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Current microfluidic platforms for reverse engineering of cornea

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ABSTRACT

According to the World Health Organization, corneal blindness constitutes 5.1% of global blindness population. Surgical outcomes have been improved significantly in the treatment of corneal blindness. However, corneal transplantation is limited by global shortage of donor tissue, prompting researchers to explore alternative therapies such as novel ocular pharmaceutics to delay corneal disease progression. Animal models are commonly adopted for investigating pharmacokinetics of ocular drugs. However, this approach is limited by physiological differences in the eye between animals and human, ethical issues and poor bench-to-bedside translatability. Cornea-on-a-chip (CoC) microfluidic platforms have gained great attention as one of the advanced *in vitro* strategies for constructing physiologically representative corneal models. With significant improvements in tissue engineering technology, CoC integrates corneal cells with microfluidics to recapitulate human corneal microenvironment for the study of corneal pathophysiological changes and evaluation of ocular drugs. Such model, in complement to animal studies, can potentially accelerate translational research, in particular the pre-clinical screening of ophthalmic medication, driving clinical treatment advancement for corneal diseases. This review provides an overview of engineered CoC platforms with respect to their merits, applications, and technical challenges. Emerging directions in CoC technology are also proposed for further investigations, to accentuate preclinical obstacles in corneal research.

1. Introduction

The cornea is a highly transparent structure located at the outermost fibrous layer of the eyeball, allowing precise light penetration into the intra-ocular space (Fig. 1A). The cornea is directly exposed to air, therefore becomes susceptible to bacterial and viral infections, which can impair vision significantly [1]. Cornea-related disease is now the second leading cause of blindness [2], and around 12.7 million patients require the corneal transplantation [3]. However, only 1/70 of these patients receive transplantable corneas [3]. The extreme scarcity of corneal donor grafts is a severe global concern [2,4]. Therefore, there is a pressing need to develop corneal models of human physiological resemblance to aid investigations of underlying pathogenesis and treatments of corneal blindness.

For decades, traditional *in vitro* two-dimensional (2D) cell culture, advanced three-dimensional (3D) culture systems, and *in vivo* animal models have been extensively employed in corneal research. However, conventional 2D *in vitro* culture systems are not capable of replicating the complex architecture and microenvironment of the *in vivo* ocular surface. For example, compared with the 3D *in vitro* culture model, human trabecular meshwork cells in the traditional 2D model show a lower sensitivity to glaucoma-related oxidative stress [5]. Although 3D hydrogel matrix can address the needs from basic cell culture, the physiological conditions *in vivo* cannot be controllably reproduced. Animal models are still widely used, while they cannot fully mimic the physiological conditions of the human eye owing to species differences. For instance, the Bowman's layer, which might facilitate stromal wound healing and recovery of epithelial innervation after injury [6], only exists in human corneas rather than in commonly used animals such as murine and piscine corneas [7], hindering the development of animal research on corneal wound healing. Moreover, the increasing drug permeation through the ocular surface in rabbits can be caused by the reduced blinking rate, and may consequently misguide researchers in follow-up translational studies and thus clinical practice [8]. In contrast, *in vitro* corneal models have been adopted to make up for these limitations by means of building Bowman's layer and simulating blinking with controllable frequency [9,10].

The organ-on-chip (OoC) technology - a strategic combination of

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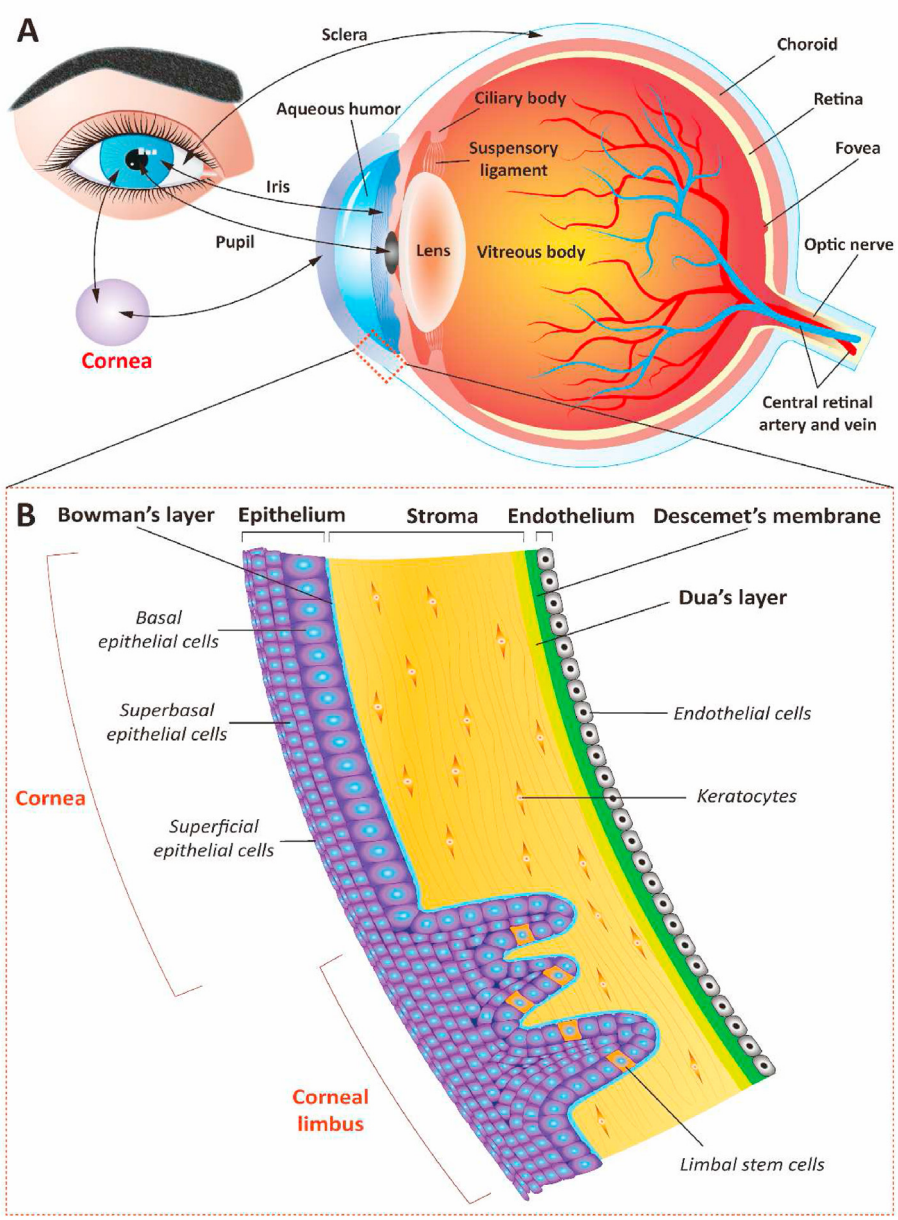

Fig. 1. (A) Schematic anatomy of the human eyeball structure, with the layered structure of the cornea highlighted. (B) Detailed illustration of the five tissue layers within the cornea.

tissue engineering and microfluidics, is attracting growing attention in biomedical research [11]. Numerous OoC platforms aim to actualize the *in vitro* remodeling of human organs in a time-saving and low-cost manner. Tissue reconstruction is generally achieved by growing cells into microfluidic devices with an *in vivo*-like biological environment. Not only single-organ systems such as the lymph and microvascular network, but also multi-organ platforms can be created to capture the biological nature of organ interactions in human body [12–14]. To predict drug permeability, the biological barriers built *in vitro* using various types of cells (e.g. human brain microvascular endothelial cells, alveolar cells, intestinal epithelial cells, etc.) have been widely discussed [15,16]. In ophthalmology, the engineered platforms that are clinically relevant to blood-retinal barrier (BRB) and age-related macular degeneration (AMD) have been put forward to improve the understanding of retinal biology [17–19]. Microfluidics also provides an effective approach for *in vitro* simulation of non-ideal silicone oil emulsification in the eye cavity [20, 21]. The recent reviews regarding eye-on-a-chip provide cutting-edge schemes for disease modeling and indicate its great potential in pharmacological studies on drug efficacy and safety [22–24]. However, few cornea-on-a-chips (CoC) using miniature corneal cells/tissues and microfluidic channels have been developed so far. To better investigate the corneal pathophysiology and underlying drug action mechanisms, CoC models are becoming increasingly important for basic science research and preclinical trials.

In this review, recent progresses in CoC aiming at simulating physiology of the human cornea are highlighted. The objective of this review is to narrow the research gap between cell/tissue basic research and clinical research on corneal diseases through putting forward current strengths, drawbacks, and the potential technological trend on CoC development in future. The physiological structure of the cornea and the role of the individual tissue layers are firstly introduced, followed by illustration of the CoC studies in order of corneal epithelial barrier, stroma, endothelium, and the representative blinking model with eyelid movement. The advantages and limitations of existing CoC platforms are listed in the

discussion section. Future improvements of CoC functions might be fulfilled with advanced 3D printing, organoids culture and specific extracellular matrix (ECM). The integration of corneal nervous system or microbiology with progressive platforms will deepen the understanding of corneal nerve regeneration and microbial infections.

2. Anatomical structures of the human cornea

The avascular and transparent human cornea is the most essential biological barrier that protects the inner tissues of the eye against injuries and infections [25]. Together with the outermost tear film, the cornea maintains the vision not only by its excellent transparency, but also its contribution to 70% of the total optic refractive power [25,26]. Oxygen molecules from air dissolve in tears followed by transportation through the corneal barrier to sustain aerobic metabolism and inhibit hypoxic swelling [27]. Furthermore, the human cornea contains large quantities of sensitive unmyelinated nerve endings, which give rise to conditioned response of eye closure or lacrimation upon direct physical collision or chemical stimulation to the cornea. The diameter of cornea is about 11 mm while its average central thickness is approximately 0.52 mm, with horizontal corneal diameter around 1 mm longer than the vertical diameter [26,28]. The human cornea consists of 3 cellular tissue layers (epithelium, corneal stroma, endothelium) and 3 acellular interfaces (Bowman's layer, Dua's layer, Descemet's membrane). Moreover, the tear film from the lacrimal system plays a critical role in protecting cornea from toxins and dehydration, thus acting as the first barrier at the anterior ocular surface [29]. A detailed schematic diagram of the tissue structures inside the human cornea is shown in Fig. 1B.

The corneal epithelium, an ultra-thin 50 μm stratified cellular layer with consistent tear lubrication, maintains direct contact with the external environment [30]. Non-keratinized squamous epithelial cells constitute the primary component of epithelium, which exhibit fast-growing and robust self-regeneration capabilities. This is mainly contributed by the limbal stem cells at the corneal periphery that can differentiate into corneal epithelial cells (CEpCs) to support corneal re-epithelialization [31,32]. In addition, the corneal permeability of various drugs is dependent on physical and biochemical characteristics of the cornea. For example, the epithelial barrier effectively restricts permeability of bio-macromolecules and hydrophilic drugs, whereas lipophilic substances are primarily limited by the stromal barrier [33,34].

Bowman's layer consists mainly of laminin and collagen fibrils with typical thickness ranging from 10 to 20 μm. It adheres to the superficial epithelium and protects the underlying corneal stroma from physical injuries [35]. This acellular layer is relatively stiff without self-regenerative capability and exists in nearly all primate species [36].

Corneal stroma occupies around 90% of corneal thickness, composing of nearly 200 parallel lamellae of collagen type I and type V arranged in a uniform pattern with superior optical transparency [37,38]. Keratocytes - the specialized stromal cells - are crucial to both the lamellar structural integrity and corneal wound healing capability after injuries [39]. Generally, typical dendritic morphology can be observed among these interconnected cells in healthy populations [40]. Nevertheless, corneal dysfunction such as keratoconus may appear under the unexpected exacerbation in keratocytic apoptosis [41]. During later stages of clinical treatment, localized opaque corneal scars could, undesirably, remain, leading to further loss of vision.

The Dua's layer (pre-Descemet's layer) with thickness of 10–20 μm posterior to cornea stroma was recently identified, which is primarily made of compactly arranged collagen type I and abundant collagen type VI [42–44]. This layer is almost acellular with high tensile strength, and its extension becomes the collagen matrix in the trabecular meshwork [45]. In keratoconus patients, corneal hydrops might result from a tear in Duas layer, causing acute pain and vision loss [42]. The finding of this layer has contributed to the surgical innovation such as pre-Descemet's endothelial keratoplasty and suture operation in hydrops [44].

The Descemet's membrane - basement membrane for the inner endothelium - is around 10–12 μm in thickness and mainly composed of collagen type IV, laminin, nidogen, and perlecan [25,46]. This membrane consists of distinct banded and unbanded layers with varying composition and morphology. Several unique components such as collagen type VIII, fibronectin, keratan, and dermatan sulfate exist in the Descemet's membrane instead of other types of basement membranes [47]. This acellular and transparent membrane maintains corneal transparency and homeostasis by mediating the exchange of nutrients and various molecules between the corneal stroma and aqueous humor. The ultrastructure of the membrane also enables its structural role in strengthening corneal integrity, maintaining corneal curvature [46].

The corneal endothelium is formed by a monolayer of hexagonal corneal endothelial cells (CEdCs) with uniform thickness, appearing as a honey-comb mosaic [25]. CEdCs possess gap and tight junctions along its borders, whereas the basal surface contains hemidesmosomes to facilitate endothelial adhesion to the Descemet's membrane. Corneal transparency is maintained by the pumping of excess fluid from corneal stroma into the aqueous humor via ion pumps. This establishes an osmotic gradient across the partially permeable endothelial membrane [26,48,49].

Additionally, the cornea is densely innervated. Among corneal diseases, bilateral blindness normally results from corneal opacity, currently affecting 7 million people worldwide, with one-third suffering from disturbance or degeneration of the nervous system, even resulting in corneal sensory loss in severe cases [50,51]. Corneal innervation loss in inflammation-related corneal diseases may further cause neurotrophic keratopathy [52]. A mass of nerve endings from the corneal limbus pass through the corneal stroma and is densely distributed across the corneal epithelium [53]. The human cornea is primarily innervated by the ophthalmic branch of the trigeminal nerve, specifically the nasociliary branch. Many of the nerve fibers also terminate as free nerve endings, becoming nociceptors [54]. This explains why the human cornea is extremely sensitive to pain, as even a light corneal stimulation may induce pain [55].

3. Reconstruction of physiologically relevant corneal microstructures using *in vitro* microfluidic technologies

Various CoC platforms have been introduced to replicate the above-mentioned corneal tissue layers in the *in vitro* setting over the past decades. Recent advances in bio-engineered cornea models have been made, covering corneal wound healing, drug evaluation across the epithelial barrier, single corneal tissue layer culture with improved ECM, and dynamic blinking microfluidic system. The following sections will present representative *in vitro* CoC models in corneal research, which their primary biological functionality, potential merits or demerits discussed. Table 1 summarizes the published CoC models and their relevant cell types, materials, fabrication approaches, primary features and study objectives.

3.1. Corneal epithelial barrier models

As the primary physical barrier, the corneal epithelium constantly maintains both the metabolism and homeostasis of the human ocular surface through transporting nutrients and metabolites between tear film and corneal stroma [65,66]. This barrier also blocks harmful organisms and chemicals from the external environment entering the intra-ocular structure, which may disrupt the corneal physiological stability and cause dystrophy [67,68]. However, at the same time corneal epithelium is vulnerable to injury when subjected to external physical shocks or scratches. Multiple integrated CoC platforms with cell co-culture mode have been put forward so far to study ocular drug delivery and permeability across corneal epithelial barrier.

The first microfluidic CoC platform with co-culture of cells was developed more than a decade ago, which utilized the collagen vitrigel (CV) as a sacrificial membrane incorporated into the PDMS-based device

Table 1
A summary of representative CoC models.

Model types	Cell types	Materials/ fabrication methods of the device	Primary model features	Key findings	Ref.
Corneal epithelial barrier	Immortalized human CEpCs	PC membrane, PDMS/Xurography technique, cast molding	Human CEpCs on a fibronectin functionalized PC membrane, mimicking anterior basement membrane and Bowman's layer (3 conditions: static, continuous flow and pulsatile flow)	The chip was capable of simulating tear volume, tear flow, shear, friction, air exposure and blinking associated tear movement	[34]
	Primary rabbit CEpCs and keratocytes	CV membrane, PDMS/Soft lithography	Bilayered culture of epithelium and stroma through fluidic connectivity to both the apical and basal side	CV served as a natural scaffold for corneal tissue culture, and the vacuum integration enabled fluidic access to bilayered culture	[56]
	Human CEpCs	PET membrane, PDMS/3D printing, cast molding	Spatiotemporal collection and analysis of extracellular metabolites	Antioxidants (e.g., glutathione and uric acid) can be secreted from CEpCs recapitulating similar vivo secretion conditions	[57]
			A bidirectional flow in the upper channels, while a unidirectional flow in the lower channel	The shear stress forces would signal CEpCs of the corneal barrier to adapt to the frequent mechanical stimuli during eye blinking	[58]
	Human keratocytes and CEpCs	PC, stainless steel/N/A	Cell culture inserts can be integrated with dynamic flow, forming a 3D HC construct	The system realized automation, real-time assessment of substance barrier interaction and evaluation of tissue permeability	[59, 60]
Corneal stroma	Human corneal keratocytes	PDMS/3D printing, cast molding	Keratocytes in a hydrogel matrix of collagen type I to simulate *in vivo* human stroma	LBP reduced both pro-fibrotic proteins and pro-inflammatory cytokines on corneal injury *in vitro*	[61]
	Primary rabbit corneal keratocytes	PDMS/Soft lithography	Simultaneous coating of up to 8 substrates with aligned collagen fibrils	This platform enabled to study how simultaneous exposure to topographical and soluble cues influence cell behavior.	[62]
Corneal endothelium-related model	Immortalized human CEpCs and CEdCs	PC membrane, PDMS/Soft lithography	3D culture of CEpCs and CEdCs on the upper and lower side of the membrane, respectively	MSC-derived EVs could accelerate the scratch wound healing of corneal epithelium	[63]
	Primary mouse CEpCs and CEdCs	PDMS/Soft lithography	Epithelial/endothelial cells in the two peripheral channels, collagen matrix in the central channel, a condensed collagen layer in the epithelium channel	The microfluidic coculture device provided a new strategy to investigate corneal function and drug delivery	[9]
Eye blinking on the ocular surface	Primary human CEpCs, keratocytes and immortalized human conjunctival epithelial cells	PDMS, PS scaffold/ 3D printing, cast molding, heat embossing	Eyelid could be actuated to slide back and forth on the scaffold surface to mimic blinking; DED model *in vitro*	The platform could replicate the key biological characteristics of the ocular surface at the cellular, tissue and whole organ level and simulate spontaneous eye blinking	[10, 64]

(Fig. 2A) [56]. Apical CEpCs growth was supported by CV membrane, in which enzymatic degradation contributed to sacrificial etching of CV, thus allowing for the subsequent seeding and culture of underlying keratocytes to construct the bilayer corneal tissue. This model introduced a new technique using microfluidic culture for studying epithelial barrier and the interaction between keratocytes and CEpCs, as well as for modeling corneal damage and its healing process.

To investigate metabolic and transport activities of corneal epithelial barrier in a spatiotemporal manner, a corneal-epithelium-on-a-chip (CEpOC) was developed, composing of upper and lower PDMS chambers separated by a transparent polyethylene terephthalate porous membrane (Fig. 2B) [57]. This CEpOC enabled the spatiotemporal collection of metabolites between the apical and the basolateral sides, as well as metabolite analysis with untargeted liquid chromatography–mass spectrometry (LC-MS). The human CEpCs growing on this CEpOC could also secrete *in vivo*-like antioxidants (e.g. uric acid, glutathione). Because the human cornea and conjunctiva possess significant roles in cell detoxification, these antioxidants in the CEpOC could further be considered as indicators of cytotoxicity and stress response in corneal epithelial homeostasis after chemical stimuli. Unique spatial distribution within the device potentially sheds light on studying the secretion and delivery of exogenous and endogenous biomolecules from tear film to aqueous humor.

A majority of CoC models were designed to construct a specific environment with *in vivo* shear stress and barrier properties, which inevitably cause a high resistance against drug permeation and increasing transepithelial electrical resistance (TEER) [69]. Although TEER has been a highly sensitive index to evaluate the integrity and permeability of the single tissue layer, few measurement methods have been put forward to monitor real-time TEER values in CoC systems [34, 59,60,63,70]. A microfluidic Dynamic Micro Tissue Engineering System (DynaMiTES) was proposed and established as the first platform integrated with TEER (Fig. 2C) [59]. In this system, the combination of tissue inserts and controllable dynamic microfluidic conditions enabled a 3D hemi-cornea (HC) reconstruction using human keratocytes and human CEpCs. Moreover, a tailor-made inert polycarbonate (PC) component is integrated with commercial inserts, hence overcoming the limitation of small-molecule absorptions by PDMS materials [71]. Furthermore, TEER was continuously monitored via built-in electrodes, allowing the collection of real-time information about drug influence on the barrier between CEpCs. Based on this automated microfluidic system, another study improved the protocol in culturing inverted HC that allowed highly controlled fluidic exposition to the corneal epithelium [60]. Further investigation revealed that inverted HC showed similar biological functions as the normal HC tissue, including cell viability, morphology, TEER value, and permeability. Sodium fluorescein and benzalkonium chloride were used as small molecules to evaluate epithelial barrier properties, and results showed that compared to a static microenvironment, permeability in dynamic conditions has higher similarities with the *in vivo* conditions. Although the DynaMiTES platform potentially contributes to improving the prediction of drug absorption and permeation, the improvement scheme in downsizing the testing compartments will be imperative to the reduction of drugs and reagents consumption, which are not always available. In addition, highly sensitive built-in sensors, instead of via additional collection and analysis, can be embedded into the system for online analysis of molecular concentrations in the future.

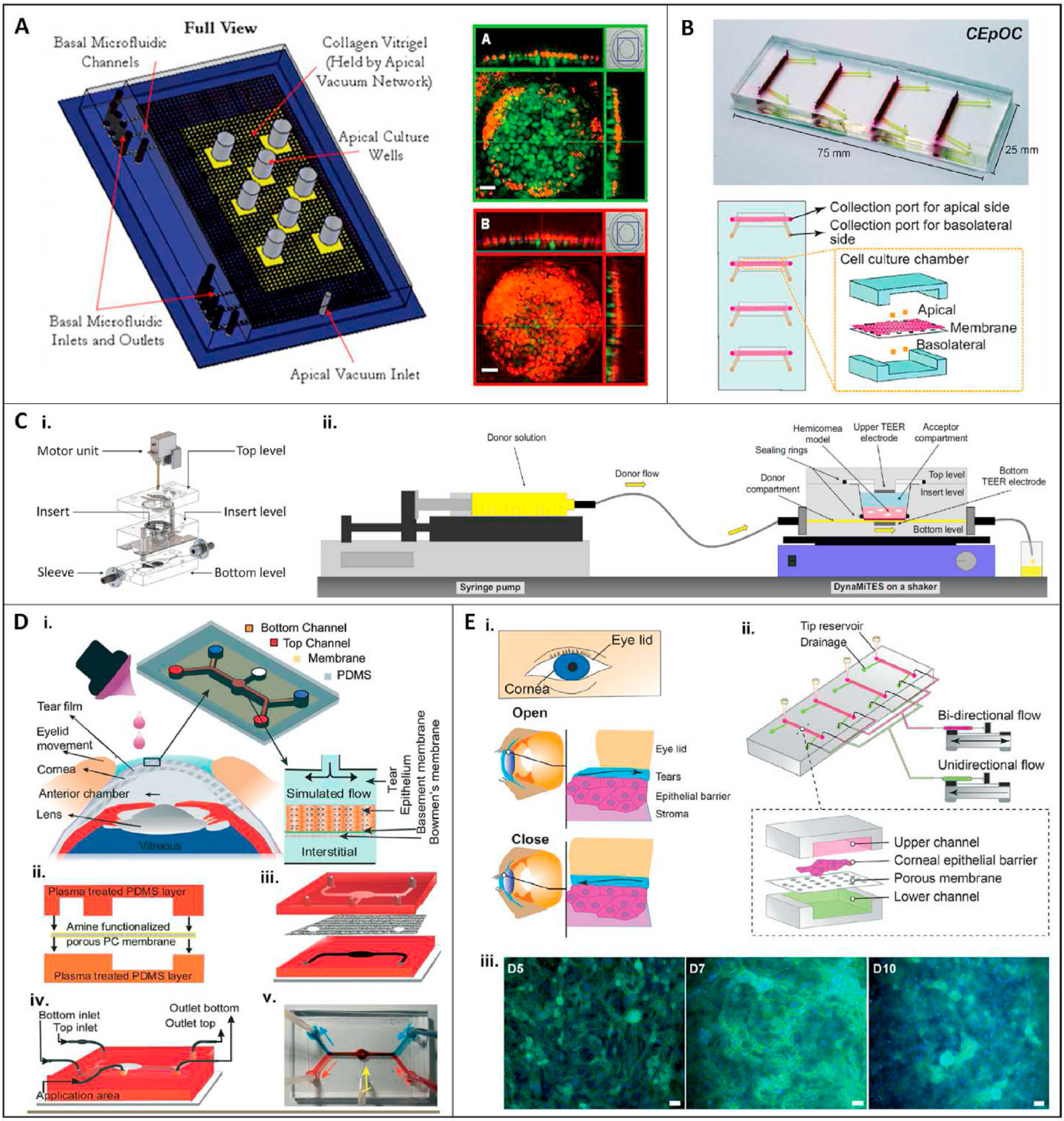

Fig. 2. Representative designs of CoC platforms for corneal epithelial barrier studies. (A) (i) Schematic overview of the hybrid microfluidic device and (ii) Confocal fluorescence images of corneal epithelial cells (green) and keratocytes (red) (Reproduced Ref. [56] with permission of Royal Society of Chemistry, Copyright 2009 Royal Society of Chemistry). (B) The photo and structural illustration of CEpOC, and its applicability using untargeted LC-MS equipment (Reproduced Ref. [57] with permission of Elsevier, Copyright 2021 Elsevier). (C) (i) Exploded assembly diagram of the DynaMiTES unit (Reproduced Ref. [59] with permission of Elsevier, Copyright 2017 Elsevier), and (ii) detailed components of the systematic ocular DynaMiTES (Reproduced Ref. [60] with permission of Elsevier, Copyright 2017 Elsevier). (D) Schematic diagram of (i) the dynamic CoC simulation of anterior ocular architecture, (ii) device fabrication, (iii) breakdown structure and (iv) principle of operation. (v) Photograph of demonstration with two dyes in the individual channels (Reproduced Ref. [34] with permission of Royal Society of Chemistry, Copyright 2018 Royal Society of Chemistry). (E) (i) Tear flow dynamics during eye blinking, (ii) illustration of the multi-layered flow perfusion CoC, and (iii) immunofluorescence photographs of ZO-1 (green) in HCEpCs (Reproduced Ref. [58] with permission of Royal Society of Chemistry, Copyright 2020 Royal Society of Chemistry). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To further study dynamic drug transportation through corneal epithelial barrier, an integrated microfluidic device with double PDMS channels and a PC membrane with 5 μm-sized pores was presented (Fig. 2D) to reconstruct the epithelium, basement membrane and Bowman's layer [34]. Fibronectin from corneal basal matrix was coated on the PC membrane, followed by cultivation of immortalized human CEpCs on the gel matrix surface. The mass transport efficiency of two widely used commercially available eye drops - Pred Forte and Zaditor - was studied under three different flow conditions: static, continuous, and pulsatile. Results confirmed that Pred Forte resulted in longer action time on epithelium, while Zaditor elicited higher permeability. Therefore, this proof-of-concept study demonstrated the capability in dynamic quantitative measurements *in vitro*, and preliminary evaluations of new therapeutic eye drops on this microengineered model are appropriate for understanding pharmacokinetics and pharmacodynamics of ocular drugs.

The corneal epithelium, as the primary barrier of the ocular surface, is directly influenced by shear stress stimuli during eye blinking [72]. To explore the effects of the microfluidic shear stress on phenotypic alternation of corneal epithelium, a high-throughput multi-channel CoC was developed (Fig. 2E) [58]. A thin polyester (PET) membrane separates the PDMS chamber into the upper and lower channels, where bidirectional flow and unidirectional flow were applied respectively. Human CEpCs seeded on the polyester membrane formed the epithelial barrier with high expression of zona occludens-1 (ZO-1) tight junction protein on day 7. This shows that cell adhesion would not be affected under either bidirectional or unidirectional shear stress. Furthermore, continuous dynamic flow could promote the expression of cytokeratin 19 (CK-19) in human CEpC, indicating that shear stress forces under constant blinking would strengthen the barrier function. The effects of ocular drug applications on engineered epithelial barrier during repeatable mechanical stimuli will be worth exploring in the future. A further step may also incorporate tear film components such as mucin and lipid layers into the existing system to improve the accuracy of drug assessment.

3.2. Corneal stroma models

After corneal injury, keratocytes in stromal layer proliferate and irreversibly differentiate into myofibroblasts causing corneal scarring, which can only be treated by keratoplasty when the symptom worsens [73–78]. Therefore, understanding the physiology of corneal opacification, especially on prevention from differentiating into fibroblastic-like phenotype, is important to reduce blindness due to stromal scarring.

Lycium barbarum polysaccharide (LBP), a Traditional Chinese Medicine, was shown to elicit a therapeutic effect on minimizing liver fibrosis [79], and its potential efficacy in minimizing corneal stromal scarring was investigated using a PDMS corneal-stroma-on-a-chip [61]. Keratocytes were cultured in a thin collagen type I matrix that mimicked the thickness and composition of corneal stroma (Fig. 3A). The results showed that keratocytes pre-treated with LBP had significantly lowered levels of pro-fibrotic proteins (vimentin and α-smooth muscle actin) and secreted less fibrotic ECM proteins (collagen type II and type III) after

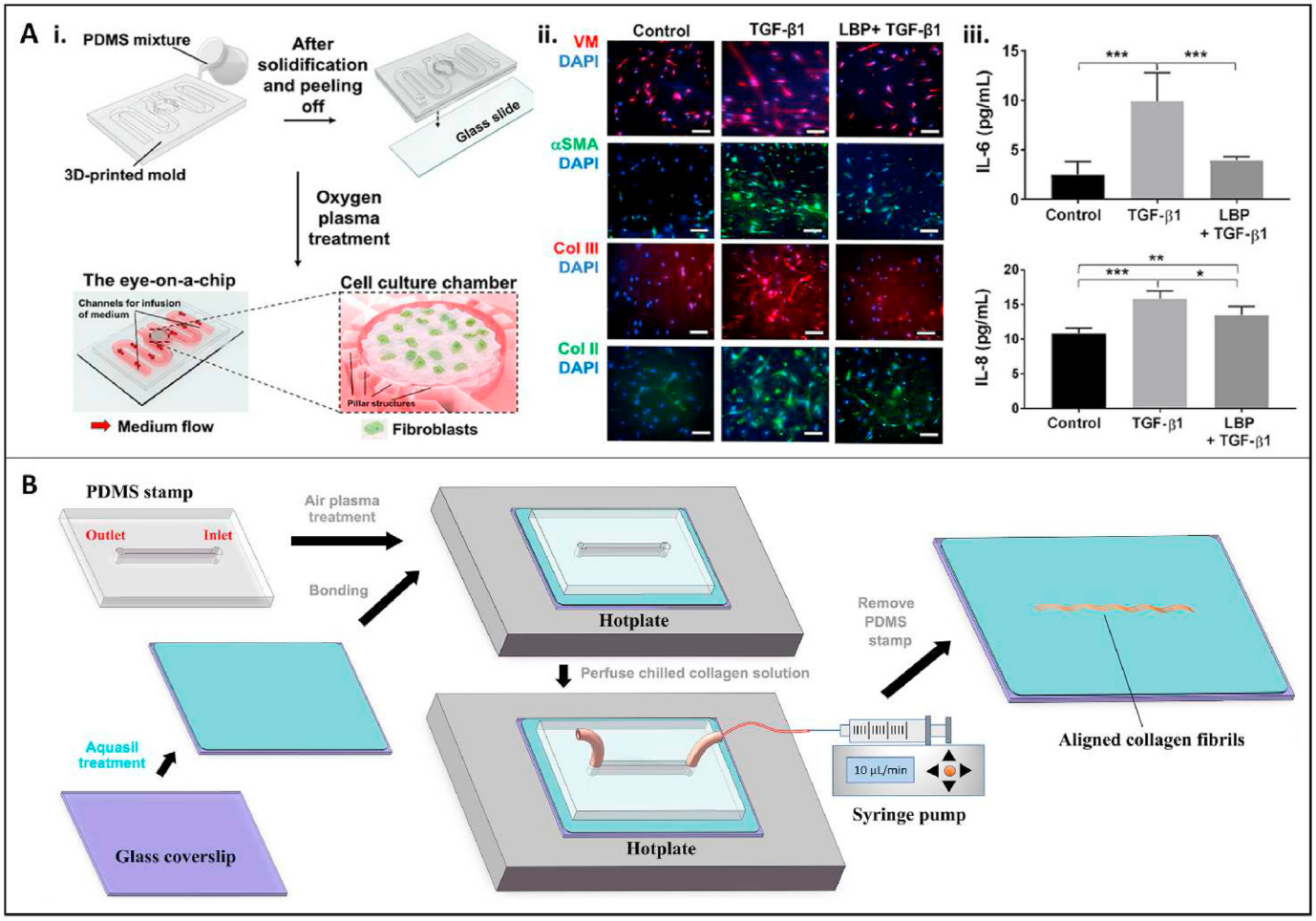

Fig. 3. Corneal stroma chips with different applications. (A) (i) Schematic procedure of chip fabrication, (ii) the immunocytochemistry results, and (iii) effect of LBP pre-treatment on secretion of IL-6 and IL-8 proteins from keratocytes (Reproduced Ref. [61] with permission of Elsevier, Copyright 2021 Elsevier). (B) Fabrication process of the PDMS microfluidic device and aligned collagen fibril formation [62].

injury with TGF-β1 stimulation, compared to control. Therefore, LBP is likely to be a promising therapeutic agent to minimize stromal scarring.

In addition, the alignment of collagen fibrils plays a crucial role in regulating keratocyte alignment and migration [80]. To explore the relationship between keratocytes behaviors and collagen fibrils arrangement in the corneal stroma, a high-throughput glass-based microfluidic platform was developed [62]. Collagen fibrils were deposited in alignment on the glass substrate, which was achieved by high-throughput collagen perfusion with multichannel syringe pump (Fig. 3B). The finding indicated that keratocytes became more prone to growth in alignment with the fibril orientation on the collagen type I coating as the concentration of collagen increases. For further investigation on corneal stroma, this platform allows direct fibril generation with controllable collagen density and specific keratocyte orientation. Future studies might be needed to reveal the relevant biochemical cues (e.g. cytokines, growth factors, inflammatory factors and kinases, etc.) in the stroma-related platform, to better support normal corneal functions and homeostasis *in vitro* [81].

3.3. Corneal endothelium-related models

In humans, CEdC would not proliferate *in vivo* and its density decreases constantly with age [82,83]. Endothelial cell dysfunction would cause corneal edema and decreased corneal clarity, which ultimately leads to corneal blindness [83]. Currently primary CEdC culture is commonly adopted for studying cell growth regulation *in vitro* and endothelial wound healing *in situ* after transplantation. However, most CEdC models are based on *in vitro* 2D culture, and no microfluidic model has been constructed using CEdCs alone so far. The presentation of few CoCs using CEdCs is typically accompanied by CEpCs to study drug delivery or evaluate permeability of the corneal barrier.

For the CoC mentioned in the previous sections, tissue structure in parallel enclosed microchannels cannot realize the interaction between the corneal surface and the external air environment (air-liquid surface). To overcome this spatial limitation inside CoC platforms, a human cornea chip with open culture zone was presented, which consisted of a 0.4 μm collagen-coated porous membrane and double microfluidic channels (Fig. 4A) [63,70]. An air-liquid interface in an open-top structure was established, in which barrier effects were validated with TEER and ZO-1 protein. Compared to normal medium culture condition, extracellular vesicles (EVs) derived from the mesenchymal stem cells (MSCs) can stimulate mild corneal epithelial wound healing, meanwhile reducing matrix metallopeptidase-2 (MMP-2) expression, which efficiently suppressed the corneal inflammation [63]. However, in addition to endothelium and epithelium, corneal injury and subsequent wound healing response may also include corneal stroma layer. Wound healing in more severe corneal trauma still cannot be simulated in this cell coculture model.

To improve the integrity of the CoC with more tissue layers, a representative static culture model adopted multichannel design in PDMS device (Fig. 4B) [9]. Primary CEdCs, CEpCs, and collagen type I were applied to reconstruct corneal epithelium, stroma, and endothelium inside three individual chambers respectively. A thin Bowman's layer was built using condensed collagen. The results showed that this CoC was able to maintain corneal structural integrity for up to two weeks. CEpCs in the microchannel can form a structure simulating the *in vivo* corneal epithelial sheets with 5–7 cell layers. In addition, corneal-like functions on chip were evaluated by measuring the diffusion permeability of dextran from 10 kDa to 70 kDa, which validated the decisive role of the corneal epithelium in drug transportation rates [34]. In general, corneal endothelium can be co-cultured with other types of corneal cells and retain its structure and functionality in microfluidic chips. Before constructing more advanced CoC systems involving endothelium, some issues need to be addressed such as the demanding manual isolation of primary CEdCs, relatively low available CEdC number and limited proliferation capacity.

3.4. Eye blinking model with eyelid movement

Spontaneous eye blinking induces dynamic microenvironment on the ocular surface. The tear film is frequently replenished to keep the cornea and conjunctiva away from dehydration and discomfort. Despite recent advances in CoC platforms mentioned in the previous sections, challenges still exist in mimicking eyelid movement in an integrated *in vivo*-like context. Static cultivation conditions sometimes might lead to either efficient drug candidates being wrongly excluded, or an incorrect selection of molecular substances for subsequent clinical analysis [84,85]. To better simulate the complex dynamic microenvironment at the ocular surface, a representative human CoC with 3D polystyrene scaffolds and PDMS slabs was presented [64]. This realized combination of corneal cells and conjunctiva was closely similar to *in vivo* spatial distribution of the ocular surface, covering biological, physical and mechanical features. Primary human CEpCs, conjunctival epithelial cells, and keratocytes were distributed selectively across the 3D arcuate surface. The embedded scaffold was set to have a 5 mm radius of curvature to match the size of the human cornea [86]. More importantly, spontaneous eye blinking was achieved through the mechanical motion of the 3D-printed eyelids actuated by a direct current driven micro-motor, providing a smooth and lubricated surface. Furthermore, a more advanced blinking human CoC can be interfaced with human-scale diagnostic tools and standard clinical tests on ocular surface like tear film break-up and keratography (Fig. 4C) [10]. The application value of lubricin was verified in terms of its therapeutic anti-inflammatory capability. This distinctive feature makes it a physiologically representative *in vitro* model of evaporative dry-eye disease (DED) for the testing of drugs, consumer products, environmental allergens, and even contact lenses. Although this microfluidic CoC serves as a ground-breaking physiologically relevant *in vitro* model for drug screening, significantly different characteristics compared to the *in vivo* counterpart still exist. For example, in terms of human DED model, the absence of vascular system and immune components inside the conjunctiva may give rise to less representative immune responses [87]. The tear volume and composition inside the artificial channel is also markedly different from the tears secreted by the lacrimal gland *in vivo*.

Up to now, the use of CoC to construct *in vitro* models that simulate dynamic blinking on curved ocular surfaces is still limited in literature. Multiple models with physiological blinking mechanism are based on the 3D printed eyeball for the study of drug delivery [88–90]. However, limitations such as the evaporation of fluid and absence of corneal cells still hinder further development of these models. Native ocular surface could also be simulated by the more advanced mechanical model, which integrates CEpC layer with controllable eyelid movement speed and distance, as well as blinking frequency [91]. Nevertheless, *in vitro* tear fluid conditions differ widely from *in vivo*. For example, the solution volume *in vitro* is difficult to be controlled to 7 μL as *in vivo* environment [92], and culture medium is considerably different from protein-containing tears. Microfluidic channels with regulable flow rate may help improve the accuracy of tear volume in the eye blinking model. Corneal stroma and endothelial layer are also required to be constructed under epithelial layer as well to better evaluate drug delivery across the epithelial barrier. Apart from the *in vitro* corneal tissue culture, the *ex vivo* model such as isolated eyeball might also be fixed to the microfluidic blinking platform as an alternative for drug toxicity testing.

4. Discussion

The transparent cornea is prone to infections and injury, resulting in visual disturbance and even blindness. However, the development of a new drug to treat corneal diseases normally requires huge financial investment with long-term efficacy and safety tests. To improve such intractable situations, CoC platforms have been developed over the past decade, and recent studies have demonstrated the desirable biomimetic capability of these platforms. The advantages of CoC are multifold. Firstly, continuous improvement of manufacturing technology such as

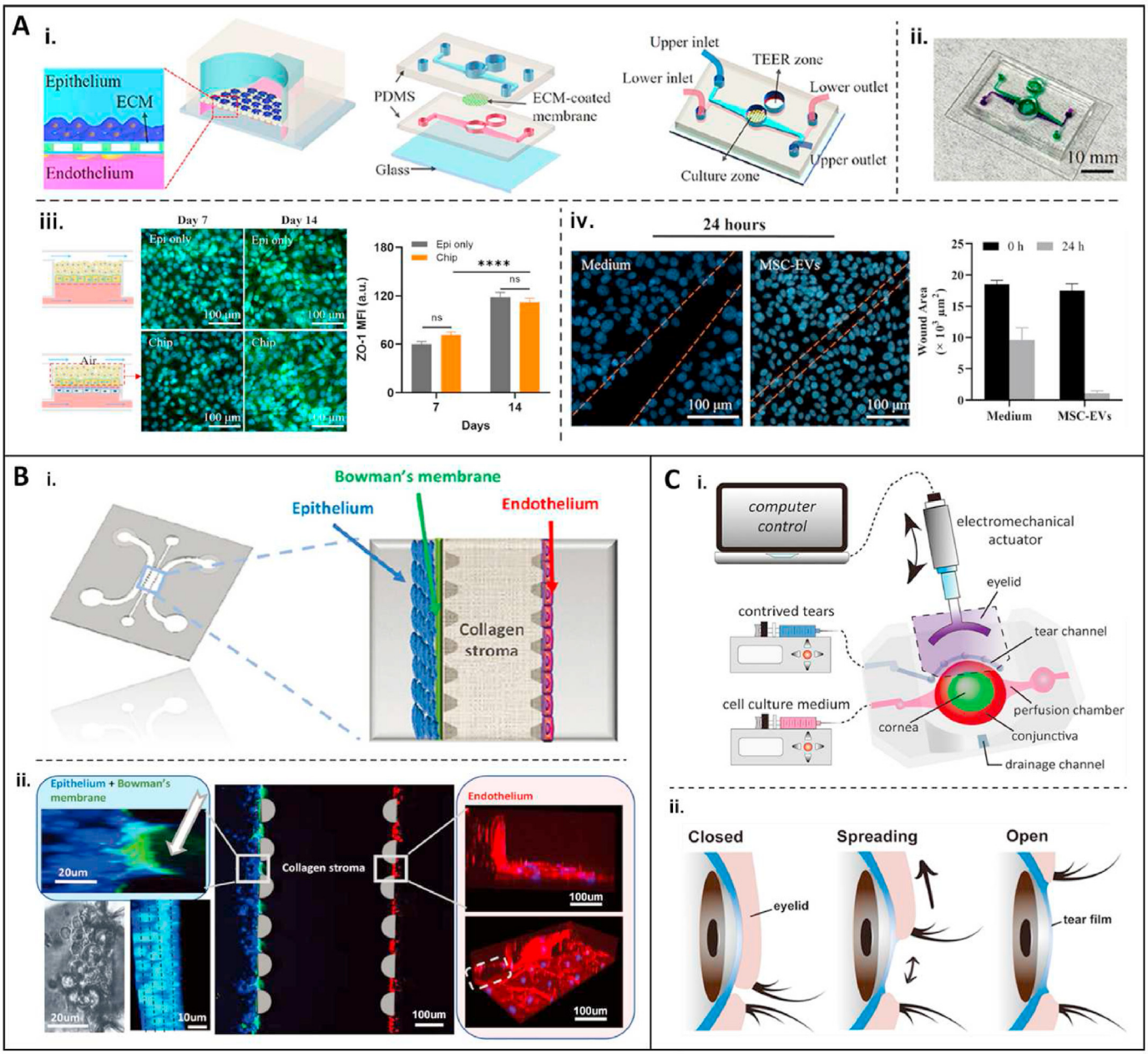

Fig. 4. Corneal endothelium-related CoC and representative eye blinking model. (A) (i) Schematic diagrams of cross-sectional culture zone, exploded view and the top view of the device with double-channel structure; (ii) photograph of the cornea chip; (iii) ZO-1 (green) and DAPI (blue) staining results in different conditions ('Chip' means the coculture of CEpCs and CEdCs; MFI: mean fluorescent intensity); (iv) CEpC migration results during wound healing under two different culture conditions (Reproduced Ref. [63] with permission of Elsevier, Copyright 2022 Elsevier). (B) (i) The diagram of the microfluidic device with multichannel; (ii) Microscopy images of different layers at 24 h after seeding (Reproduced Ref. [9] with permission of Creative Commons Attribution License, Copyright 2020 Creative Commons Attribution License). (C) Schematic of the (i) representative CoC setup to simulate eye blinking with an electromechanical actuator, and (ii) eyelid movement and changes of the tear film during spontaneous eye blinking [10]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

high-precision lithography, 3D-printing, and thermo-pressing actualizes rapid production of high throughput chips, which allow the size miniaturization of these chips in a reagent-saving manner. PDMS has been used extensively because of its various advantages including superior optical transparency, desirable air permeability, and biocompatibility. The special corneal curvature radius can be built with advanced 3D-printed scaffolds. Secondly, CoC can partially simulate 3D structures and functions of the human cornea, providing a powerful *in vitro* research platform for advancing precision medicine. Micro-physiological systems in CoC enable the maximization of physiological relevance with human-derived cells, and thus contributes to subsequent bio-analysis. Thirdly, the higher flexibility in CoC device design paves the way for establishing specific disease models in a controllable and reproducible manner, regardless of static or dynamic conditions. In addition, the establishment of CoC would require comparatively lower cost, fewer ethical constraints than current *in vivo* models. These advantages allow pharmaceutical industries to use engineered cornea to achieve potential large-scale high throughput drug screening, and thus eventually accelerate the pace of clinical trials.

The existing platforms also have several shortcomings, and extensive

research is required to break the technological bottleneck. Above all, PDMS absorption of small hydrophobic molecules always negatively impacts preclinical drug evaluation [71]. In addition, most CoC devices are relatively simple and only concentrate on studies of single cell/tissue subtypes from the cornea, which makes experimental results less representative for pharmaceutic studies. For example, keratitis may affect all tissue layers of cornea [93–95], while corneal neovascularization primarily influences corneal limbal vascular endothelial cells and the stromal layer [96]. Moreover, current CoC models lack the integration with neural cells, hindering the exploration on neuronal regenerative mechanisms and corneal sensitivity after physical trauma or medicine stimuli. Another insurmountable obstruction exists in the differences between the culture medium required for each cell type, preventing the co-culture of multi corneal tissue layers. Besides, pharmaceutical effects between immortalized corneal cells and primary corneal cells might be different, which could cause inaccurate drug evaluation.

Nevertheless, 3D bio-printing technology that actualizes the printing of multiple cell types may enable the spatially realistic *in vitro* construction of corneal tissues through layer-by-layer fabrication [97]. The stereolithography 3D-printing has been reported for producing corneal keratocyte-laden collagen, which can maintain more than 80% cell viability for 1–2 weeks after printing [98,99]. In addition to the collagen, gelatin methacrylate (GelMA) scaffolds have also been successfully demonstrated as the promising bio-ink with elevated light transmittance for dome-shaped structure of cornea [100]. A series of technological obstacles, however, still require to be resolved, such as the low precision of the printing nozzle and cell damage during the extrusion process of bio-ink. The light transmittance of hydrogel scaffold might also potentially decline under incubation due to the continuous cell proliferation [101,102].

In addition to the CoC fabrication technology, the *in vitro* culture conditions of the corneal cells can be possibly improved through providing ECM-like culture environment and relevant biophysical stimuli. Current publications reported that corneal cell growth and functions could be regulated using external electric field, topographical cues, and specific ECM-related materials (e.g. silk film substrates) [102–108].

Directed CEpC migration initiates corneal wound healing to maintain homeostatic balance after injury. Researchers have revealed that CEpC migration rate was significantly higher on topographically patterned substrates compared to flat surfaces, and the increasing electric field could stimulate cell migration towards the cathode (Fig. 5A) [103]. Moreover, during corneal inflammation or after corneal refractive surgery, TGF-β-induced keratocytes differentiation to myofibroblasts might occur, which can be inhibited by culturing keratocytes on nano-patterned ridges and grooves (0.4–4 μm width) coated with collagen type I and type III [104]. This physiological finding may provide a nano-topographical clue towards stabilizing the keratocyte phenotype for corneal stromal reconstruction. A recent study showed orthogonally topographical stacked silk film layers could support the growth of human corneal stromal stem cells (hCSSCs) for 9 weeks (Fig. 5B) [102]. With such multi-lamellar silk film architecture, hCSSCs, compared with human keratocytes, could improve the elastic modulus and light transmittance of the silk film, which was comparable to natural cornea stromal tissue.

The expansion of primary CEdCs could also be promoted with patterned topography. The nanopillars, compared with other geometrical patterns, could enhance endothelial functionality (Na^+/K^+-ATPase activity) and significantly increase the cell density (Fig. 6A) [105]. In addition, heat-embossed polystyrene surface with pillars (diameter: 1 μm) can facilitate the formation of cell-cell tight junction and greatly promote the CEdC proliferation [106]. Notably, topography-exposed cell

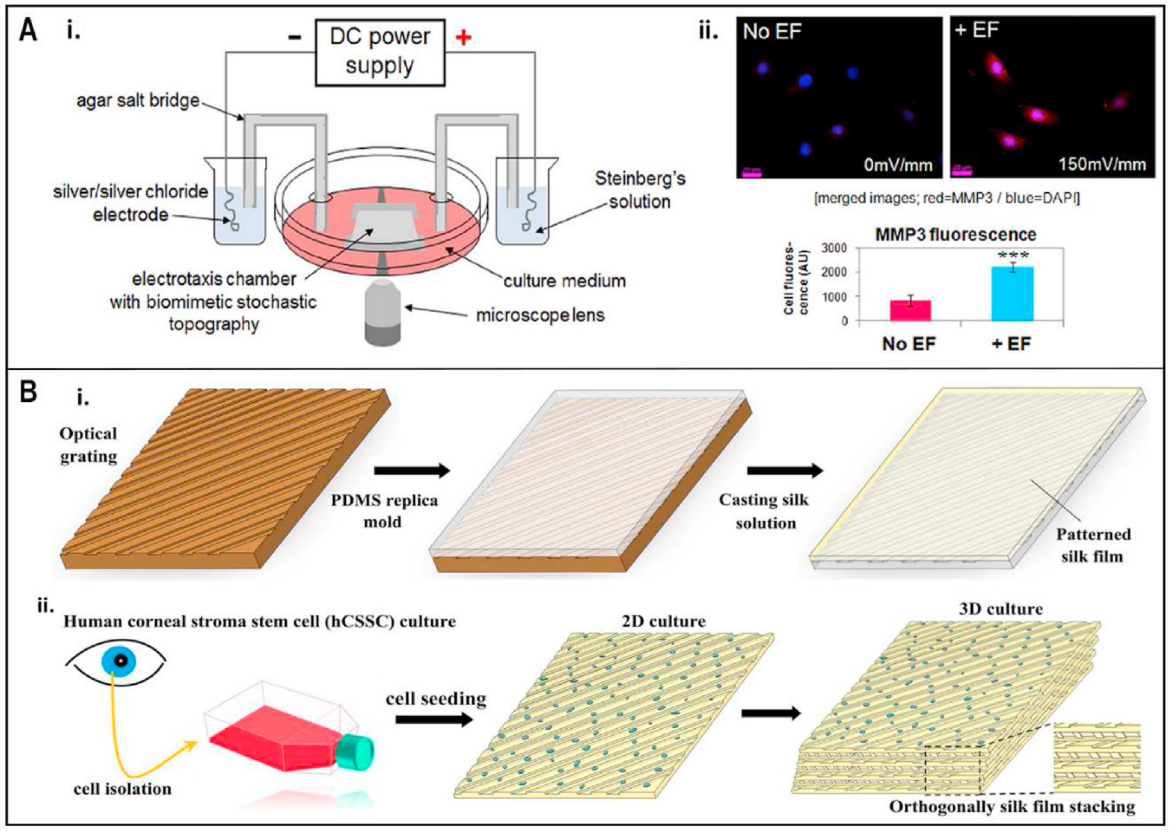

Fig. 5. Different corneal cell cultures using specific ECM environment. (A) (i) Schematic diagram of overall CEdC culture setup with biomimetic topography and electric fields (EF), and (ii) analysis of the protein with and without EFs (Reproduced Ref. [103] with permission of Elsevier, Copyright 2014 Elsevier). (B) (i) Silk film functionalization and (ii) preparation of 3D functional corneal stroma on the silk film [102].

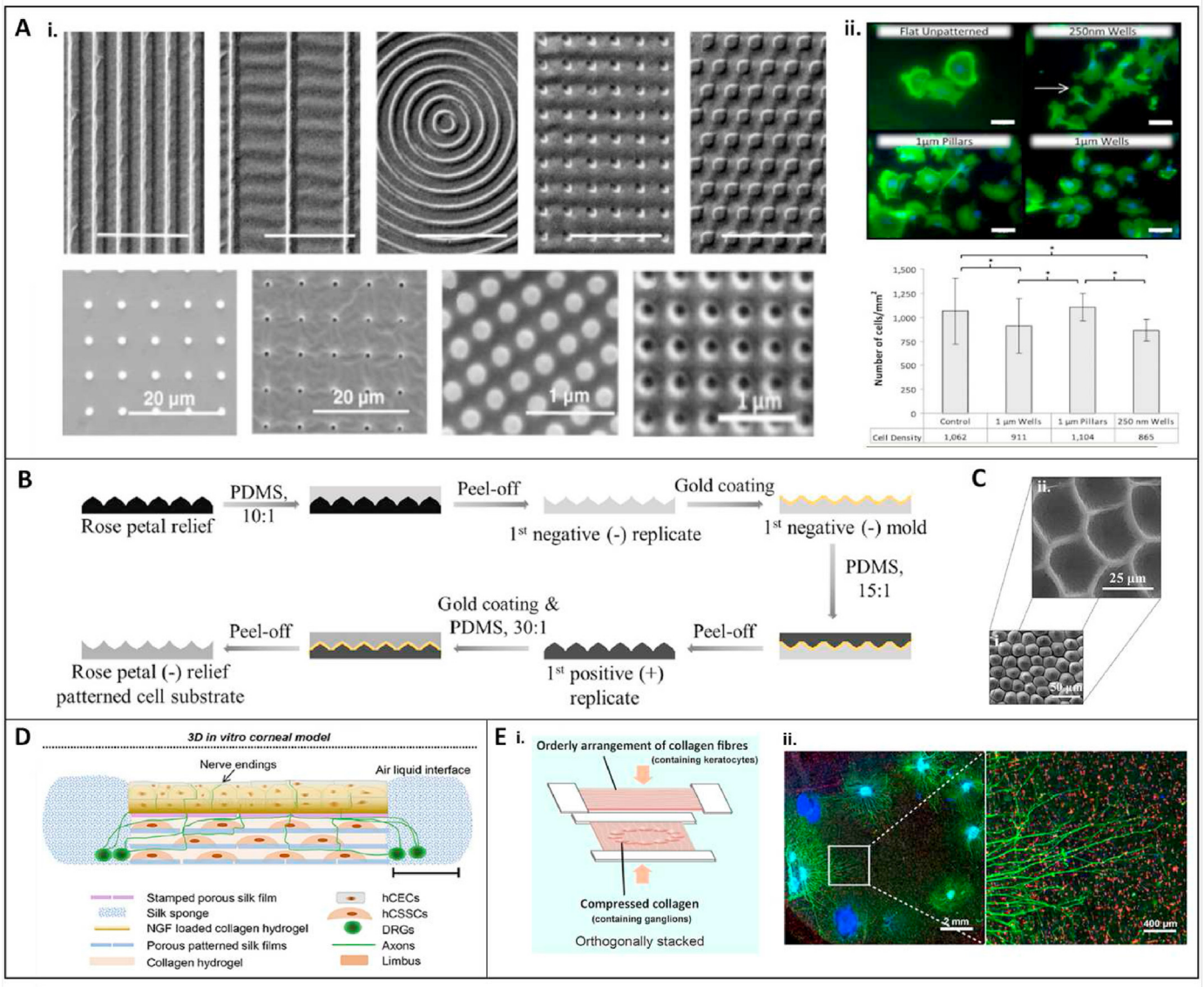

Fig. 6. Representative CEdC-related research with various topographical patterns and *in vitro* corneal nerve models. (A) (i) Scanning electron microscope characterization and (ii) effect of topographical features on endothelial cell morphology (Reproduced Ref. [105] with permission of Elsevier, Copyright 2012 Elsevier) (B) Preparation process of PDMS substrate and (C) (i) patterned mold of natural white rose and (ii) substrate for cell culture (Reproduced Ref. [108] with permission of Elsevier, Copyright 2021 Elsevier). (D) Schematic diagram of 3D engineered corneal nerve model (Reproduced Ref. [109] with permission of Elsevier, Copyright 2016 Elsevier). (E) (i) Sketch map and picture of the corneal nerve-stroma model and (ii) fluorescence images at day 7 (Green: β tubulin-III labeled nerve bundles; Red: vimentin labeled CSCs; Blue: DAPI labeled nuclei) (Reproduced Ref. [110] with permission of Elsevier, Copyright 2022 Elsevier). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phenotypes could be maintained after detaching CEdCs from the patterned substrate. More interestingly, high similarities between rose petal and hexagonal CEdC morphology were discovered, and natural rose petal topography could preserve CEdC phenotype *in vitro* (Fig. 6B and C) [108]. Topography with hexagonal structures could also induce human mesenchymal stem cells (hMSCs) to differentiate into CEdCs [107]. These findings can provide potential directions towards *in vitro* corneal endothelium culture in CoCs, contributing to drug evaluation in treatment of endothelial dysfunction. Future investigations might need to focus on the subsequent surgical procedures, such as how to transfer the *in vitro* matured monolayer endothelium into aqueous humor and attach endothelium to Descemet membrane.

Beyond that, CoC platforms for studying corneal nerve regeneration would also be of high priority, because in most conditions that lead to corneal blindness, the sensitivity of corneal nerve tracts decreases due to neural dysfunction or degeneration [51]. Current studies have validated the feasibility to reconstruct corneal nerve *in vitro*. For example, a silk protein-based platform showed the engineered air-liquid interface played a crucial role in supporting neuronal innervation, which paves the way for further CoC research on neural signal responses to environmental factors and corneal model development (Fig. 6D) [109]. Besides, a ganglion-keratocytes model demonstrated that orthogonally stacked collagen membranes could provide a crosstalk environment, in which the nerve bundle regeneration and keratocyte proliferation coexisted and mutually promoted each other (Fig. 6E) [110]. Therefore, these results provide the possibility to coculture CEpC, keratocytes and sensory nerves to construct multicellular CoC with air-liquid surface.

Last but not least, CoC combined with microbiology might become another emerging direction, which could eventually offer prevention strategies against viral infections. This is because microfluidic platforms are likely to shed light on the study of direct interplay between the microbiome and the corneal tissue through the air-liquid surface. Due to

directly exposing the cornea to the environment, the risk of corneal contamination by pathogens and irritants is greatly increased. Some viruses such as the Zika virus and SARS-CoV-2 might infect human through the ocular surface. Herpes simplex virus (HSV) could even replicate in the corneal tissue [111]. Interestingly, the limbus has a much higher SARS-CoV-2 replication level than central cornea, and is most vulnerable part of the eye to virus invasion [112]. However, owing to clinical research limitations and lack of donors, the underlying reasons of this phenomenon have not been thoroughly studied. In addition, ocular surface moisture, preventative against keratitis, is reduced due to insufficient secretion from ocular gland during HSV-1 infection, thereby aggravating keratitis symptoms [113]. Impaired corneal nerve signaling also contributes to ocular gland dysregulation during immune reaction against infection, with unknown mechanism [114,115]. Microbes such as *pseudomonas aeruginosa* may induce vision-impairing corneal diseases after corneal injury or prolonged contact lens wear [116,117]. Until now, multiple viral infection *in vitro* models have been reconstituted, including hepatitis B virus infection study with human liver model, Coxsackievirus B research with gut-on-a-chip, pseudorabies virus integrated with neurological disorder model, and recent Covid-19 therapeutics with lung airway infection model [118–122]. Therefore, CoC systems provide an excellent opportunity to understand mechanisms of microbe-related disorders, and may exert great insights on the development of novel antibacterial and antivirus medicine suited for clinical practice.

5. Conclusion

Bio-engineered *in vitro* corneas evolved into a new avenue in ophthalmic research, pointing towards promising therapeutic paradigms. Of which, CoC systems have demonstrated huge potential to recapitulate the *in vivo* corneal microenvironment and functions. These engineered corneal platforms, such as corneal wound healing models and epithelial barrier models, are providing novel schemes for medicine screening that expedite drug development process with more efficient transition into the clinical stage. However, current progress is slow since not all CoC systems are applicable for modeling ocular disorders and screening pharmacological treatment options. Most devices published are relatively minimalistic despite their success in studying cellular behaviors and establishing cornea-related models. The advanced platform for integrated tissue engineering, drug testing, and sensor detection needs to be further developed. In the future, microfabrication technology may assist in improving micro-architecture of *in vitro* models. Besides, the investigation of microfluidic models may further shed light on the study of interaction mechanisms between corneal tissues and microbes. Antibiotic discovery for treatment of corneal infections will be further accelerated, which would be of great value for clinical application and translational science. Moreover, the combination of higher maturity of CoC devices with multi-tissues and animal models will be a real prospective of experimental models in eye and vision science research. With the confluence of analytical biology and engineered cornea, CoC will play a pivotal role in the field of clinical translational medicine, not only for basic biological mechanism studies, but also for practical significance in transplantation for the benefits of mankind.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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