Col3a1EGFP) DJ124Gsat to simulate corneal fibrosis and scar formation. The transgenic mice exhibit similar stromal responses to corneal mechanical injury and alkali burn as WT mice. Over time after corneal injury, the expressions of both EGFP and Col3a1 are increased, consistent with the expressions of other related genes such as α-SMA, fibronectin, and Tenascin C. The number and fluorescence intensity of EGFP-expressing cells corresponds to changes in corneal thickening and scar volume. This transgenic model allows for *in vivo* real-time detection of corneal fibers and scarring visibility, providing insights into the dose-phenotype association and identification of potential therapeutic targets before human trials.

To maintain the genetic integrity of transgenic mouse lines, it is important to produce them in a pure genetic background or repeatedly backcross them to standard inbred lines to avoid the creation of non-homozygous lines. Additionally, to prevent gene drift in transgenic mice and ensure strict control of the genetic background, it is necessary to regularly crossbreed mutant mice with controls and heterozygotes. However, this process requires significant human and resource investments.

Currently, genetically modified animal models predominantly find utility in studying single-gene genetic diseases and tumors characterized by distinct genetic patterns. Mechanical, chemical, and other environmental factors largely contribute to corneal epithelial-stromal injury. However, the presence of non-healing, delayed healing, or scar formation subsequent to corneal injury, as well as stromal neovascularization, may be linked to pathogenic genes. The vast array and intricacy of corneal healing genes necessitate time-consuming gene screening. Additionally, there is often an interplay between environmental and genetic factors in corneal stromal defects, prompting further investigation into the potential influence of environmental factors on the regulation of transcription and expression of associated genes.

CONCLUSIONS & FUTURE PERSPECTIVES

Corneal epithelial-stromal injury is a prevalent condition characterized by extracellular matrix remodeling, abnormal protein accumulation, and localized scarring during the healing process. These scars can impair vision, reduce corneal clarity, and in severe cases, necessitate corneal transplantation. To investigate corneal stromal regeneration, the development of repair materials, and donor keratoplasty, researchers have established various animal models simulating mechanical, chemical, and refractive surgery-induced corneal injuries. However, an ideal animal model that fully replicates the occurrence and progression of corneal epithelial-stromal injury is currently lacking.

In this review, we provide an overview of commonly employed corneal epithelial-stromal injury models, focusing on four perspectives: mechanical injury, chemical burns, infection following keratoconus surgery, and genetically engineered animal models. Animal models serve as valuable tools for studying the pathogenesis of human diseases. While each model has its advantages and limitations, researchers should select the appropriate modeling method based on their specific

research goals. Moreover, they should possess a thorough understanding of animal model creation techniques that ensure high success rates, as well as similarities and consistency between the modeled disease and human corneal injury. These measures are crucial to ensure the objectivity, reliability, and reproducibility of experimental results.

In clinical practice, continuous improvement of existing models, development of new models, and conducting animal experiments are essential. These efforts aim to investigate wound healing mechanisms and the prevention of complications, such as fibrosis and infection. By timely controlling disease progression and reducing the rate of blindness, the medical community can effectively address the challenges posed by corneal epithelial-stromal injuries.

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