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A Guide to the Development of Human CorneaOrganoids from Induced Pluripotent Stem Cells in Culture

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Abstract

The cornea is the outermost transparent and refractive barrier surface of the eye necessary for vision. Development of the cornea involves the coordinated production of extracellular matrix, epithelial differentiation, and endothelial cell expansion to produce a highly transparent tissue. Here we describe the production of multilayered three-dimensional organoids from human-induced pluripotent stem cells. These organoids have the potential for multiple downstream applications which are currently unattainable using traditional in vitro techniques.

Keywords

Cornea; Organoids; iPSC; Corneal epithelium; Corneal stroma

1 Introduction

The cornea is the outermost barrier tissue of the eye and may be viewed as a specialized skin to the eye. From a tissue engineering point of view, made up of three major resident cell types, it is a relatively simple tissue. The outer most layer of the cornea consists of a stratified epithelium, a central stroma of orthogonally stacked collagen fibril lamellae interspersed with flattened cells, the keratocytes, and an innermost single cell layered endothelium. The self-renewal potential is highest in the basal epithelial cells of the stratified epithelium, decreasing dramatically in the stromal and endothelial cells [1–3].

In development, the cornea arises from the cranial ectoderm and neural crest cells which subsequently differentiate into the Pax6 positive ocular surface ectoderm (giving rise to the corneal epithelium) and the neuroectoderm (keratocytes and endothelial cells) [4, 5]. These cell types must then coordinate to produce the highly aligned collagenous extracellular matrix necessary for the unobstructed passage of incident light [6].

The generation of induced pluripotent stem cells (iPSC) has revolutionized the field of regenerative medicine and has allowed for investigations into development and disease and

opened new avenues for therapeutic intervention [7]. In the eye, the focus has often been on the development of the retina and in this the development of optic cups has been a major advance [8–12]. Since these early investigations the field has progressed to produce ever more specific cell types [10, 13–15]. In the cornea field, there has been progress in the generation of iPSC-derived corneal epithelial cells [16], keratocytes [17], endothelial cells [18], and all cell types in 2D culture [19]. Thus, we sought to leverage the development of iPSC-derived corneal progenitors in a self-assembly model of corneal development to produce stem cell-derived corneal organoids [20]. Here we describe these techniques in detail.

2 Materials

2.1 IMR 90.4 iPSC Cell Line Culture

IMR-90.4 iPSC cells are maintained by clonal propagation on growth factor-reduced Matrigel in mTeSR1 medium under hypoxia (10% CO_2, 5% O_2) in a copper-lined incubator.

2.2 Small Molecules and Supplements

1. 10 mM all-trans-retinoic acid (ATRA): 50 mg ATRA, 16 mL of dimethyl sulfoxide (DMSO). Store at −80 °C for up to 4 months.
2. 10 mM (−)-Blebbistatin: 1 mg blebbistatin, 340 μL DMSO. Make 10 aliquots and store at −80 °C.
3. E6 supplement: 100 mL water, 7.5 g $NaHCO_3$, 97 mg insulin, 53.5 mg holo-transferrin, 320 mg L-ascorbic acid, 70 mg sodium selenite. Mix and make 10 mL aliquots into 15 mL tubes. Store in designated box at −80 °C.
4. 3 mM IWR-1-endo: 10 mg IWR, 8.1 mL DMSO. Make 50 μL aliquots and store at −80 °C.
5. 3.7 M sodium chloride (NaCl): 10.95 g NaCl, 50 mL cell culture grade water. NaCl stock solution cannot be easily filter sterilized.
6. 100 μM smoothened agonist (SAG): 1 mg SAG, 16 mL DMSO. Make 100 μL aliquots and store at −80 °C.
7. 400 mM Taurine: 500 mg taurine, 10 mL sterile cell culture grade water. Make 1.25 mL aliquots and store at −80 °C.
8. Matrigel-Growth Factor Reduced (GFR).
9. Accutase.
10. Hanks buffered saline solution (HBSS).

2.3 Medium Solutions

1. mTeSR1 medium.
2. Neural induction medium (NIM): Dulbecco’s modified Eagle medium (DMEM), 1% B27 minus vitamin A, 1 mM pyruvate, 1× nonessential amino acids

(NEAA), 1× Glutamax, 2× E6 supplement, 0.88 g/L NaCl. NaCl should raise the osmolarity by +30 mOsm to ~330–340 mOsm. Sterilize using a 0.22 μm filter.

3. Optic vesicle induction medium: NIM, 100 nM of the smoothened agonist (SAG).

4. Long-term retina maturation medium (LTR): 3:1 mix of DMEM:F12, 1% B27 supplement, 10% heat-inactivated fetal bovine serum (FBS), 1 mM pyruvate, 1× NEAA, 1× Glutamax, 1 mM taurine. LTR is sterilized with a 0.22 μm sterile filter. (For inclusion of 500 nM all-trans-retinoic acid, *see* Note 1).

2.4 Tungsten Needles for Neural Vesicle Excision

Tungsten needles are made by embedding a 0.64 mm diameter tungsten wire into a hollow glass rod cut to size with a glass cutter and mounted using epoxy resin. The tungsten wire is connected to the cathode of a gel electrophoresis DC power supply; a carbon rod is connected to the anode. Tips are immersed in a 0.5 M NaOH solution and low voltage (30 V) applied. Bubbles forming at the tip of the tungsten wire indicate active electrolytic sharpening. The tip of the tungsten wire should be repeatedly immersed in an up/down motion until the tip becomes very sharp. The first-time electrodes are sharpened it can be helpful to bevel the edges with a sharpening stone. Sharpening is recommended prior to each cutting session.

3 Methods

This method is permissive for generating retinal organoids, corneal organoids, and hybrids. It is at the discretion of the investigator to select for those organoids which meet their needs. A visual representation of this method is provided in Fig. 1.

3.1 Stem Cell Culture

1. IMR-90.4 iPSCs should be maintained in mTeSR1 medium on dishes coated with 1% (v/v) Matrigel-GFR™ at 37 °C under hypoxic conditions prior to reaggregation.

2. Replace medium every day as per standard cell culture conditions.

3. Once colonies reach 70% of a field of view under 10× magnification, treat cells with Accutase for 8–10 min.

4. Dissociate into a single cell suspension and quench Accutase activity with mTeSR1 plus 5 μM blebbistatin (B) to improve single cell survival.

5. Spin cells at 80 × *g* for 5 min.

6. Resuspended in mTeSR1 + B and plated at 5000 cells per35 mm well.

7. After 48 h, feed cells with mTeSR1 alone.

8. The entire protocol is carried out without antibiotics to minimize cell stress.

[1]·ATRA is not added to LTR between day 15 and 20; it is added fresh for each feeding after day 20.

3.2 Forced Aggregation

1. On day 1, passage cells as described in Subheading 3.1.
2. Resuspend cells in mTeSR1 containing 5 μM blebbistatin and count with a hemocytometer.
3. For a full 96-well plate, 3.0×10^5 cells are diluted in 5.0 mL of media (60,000 cells/mL) and mixed by inversion 10 times.
4. Transfer 50 μL of cell suspension (corresponding to 3000 cells) to a ChannelMate reservoir and add to each well of an ultralow attachment U-bottom 96-well plate using a multichannel pipette. This approach quickly dispenses cells and minimizes variability.
5. After plating, place cells back in hypoxia overnight at 37 °C to facilitate cell survival and aggregate formation (*see* Note 2).

3.3 Neural Induction

1. On day 2 induce forced aggregates to a neuronal phenotype by supplementing with an equal volume (50 μL) of NIM supplemented with 2% Matrigel and 6 μM IWR1-e.
2. Transfer organoids to normoxia on day 2 (5% CO_2, 20% O_2) and maintain for the duration of the experiment.
3. On days 3 and 4, feed aggregates daily by addition of 50 μL of NIM supplemented with 1% Matrigel and 3 μM IWR1-e.
4. On days 5–7, feed aggregates daily with a 50% media change (100 μL) with NIM supplemented with 1% Matrigel and 3 μM IWR1-e.
5. On day 9, feed aggregates with a 50% media change (100 μL) with NIM supplemented without matrigel or 3 μM IWR1-e. Media is changed every other day at this point.
6. On day 11, pool aggregates into 15 mL tubes and are allowed to sink (*see* Notes 3 and 4).
7. Rinse the aggregates three times with Hanks buffered saline solution (HBSS) to remove Matrigel, cell debris, and other components of NIM.
8. Resuspend in 20 mL of optic vesicle medium in ultralow attachment T-75 flasks or 15 mL of medium in untreated deep dish 10 cm polystyrene petri dishes and keep in a standard tissue culture incubator.
9. Feed vesicles every other day with >75% media exchange (*see* Note 5).

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2. It is strongly recommended to tap the sides of the 96-well dish intermittently for up to 1 h after plating to ensure that solitary organoids form.
3. To minimize damage to the aggregates we recommend using large diameter 1000 μL tips for collection or 200 μL tips which have had the lower ¼ removed to expand their smallest diameter.
4. Failure to form well-organized vesicles from between days 6 and 12 indicates a failure of differentiation which can lead to unreliable corneas. These aggregates should be discarded.

3.4 Neural Vesicle Excision

1. On days 11 and 13, feed organoids with NIM media + 100nM SAG. On days 11–15, visually identify and manually excise vesicles from the rest of the organoids using a microscope and a scissoring motion with fine-tipped tungsten needles (*see* Note 6).
2. Once isolated from the central mass, place the presumptive neural vesicles back in optic vesicle medium containing 100 nM SAG in ultralow binding T-75 flasks or untreated 10 cm polystyrene petri dishes in a standard normoxic incubator at 37 °C (*see* Note 7).

3.5 Organoid Maturation and Selection

1. On days 15–19, feed vesicles with LTR media + SAG every 2 days.
2. On day 21, feed vesicles with LTR + ATRA every 2–3 days for the duration of the experiment (*see* Note 8). By day 31, corneal organoids should be apparent by their translucent cystic appearance. These structures differ from retinal organoids or RPE organoids that have distinctive cup-shaped or darkly pigmented appearances, respectively (*see* Note 9).

3.6 Validation

Corneal organoids can be differentiated from retinal progenitors by their lack of pigmented cells, transparent appearance, and larger cystic morphology. For molecular validation we recommend assaying by immunofluorescence where good antibodies are available or a transcript level assessment by RT-PCR:

1. PAX6—Ocular phenotype.
2. P63α—Limbal epithelium.
3. KERA—Stromal keratocyte.
4. KRT14—Basal epithelium.
5. COL8A1—Bowman’s membrane.
6. Six6—Retinal phenotype (should be absent as tested by RT-PCR).
7. OCT4 and Nanog—Should be absent.

Additional information on assays and antibodies for these markers are provided in our previous work [15, 20].

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5. Organoids are visualized every day until day 10, to verify that neural vesicles budding from the central mass have formed.
6. Detailed images of the budding neuronal vesicles can be found in “Wahlin, K. J. et al. Photoreceptor Outer Segment-like Structures in Long-Term 3D Retinas from Human Pluripotent Stem Cells. Sci. Rep. 7, 766 (2017)”.
7. Too many organoids can adversely affect cell survival; thus, it may be necessary to titrate different numbers of organoids for long-term survival if other cell lines are used. A range from 20 to 50 vesicles per 10 cm dish is recommended.
8. Corneal organoids can be allowed to mature at the discretion of the researcher. It is possible to mature these organoids >120 days if so desired.
9. It is common for organoids to contain areas of dense pigmented cells and nonpigmented cells which correspond to presumptive RPE and corneal regions, respectively.

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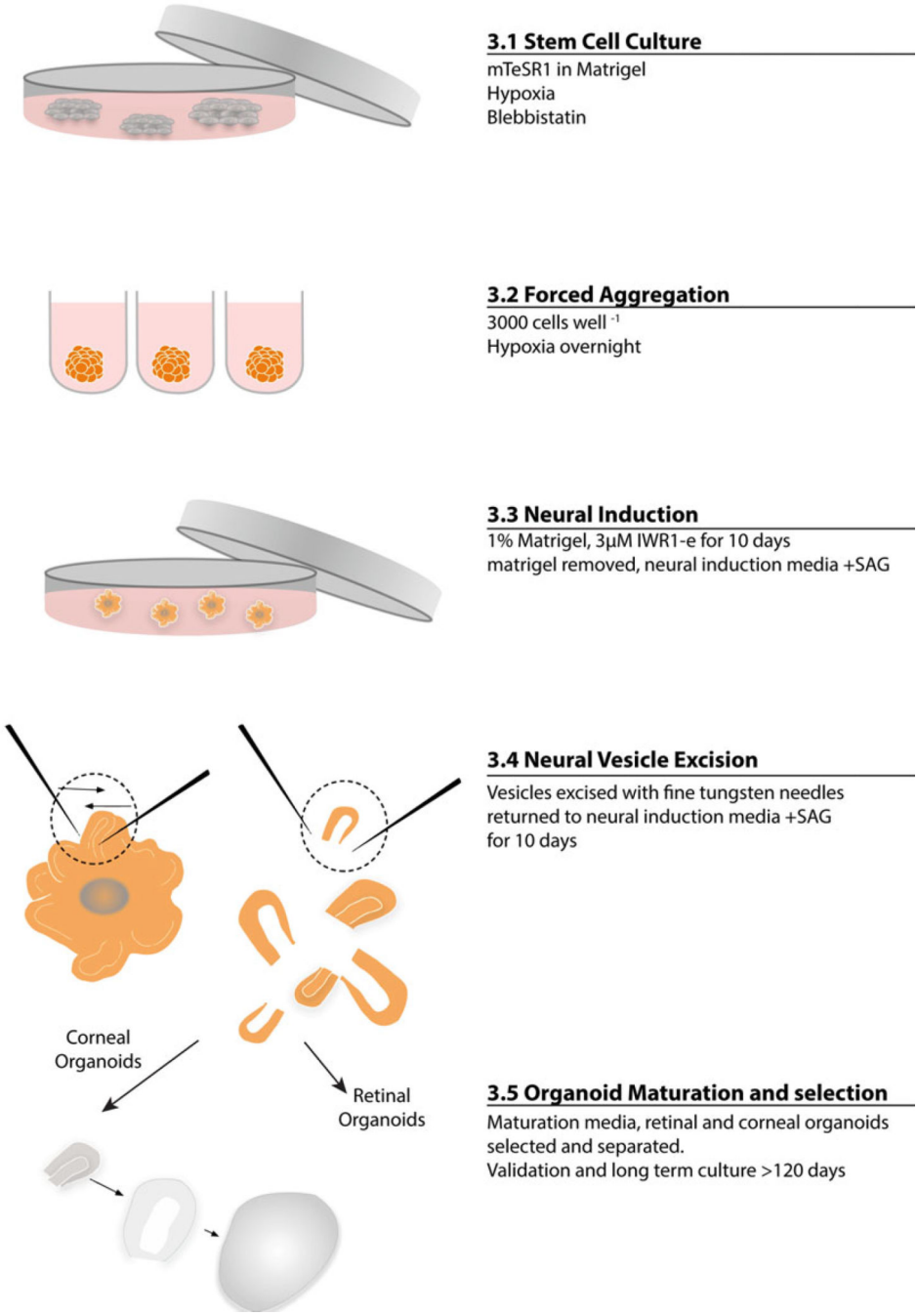

Fig. 1.

Visual representation of corneal organoid methodology. IPS cells are taken through sequential stages of differentiation from pluripotency, neural phenotype, optic induction, and finally corneal selection over 30 days