• Review Article •

Corneal stromal mesenchymal stem cells: reconstructing a bioactive cornea and repairing the corneal limbus and stromal microenvironment

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Abstract

● Corneal stroma-derived mesenchymal stem cells (CS-MSCs) are mainly distributed in the anterior part of the corneal stroma near the corneal limbal stem cells (LSCs). CS-MSCs are stem cells with self-renewal and multidirectional differentiation potential. A large amount of data confirmed that CS-MSCs can be induced to differentiate into functional keratocytes *in vitro*, which is the motive force for maintaining corneal transparency and producing a normal corneal stroma. CS-MSCs are also an important component of the limbal microenvironment. Furthermore, they are of great significance in the reconstruction of ocular surface tissue and tissue engineering for active biocornea construction. In this paper, the localization and biological characteristics of CS-MSCs, the use of CS-MSCs to reconstruct a tissue-engineered active biocornea, and the repair of the limbal and matrix microenvironment by CS-MSCs are reviewed, and their application prospects are discussed.

● **KEYWORDS:** corneal stroma-derived mesenchymal stem cells; bioactive cornea; corneal limbus; tissue-engineered active biocornea; stromal microenvironment

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INTRODUCTION

Trauma, infection, inflammation, immunity, and other factors can cause corneal damage, and severe cases can affect vision and appearance. Statistics show that corneal disease is the second most common blinding eye disease after cataracts. There are approximately 60 million corneal blindness patients in the world, and there are approximately 4 million in China. Infection is still the main cause of corneal blindness in China[1]. Trauma and infective keratopathy may lead to corneal perforation. According to a survey by the Shandong Institute of Ophthalmology, ocular trauma is the most common cause of corneal perforation, accounting for 67% of cases[2]. Corneal perforation will destroy the integrity of the eyeball and change the optical structure of the cornea and the microenvironment. If treatment is not timely, corneal perforation may lead to serious complications such as secondary glaucoma, complicated cataracts, and endophthalmitis, eventually causing visual impairment. In severe cases, eyeball removal must be performed[3]. The scar repair mechanism and the nonrenewable mechanism of the corneal endothelium after corneal perforation seriously affect the treatment and recovery of the cornea. The structure of corneal tissue cannot be reconstructed with drugs alone, and surgery must be performed to rebuild the severely damaged tissue structure during corneal perforation. For patients with a small corneal perforation, although eyeball integrity can be maintained by conservative treatments such as corneal suture, fibrin glue closure, eyelid margin suture, conjunctival flap coverage, and amniotic membrane transplantation, these treatments may also be accompanied by various complications[3-6]. For corneal perforations greater than 3 mm, keratoplasty is required. Unlike other human organs, due to the absence of blood vessels in the corneal tissue and the presence of the blood-eye barrier mechanism, the corneal

tissue is relatively deficient in antigen-presenting cells, and the immune response to foreign and autoantigens is weak; thus, the cornea is an immune-privileged tissue[7-8]. Corneal transplantation is the only reliable and effective means of recovery for patients with corneal disease.

According to statistics, the one-year failure rate of corneal transplantation is 9.4%, and the 5-year failure rate is 23.5%[9]. The cornea has one of the highest success rates for body tissue transplantation. In addition, although the cornea is an immune-privileged tissue, approximately half of all patients after allogeneic keratoplasty still require immunosuppressive therapy[10-11]. Even with lamellar keratoplasty, 3%-24% of patients will develop immunological rejection after surgery[12]. Due to the lack of corneal donors, only approximately 5000 corneal transplants can be performed each year in China. Therefore, how to obtain corneal graft materials has become an urgent problem to be solved. For these reasons, there is increasing interest in alternative treatments for corneal transplantation, including corneal transplantation stem cell therapy, acellular collagen scaffolds, tissue-engineered artificial corneas, and corneal prostheses[13-15]. Tissue-engineered artificial corneas have made great progress in recent decades[16]. Seed cells (including corneal epithelial cells, keratocytes and corneal endothelial cells) are cultured *in vitro* on a scaffold material with good optical transparency, biocompatibility and mechanical stability. Through an *in vitro* three-dimensional culture system, a construct that is structurally and functionally close to the normal cornea is ultimately obtained. The ideal tissue-engineered artificial corneal scaffold material should have structural and physiological characteristics similar to those of the natural cornea. To find the ideal tissue-engineered artificial corneal scaffold material, scholars at home and abroad have explored the feasibility of using synthetic materials and natural biomaterials, from classic collagen gels, films and sponges to other components such as silk, fish scales, gelatin and polymers[16-17]. Subsequent studies have found that although various synthetic materials have good biocompatibility and can maintain the intact structure of the eyeball after transplantation, they restore the transparency of the cornea only partially due to the absence of a natural corneal structure and microenvironment. There are still many shortcomings, such as low transparency, rapid degradation after transplantation, poor biomechanical strength, and inability to withstand suture cutting, which limits clinical application[18-20]. Although the study of artificial biological corneas is a global hotspot in ophthalmology research, the endothelial cell culture system is still immature and has become a bottleneck that seriously restricts the construction of full-thickness artificial corneas. In China, because infection is the main cause of blindness, lamellar keratoplasty is the main method to treat the disease. Therefore, active lamellar artificial corneal research has great clinical significance. The heterogeneous corneal stroma has optical properties, toughness and a tissue structure close to those of the human cornea. It has a wide range of sources and has become a research hotspot in the preparation of matrix scaffolds in recent years[21-25]. Among the investigated sources, acellular porcine corneal stroma has good light transmission, mechanical properties and biocompatibility. The currently developed acellular porcine corneal stroma has been initially applied to clinical lamellar keratoplasty[26-27]. However, due to the lack of human biological activity in the acellular porcine corneal stroma, its indications are limited. We believe that the corneal graft and recipient bed of the recipient cornea may lack a healthy limbus and matrix microenvironment, which may result in the inability of the donor cornea to be effectively integrated, affecting the repair of damaged corneal tissue and the recovery of transparency. In addition, the acellular porcine corneal stroma is a xenograft, and the extracellular matrix retained after decellularization may be antigenic, leading to immunological rejection, and carries the risk of transmitting potential animal-related diseases. Therefore, the safety of this approach still requires long-term follow-up studies[28-31]. In addition, religious beliefs and other factors limit the clinical application of the acellular porcine corneal stroma[32-33]. However, for other corneal diseases, such as keratoconus and large-scale perforation of the cornea, penetrating keratoplasty is still the most widely used surgical procedure, and the residual cells in the donor cornea can be removed by a suitable decellularization method, which is closely related to immune rejection[34-37]. In recent years, some scholars have attempted to construct artificial corneas. These materials are obtained through decellularized donor corneas that come from residues of the corneal tissue after lamellar keratoplasty and from poor-quality corneal tissues obtained at eye banks[38]. Human corneal tissue is an allograft, and there are no concerns about using a heterogeneous acellular corneal stroma. Moreover, after decellularization, the risk of postoperative immunological rejection is further reduced. However, due to the influence of traditional beliefs, the corneal donation rate in China is relatively low. Currently, corneal donors in China are seriously deficient, and the development of eye banks is lagging. The use of donor corneal tissue to construct artificial biokernels has a long way to go.

Mesenchymal stem cells (MSCs) are adult stem cells with strong self-renewal and multidirectional differentiation potential. They are relatively abundant, easy to isolate *in vitro* and low in immunogenicity. They can regulate the immune system and exert an anti-apoptotic effect. MSCs can also provide cytokines to promote extracellular matrix deposition, support the reconstruction of the cell microenvironment and

play a significant role in corneal repair and reconstruction of tissue-engineered active biocornea[39-40]. According to the classification of tissue sources, MSCs can be divided into bone marrow, fat, heart, umbilical cord, alveolar, amniotic membrane, fallopian tube, menstruation and limbal matrix MSCs[41]. This review focuses on the localization of corneal stromal-derived mesenchymal stem cells (CS-MSCs) and their biological properties, tissue-engineered active biocornea, repair of the limbus and matrix microenvironment, *etc*. We also explored the application prospects of CS-MSCs.

LOCALIZATION OF STROMAL-DERIVED MESENCHYMAL STEM CELLS

The cornea is generally divided into five layers: the corneal epithelial layer, Bowman's membrane, the corneal stroma layer, Descemet's membrane and the corneal endothelium, among which the stromal layer accounts for more than 90% of the cornea, and the corneal stroma is composed of highly organized collagen fibers. The transparent layer is interspersed with keratinocytes and a small fraction of stationary MSCs, which, together with the corneal epithelium and corneal endothelial cells, maintain the transparency and stability of the matrix. CS-MSCs originate from the embryonic neural crest. They can be cloned *in vitro*, have multidirectional differentiation potential, carry stem cell markers and are located in the anterior part of the limbal matrix near the limbal stem cell (LSCs). CS-MSCs are a component of the limbal microenvironment and play an important role in maintaining the proliferation potential of LSCs[42-43].

BIOLOGICAL CHARACTERISTICS OF STROMAL-DERIVED MESENCHYMAL STEM CELLS

Through studies on the culture method and nutritional conditions of *in vitro* cell culture, it has been shown that the corneal stroma contains functional MSCs that meet the standards of the International Society for Cell Therapy[44]. Isolation and culture of CS-MSCs *in vitro* is of great significance for the treatment of corneal diseases, such as tissue-engineered active biocornea construction, transplantation and stem cell injection[45-47]. The Pinnamaneni group[14] and the Takács group[48] found that human keratocytes contain cells expressing the stem cell markers PAX6 and ABCG2.

CS-MSCs express MSC markers, such as CD73, CD90, CD105 and CD140b/PDGFRβ, but not CD34, CD45, CD133 and HLA-DR[49]. The genes expressed by CS-MSCs also include MSC genes such as ABCG2, BMi1, CD166, cKIT, and Notch1, as well as genes for early corneal development, PAX6 and Six2. When CS-MSCs differentiate, they express high levels of keratocan, ALDH3A1, CXADR, PTDGS, and PDK4[14]. PAX6 is the master controller for early development of the eye. The expression of PAX6 in the corneal stroma can be used to recognize CS-MSCs, while keratocytes do not contain the PAX6 gene[50-51]. In addition, CS-MSCs do not express CD33 and CD133, which are positive markers of keratocytes[52-53]. Under certain culture conditions, human CS-MSCs can differentiate into keratocytes and produce a large amount of cornea-specific extracellular matrix (ECM)[15,54].

In 2009, Du *et al*[55] used flow cytometry to obtain cells in the human corneal stroma and injected the cells into the corneal stroma of lumican-deficient mice. After 12wk, the corneal stroma thickness and collagen fiber arrangement of the mice were similar to those of the normal cornea, and the transparency of the cornea was also significantly improved. In 2010, Watson *et al*[56] cultured human corneal stromal fibroblasts with MSC-conditioned medium. This culture method enhanced the damage repair ability of stromal fibroblasts. In 2018, Samaeekia *et al*[45] used isolated CS-MSCs to prepare and obtain exosomes *in vitro* to perform scratch tests and mouse corneal injury repair experiments. The results showed that human CS-MSC exosomes can promote corneal epithelial wound healing and provide a treatment for ocular surface damage.

CORNEAL STROMA AND THE LIMBAL STEM CELL MICROENVIRONMENT

The homeostasis of the ocular surface is subtle and complex. We believe that the corneal tissue, extracellular matrix and cytokine network of the donor and recipient after corneal transplantation interact to form the posttransplant corneal microenvironment. The most important ones are the limbal epithelial stem cell microenvironment and the donor matrix microenvironment.

LSCs are the source of corneal epithelial proliferation and renewal[57], and they are essential for the preservation of corneal transparency. The absence of LSCs can cause corneal inflammation, opacity, and vascularization[58].

The LSC microenvironment is located in a special anatomical structure called the "Vogt Fence", which contains a large number of pigment cells[59-60], and the basement membrane here is wavy[61-62]. This particular feature suggests a close interaction among the limbal epithelial cells, the basement membrane and the stromal cells beneath it. Melanocytes are distributed at the base of the limbus, surrounding the basal epithelial cells and reducing the stimulation of ultraviolet stem cells, and are therefore considered to be a microenvironmental cell[42]. This limbal cell microenvironment can provide stem cells with a protective environment that reduces external stimuli.

CS-MSCs in the corneal stroma[63], mainly present in the anterior part of the corneal stroma near the LSCs, can be induced to differentiate into keratocytes *in vitro*, producing collagens of type I, V, and VI; chondroitin sulfate; glucosamine; and ALDH. CS-MSCs are the main cells of the matrix microenvironment. *In vitro* laser confocal microscopy

revealed that the limbal stromal cells were very close to the basal epithelial cells, which occurred mainly in the corneal frontal subcutaneous matrix[64]. Research shows that the results achieved by culturing the limbal epithelial slices with MSCs are better than those obtained by culturing the limbal epithelial slices alone under the same culture conditions, indicating that the close physiological interaction between LSCs and adjacent CS-MSCs can promote the cloning of LSCs[41]. LSC and CS-MSC suspensions are cultured in 3 D gels, where they reaggregate into clusters that grow as globular clones. This repolymerization is mediated by the SDF-1/CXCR4 signaling pathway[65]. Based on research data, we believe that CS-MSCs are the most important component of the corneal stroma and LSC microenvironment.

FEASIBILITY OF THE SEPARATION AND CULTURE OF CS-MSCs FOR REPAIRING CORNEA

The corneal stroma is the most structured and transparent human tissue. It consists of a highly regularly arranged collagen layer and ECM components secreted by human keratocytes. The main components are collagens such as types I, V, and VI[66]. Keratocytes are a group of CS-MSC-derived dormant cells distributed between the collagen layers of the mature collagen matrix. Human corneal keratocytes (HCKs) are very slow to renew and occupy only 10% of the matrix. Although they are at rest, they play an important role in maintaining the transparency and integrity of the cornea. Upon trauma, a proportion of HCKs immediately undergo apoptosis, while other HCKs are converted to human corneal fibroblasts (HCFs) and myofibroblasts. Myofibroblasts express α-smooth muscle actin (α-SMA) and are highly contractile cells that give the wound a strong contractile force when the cornea is damaged[67]. In most cases, any damage or interruption in the precise arrangement of the healing process can cause corneal scarring, leading to activation of HCKs. These activated HCKs are often referred to as “activated corneal cells” or HCFs, and they secrete a messy ECM that disrupts the cellular microenvironment of the corneal stroma and ultimately affects the transparency and integrity of the corneal stroma.

Therefore, it may be better to use more keratocytes when constructing tissue-engineered active biocornea. There are three subtypes of transforming growth factor in the human body: TGF-β1, -β2, and -β3 (T1, T2, and T3, respectively). Although they share an amino acid sequence identity of 70%-80% and the same receptors, they function differently. These three subtypes play an important role in key biological processes that occur after cell proliferation, migration, and differentiation. During corneal repair, TGF-β can promote the secretion of more ECM. At the same time, TGF-β can reduce the decomposition of collagen and accelerate the deposition of ECM by reducing the synthesis of proteases. TGF-β can also promote the synthesis of cell adhesion receptors. Although these effects accelerate wound healing, they also promote the formation of scars, leading to a decrease in the transparency of the corneal tissue[68]. T1 and T2 are known to play a major role in reducing the synthesis of proteases and promoting fibrosis, while T3 has anti-fibrotic activity. Karamichos *et al*[69] showed that T3 can stimulate HCFs to secrete a large amount of ECM while maintaining nonfibrotic characteristics. When HCKs are stimulated by T3, they maintain important corneal cell markers, corneal proteins and their morphology. In addition, HCKs secrete a large amount of neatly arranged ECM, maintaining the expression of fibrotic markers at a minimal level, which can lead to new alternatives and cell therapy research. This is critical for constructing tissue-engineered active biocornea.

Wu *et al*[70] obtained limbal stromal tissue and used collagenase digestion. They successfully cultured CS-MSCs by adding cholera toxin-derived MSC culture medium. At the same time, corneal matrix fibroblasts (HCFs) were obtained by collagenase digestion using 10% fetal bovine serum differentiation medium (DMEM)/F12 medium. They cultured two successful cells in DMEM supplemented with cytokines and changed the solution twice a week for 9wk. The results showed that CS-MSCs can differentiate into keratocytes with normal corneal stroma components (collagen I, V, and VI; sulfate proteoglycan; orthogonal collagen lamellar structure; and a-SMAD protein negative), and the human cornea matrix is successfully obtained *in vitro*. Control cells differentiate into myofibroblasts (expressing a-SMAD protein; no type 1, 5, 6 collagen and proteoglycan)[69-70]. This study also lays a foundation for cell repair therapy of CS-MSCs cultured *in vitro.*

CS-MSCs were isolated and cultured *in vitro* and plated on acellular corneal stroma from ostrich. Vitamin C, T3, and other cytokines were added to induce the culture *in vitro*. At 8wk of culture, we observed the samples under transmission electron microscopy. The results showed that a well-arranged human corneal stroma collagen was formed on the surface of the xenogeneic acellular corneal stroma, which further proved the feasibility of constructing tissue-engineered active biocornea by human CS-MSCs (results not yet published).

PROSPECTS OF CS-MSCs IN THE TREATMENT OF CORNEAL DISEASES

Corneal Chemical Burns In the treatment of corneal chemical burns, how to inhibit the inflammatory response, protect LSCs, promote wound healing and reduce scarring and neovascularization has always been the core of corneal wound repair research. Corneal scar formation is due to the proliferation of activated keratocytes. The above studies showed that CS-MSCs significantly inhibited the proliferation and migration of fibroblasts and induced apoptosis *in vitro*, suggesting that CS-MSCs have the potential to inhibit corneal

scar formation. Zhang *et al*[71] inoculated rat LSCs and CS-MSCs isolated and cultured *in vitro* on decellularized dog corneal stroma. The results showed that MSCs secreted cytokines and keratocan that are beneficial to corneal tissue repair in the the acellular corneal matrix (ACM) environment. The ability of MSCs to repair corneal damage is superior to that of LSCs[71]. This experiment laid the foundation for CS-MSC reconstruction of heterogeneous decellularized corneal stroma for corneal transplantation for corneal chemical burns and other corneal diseases.

Various Types of Corneal Neovascular Disease Corneal neovascularization is usually the end result of severe corneal infection or inflammation. In most pathological conditions leading to corneal neovascularization, inflammatory cells, including neutrophils and macrophages, infiltrate the cornea, especially macrophages, and play a central role in the process of inflammatory corneal neovascularization by releasing proangiogenic and proinflammatory cytokines[72-74]. The depletion of macrophages largely hinders the development of corneal angiogenesis and lymphangiogenesis[75]. Studies have shown that CS-MSCs can induce macrophage apoptosis by secreting factors such as PEDF, thereby inhibiting the formation of new blood vessels[75]. The use of CS-MSCs to reconstruct biological corneal transplantation or local subconjunctival injection and other cell therapies has great application prospects.

Limbal Stem Cell Defects Stabilization of corneal epithelial cells is essential for maintaining ocular surface transparency, and corneal epithelial cells are derived from LSCs. The waterproof effect of corneal epithelial cells, the active drainage of corneal endothelial cells and the passive hydrophobic action of the stromal layer maintain a dynamic balance. When corneal epithelial cells are missing, the corneal water-repellent capacity is insufficient, and the drainage capacity of the corneal endothelial cells is limited, so corneal edema occurs[76]. Ocular surface damage caused by trauma, inflammation, *etc*. can lead to the loss of LSCs, which can cause corneal opacity and damage vision. Complete loss of LSCs leads to epithelialization of the keratoconjunctiva, neovascularization, persistent chronic inflammation, repeated epithelial defects and scarring of the corneal stroma, which ultimately leads to severe loss of vision and even blindness. Mild ocular surface damage is often treated with autologous or allogeneic LSC transplantation, which can achieve certain therapeutic effects. However, when large-area ocular surface damage occurs in both eyes, due to the lack of healthy LSCs and corneal epithelial cells and rejection caused by allogeneic transplantation, this approach is greatly limited. The foregoing studies have shown that corneal stromal MSCs have biological properties similar to those of tissue-derived MSCs from tissues such as bone marrow and fat. It was found that[65,77-78] MSCs can differentiate into corneal epithelial precursor cells, characterized by positive immunocytochemical staining or increased gene expression levels of the stem cell markers ABCG-2, P63, HesL and β1 integrin. These results indicate that MSCs have the potential for stem cell differentiation and can regulate the differentiation of MSCs into corneal epithelial cells. They can be used as a seed cell for constructing tissue-engineered active biocornea and reconstructing the limbus and matrix microenvironment after transplantation.

Congenital Corneal Diseases Human basement glycans and keratins are small leucine-rich proteoglycans that are the major sulfated proteoglycans in the corneal stroma and are essential for maintaining corneal transparency. Due to the disorder of corneal matrix collagen arrangement and the reduction of corneal proteoglycans, humanized glycosaminoglycan-deficient mice exhibit corneal thinning and cloudiness, similar to human congenital corneal diseases. Liu *et al*[79-80] found that injection of MSCs into the corneal stroma of an animal model can significantly increase corneal thickness and corneal transparency. The corneal matrix collagen fiber layer is rearranged and becomes more orderly. MSCs can survive in the corneal stroma for more than 3mo without any rejection. Transplanted MSCs exhibit the characteristics of corneal cells, appear dendritic, in a resting state, and express unique markers of corneal cells. In addition, MSC transplantation also improved the function of the recipient corneal cells.

CONCLUSION AND PROSPECTIVE OF FUTURE RESEARCH

Based on the many functions and characteristics of MSCs, CS-MSCs are expected to be the earliest cells to directly apply or reconstruct humanized active biological corneas in ophthalmology. Human corneal stroma MSCs can directly construct tissue engineering active biocornea for corneal transplantation *in vitro*. This approach great application prospects in the treatment of corneal ocular surface disease and other related eye diseases through tissue repair and promotion of corneal wound healing.

We believe that human CS-MSCs can be used to construct the microenvironment between the transplanted cornea donor and the corneal receptor. This microenvironment consists of human CS-MSCs, ECM, and cytokines, which can promote the repair of damaged corneal tissue and the restoration of corneal transparency, and such manipulations of the microenvironment through adding CS-MSCs are very helpful for corneal transplantation. However, the mechanism of interaction between CS-MSCs and the corneal limbus and matrix microenvironment and how to use CS-MSCs to better reconstruct the active cornea and repair the corneal limbus microenvironment still need further exploration.

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