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3D Stacked Construct: A Novel Substitute for Corneal Tissue Engineering

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Abstract

Corneal trauma/injury often results in serious complications including permanent vision loss or loss of visual acuity which demands corneal transplantations or treatment with allogenic graft tissues. There is currently a huge shortage of donor tissue worldwide and the need for human corneal equivalents increases annually. In order to meet such demand the current clinical approach of treating corneal injuries is limited and involves synthetic and allogenic materials which have various shortcomings when it comes to actual transplantations. In this study we introduce the newly developed, next generation of our previously established 3D self-assembled constructs, where multiple constructs are grown and stacked on top of each other without any other artificial product. This new technology brings our 3D in vitro model closer to what is seen in vivo and provides a solid foundation for future studies on corneal biology.

Lipids are known for playing a vital role during metabolism and diseased state of various tissues and Sphingolipids are one such class of lipids which are involved in various cellular mechanisms and signaling processes. The impacts of Sphingolipids that have been documented in several human diseases often involve inflammation, neovascularization, tumorigenesis, and diabetes, but these conditions are not yet thoroughly studied. There is very little information about the exact role of Sphingolipids in the human cornea and future studies aiming at dissecting the mechanisms and pathways involved in order to develop novel therapies. We believe that our novel 3D stacked model can be used to delineate the role of Sphingolipids in the human cornea and provide new insights for understanding and treating various human corneal diseases.

Keywords

3D constructs; Cornea; Extra cellular matrix; Sphingolipids; Stacking

1 Introduction

In recent years tissue engineering applications have garnered great interests across various fields of medical science in order to treat various diseased conditions. The vast implication

of tissue engineering using different biomaterials has been a great success, yet there are various limitations when it comes to actual applications due to a number of contributory factors such as immune response to foreign body or material, synthetic materials fail to respond to the changing physiological loads or biochemical stimuli which limit the lifetime of artificial body parts, graft rejections, infection, glaucoma, retinal detachment and extrusion [1, 2]. The application of tissue engineering in treating ocular dystrophies has also stimulated great interest and has been a great success over the past years [3–7].

Wound healing is one of the major challenges when it comes to treating ocular injuries. It often leads to scarring resulting in either partial vision loss or permanent blindness. The process of corneal wound healing is complex; it involves interactions between the wound-healing epithelium, a temporary “provisional matrix,” and cells present in the extracellular matrix (ECM) [8]. During this process the wounds either tend to heal in a regenerative manner, where the tissue returns to its original state, or in a fibrotic manner, where a scar is produced.

Being able to treat corneal injuries without scarring and be able to mimic the actual in vivo process remains elusive. In vitro there have been a number of models investigated and proposed [2, 8–17]. 3D in vitro models are of great interest due to their potential of mirroring cellular and physiological events that are very important during fibrosis and wound healing [8, 9, 18, 19]. In the cornea, the elucidation of using 3D in vitro systems is imperative in order to improve treatments and lead us to the identification of new therapeutic approaches. Our original 3D in vitro model has been well studied and has shown the impeccable ability of recapitulating in vivo events in vitro [18–20] but one of the biggest limitations of our model is that the cells have limited proliferative potential and can only assemble a certain amount of ECM. Such limitations have been partially overcome by stimulating with various growth factors, mainly transforming growth factor- β (TGF- β) isoforms which aid the cells in stimulating, secreting, and assembling double or triple the amount of ECM [18, 21, 22]. Even with TGF- β stimulation, however, the ECM assembled does not exceed 120–150 μm over 4 weeks, when a human corneal stroma is approximately triple in thickness [18,21,23]. Thus, a 3D in vitro model that closely mimics the corneal stroma in size would lead to more accurate results and a better understanding of cellular and ECM mechanisms. The herein described 3D self-assembled stacked model represents the latest generation of our promising in vitro model.

Sphingolipids (SPLs) are known to be involved in human diseases associated with inflammation, neovascularization, tumorigenesis, and diabetes; however, their roles associated with these diseases remain understudied and not fully understood [24, 25]. Bioactive SPLs such as Sphingosine-1-phosphate (S1P) and Ceramide (Cer) have been acknowledged as being essential mediators of many basic cellular processes such as cell migration, survival, contraction, proliferation, gene expression, and cell–cell interactions [26]. S1P and Cer actions/levels are regulated by Ceramidase enzymes; their ability to regulate diverse cellular processes has grasped the attention and interest of researchers due to their capabilities of regulating tissue fibrosis in various organ systems by utilizing S1P and/or Cer [24,27, 28]. Among the fields of interest pertaining to SPLs, the cornea remains one of the most scarcely studied. There are currently only a few publications that reported

the presence of SPLs in the cornea. Swaney et al. [28] reported the presence of Sphingosine kinase-1 (SphK1), Sphingosine kinase-2 (SphK2), and S1P_{1-3,5} receptor proteins in cultured human primary corneal fibroblasts (HCFs). Watsky et al. [29] observed expression of S1P receptor's mRNA in cultured corneal epithelial cells which mimicked wound healing responses in vivo. In a recent study, our group showed significant differences in total composition and specific SPL subspecies in the healthy cornea compared to the diabetic cornea [30].

The 3D in vitro model described in detail here can be used in order to investigate the role of SPLs in the healthy and the diseased human cornea while providing new insights in treating ocular dystrophies with better clinical results.

2 Materials

Corneal samples obtained should only be used for scientific purposes and ethical permission must be obtained prior conducting any further experiments. The corneal tissue samples should be from donors with no history of ocular trauma or systemic disease. All reagents and media used should be completely sterile and all the protocols must be initiated in a sterile Laminar flow hood. The storage temperature of the media should be at 4 °C. Waste material should be disposed as per the proper disposal regulations.

2.1 Cell Isolation and Culture

1. Healthy corneal tissue samples from donors with no ocular trauma or systemic disease.
2. Dulbecco's Phosphate Buffered Solution (1×).
3. Sterile forceps.
4. Single edge razor blades and sterile surgical scalpel blades No. 10.
5. Eagle's Minimum Essential Medium (American Type Culture Collection, Manassas, VA, USA) containing 10% FBS and 1% antibiotic. 6.0.05% Trypsin-EDTA (1×).

2.2 3D Constructs Assembly

1. Polycarbonate membrane inserts with 0.4-μm pores (Corning Costar; Corning Incorporated, Corning, NY, USA).
2. Eagle's Minimum Essential Medium containing 10% FBS and 1% Antibiotic.
3. 0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid (Vitamin C).

2.3 Stacked Constructs

1. Sterile forceps.
2. Sterile Spatula.
3. Wax block.

4. Dulbecco's Phosphate Buffered Solution (1×).

2.4 S1P Stock Preparation

1. S1P stock solution was prepared at a concentration of 125 μ M for all S1P treatments by dissolving S1P powder in 4 mg/ml of BSA in water at 37 °C in a glass vessel.
2. SphK12 is a selective inhibitor of SphK1 [31] and a stock solution was made at a concentration of 5 mM by dissolving the powder in DMSO.

3 Methods

3.1 Cell Isolation

1. On receipt of the corneal tissue samples, the tissues should be transferred into a petri dish containing DPBS (1×).
2. The corneal epithelium and endothelium should be removed from the stroma by scraping with a razor blade.
3. The corneal stromal tissues are further cut into small pieces of size $\sim 2 \times 2$ mm and placed into T25 culture flasks.
4. Explants then should be allowed to adhere to the bottom of the flask at 37 °C for about 30–40 min and then EMEM media containing 10% fetal bovine serum and 1% antibiotic needs to be added carefully without disturbing the explants.
5. The explants should be left undisturbed until the cells begin isolating and migrating through the flask and further they require passage into T75 culture flasks upon 100% confluence after 1–2 weeks of cultivation at 37 °C, 5% CO₂.

3.2 Culture of Primary Human Corneal Fibroblast Cells and Assembly of 3D Constructs

1. HCF cells isolated from explants are cultured in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 1% antibiotic.
2. Fresh media needs to be supplied every other day for the entire duration of culture. The cultures need to be passaged upon 80–100% confluence.
3. For assembly of 3D constructs about 1×10^6 cells/well of HCF cells need to be counted and seeded on polycarbonate membrane inserts with 0.4- μ m pores (Fig. 1) (*see Note 1*).
4. The constructs need to be grown in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 1% antibiotic and after 24 h of cell seeding the cultures need to be stimulated with 0.5 mM 2-O- α -Dglucopyranosyl-L-ascorbic acid (Vitamin C) (*see Note 2*).

¹ During assembly of constructs make sure to have an even cell suspension without any cell lumps and distribute evenly throughout each well by pipetting up and down in order to avoid construct contractions.

² While preparing the Vitamin C (0.5 mM 2-O- α -Dglucopyranosyl-L-ascorbic acid) media, take about 12 ml of media to which dissolve 0.5 mM 2-O- α -Dglucopyranosyl-L-ascorbic acid and incubate it for about 15 min in order to ensure even dissolving of Vitamin C. Further filter this Vitamin C solution to the entire bottle of culture media.

5. The cultures should be maintained for 2 weeks time point and fresh media should be supplied every other day during the entire study period.

3.3 Stacking of 3D Constructs

1. 3D constructs maintained for 2 weeks are used for stacking. Firstly, the media needs to be aspirated and the constructs should be washed with sterile DPBS (1×) twice.
2. Further remove the constructs on to a wax block with sterile forceps and gently detach the edges of the membrane from the plastic with the help of a sterile spatula.
3. Now, slowly peel the ECM secreted inwards from one edge of the membrane without any breakage and transfer to a 21.5 cm² petri dish containing 2 ml of sterile DPBS (1×) (*see Note 3*).
4. Aspirate media from another construct well designated to be stacked upon and wash it with DPBS (1×) twice. Transfer the detached construct on top of the second designated construct well using forceps and ensure the transferred construct is folded inwards (*see Note 4*). Spread the construct gently with the help of forceps and spatula ensuring even attachment covering the entire construct well (*see Notes 4 and 5*).
5. Incubate the construct for about 15–20 min in about 150–200 µl culture media at 37 °C to allow proper attachment to the base construct and also to prevent floating. Gently add media to the stacked constructs without disturbing or detaching the top construct from the bottom layer.
6. Repeat stacking every other day until 8 constructs are stacked all together and change the media 3 times per week (*Fig. 2*).

3.4 Sphingolipid Analysis

1. Lipid extraction from human corneas needs to follow our previously optimized protocol [30].
2. Samples should be analyzed using targeted LC MS/MS methods. Using targeted lipidomics analysis the changes in SPLs profile in the samples should be identified.

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³.While peeling off the matrix from the membrane try to slowly roll one edge of the matrix initially in order to avoid breakage. When reached half way through the membrane can be slowly peeled off with forceps in one stroke.

⁴.When the matrix is transferred to the petri dish containing PBS it spreads out flat, exactly the way it's peeled, which clearly gives an idea about the matrix initial orientation and making it easier to identify the top and bottom of the matrix. Ensure spreading the construct the same manner as that of its initial orientation.

⁵.Ensure even spreading of the corneal matrix without any creases, this would help in avoiding contraction of the corneal matrix.

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Fig. 1. 3D constructs assembly using polycarbonate membrane inserts. **(a, b)** Polycarbonate membrane plate and respective inserts with 0.4- μ m pores. **(c)** Human corneal fibroblast grown in EMEM media containing 10% fetal bovine serum and 1% antibiotic. **(d)** Cells stimulated with media containing 0.5 mM 2-O- α -Dglucopyranosyl-L-ascorbic acid (Vitamin C)

Fig. 2. Stacking of 3D constructs. In controls the cells seeded on the polycarbonate membrane secrete an ECM of about 40 µm thickness whereas in the stacked constructs the total thickness of the ECM makes about 320 µm