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Generating cell-derived matrices from human trabecular meshwork cell cultures for mechanistic studies

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Abstract

Ocular hypertension has been attributed to increased resistance to aqueous outflow often as a result of changes in trabecular meshwork (TM) extracellular matrix (ECM) using *in vivo* animal models (for example, by genetic manipulation) and *ex vivo* anterior segment perfusion organ cultures. These are, however, complex and difficult in dissecting molecular mechanisms and interactions. *In vitro* approaches to mimic the underlying substrate exist by manipulating either ECM topography, mechanics, or chemistry. These models best investigate the role of individual ECM protein(s) and/or substrate property, and thus do not recapitulate the multi-factorial extracellular microenvironment; hence, mitigating its physiological relevance for mechanistic studies. Cell-derived matrices (CDMs), however, are capable of presenting a 3D-microenvironment rich in topography, chemistry, and whose mechanics can be tuned to better represent the network of native ECM constituents *in vivo*. Critically, the composition of CDMs may also be fine-tuned by addition of small molecules or relevant bioactive factors to mimic homeostasis or pathology. Here, we first provide a streamlined protocol for generating CDMs from TM cell cultures from normal or glaucomatous donor tissues. Second, we document how TM cells can be pharmacologically manipulated to obtain glucocorticoid-induced CDMs and how generated pristine CDMs can be manipulated with reagents like genipin. Finally, we summarize how CDMs may be used in mechanistic studies and discuss their probable application in future TM regenerative studies.

1 Introduction

The extracellular microenvironment is a rich source of important cues (biophysical and biochemical), which are critical for cell survival, growth and differentiation, and modulates every facet of tissue development, homeostasis and disease (Jaalouk & Lammerding, 2009; Mammoto & Ingber, 2010). The extracellular matrix (ECM) is complex as it relates to its

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three-dimensional structure, composition, and intrinsic mechanical properties while also serving as a reservoir for numerous growth factors and ligands that mediate cellular responses. Any of these intrinsic stimuli can either act alone or in concert with other factors at any particular point in time to regulate a vast majority of behavioral biological responses of cells, be it biochemical and/or biomechanical. Further, dynamic remodeling of the ECM is a critical player in both homeostasis and disease (Acott et al., 2014; Raghunathan, Eaton, et al., 2017; Vranka et al., 2018), and thus has garnered much attention in biomedical research. Considering that the interactions between cells and tissues are bidirectional, an effect on the former has direct consequences on the latter and vice versa (Acott & Kelley, 2008; Vranka, Kelley, Acott, & Keller, 2015). Thus, a better understanding of cell-ECM interactions has functional consequences in disease pathophysiology, drug discovery, preclinical evaluation, and tissue engineering.

Dissecting the individual role of either cells or their ECM using a myriad of animal models, with or without genetic manipulation *in vivo* is complex, may have undesired signaling consequences, is expensive, and is rife with reproducibility issues. Thus, an *in vitro* approach is often the first step to investigate the molecular basis for ECM-cell interactions and consequences. However, most of the *in vitro* studies have focused on culturing cells either on polymeric substrates that (i) have various elastic moduli, (ii) have topographical cues, (iii) are a combination of rigid or soft polymers coated/conjugated with isolated ECM proteins, or (iv) by embedding cells within isolated polymerized hydrogels (synthetic or biopolymers) supplemented with ECM molecules (Du et al., 2016; Dupont et al., 2011; Elosegui-Artola et al., 2017; Lachowski et al., 2017; Nguyen-Ngoc & Ewald, 2013; Raghunathan et al., 2013; Schlunck et al., 2008; Wood et al., 2011). Notwithstanding, these substrates are far removed from their complete recapitulation of *in vivo* ECM. There is increasing evidence in the literature regarding the effects of biological scaffolds on numerous cellular phenotypes including but not limited to proliferation, differentiation (Engler, Sen, Sweeney, & Discher, 2006), cell division (Klein et al., 2009), cancer progression (Kai, Laklai, & Weaver, 2016), and acquisition of resistance to specific therapies (Hirata et al., 2015). Thus, an *in vitro* microenvironment that imparts the complexity of *in vivo* ECM will certainly enhance our knowledge of tissue homeostasis/disease, in improving therapeutic outcomes, and in the development of bioengineered cells/tissues/organs.

Analogous to the *in vivo* cellular microenvironment, cell-derived matrices (CDMs) represent a complex array of higher-ordered ECM molecules or structures that have accumulated/been assembled over time *in vitro*. Thus, CDMs are expected to be more faithful mimics of the complex *in vivo* ECM (Vlodavsky, 2001; Yamada & Cukierman, 2007). CDMs can be generated from different cell sources including mesenchymal stem cells (Soteriou et al., 2013), podocytes (Byron et al., 2014), hepatic stellate cells (Rashid et al., 2012), and disease-associated cells (e.g., glaucoma) (Raghunathan et al., 2018). Further, cells can also be seeded on different substrates or cultured under different conditions to simulate the altered microenvironment; for example, by utilizing cells from genetically modified animal models, small molecules/chemicals that promote pathology or homeostasis (e.g., glucocorticoids or L-ascorbic acid) (Raghunathan, Morgan, Park, et al., 2015) or growth factors (e.g., transforming growth factor β) (Raghunathan, Morgan, Chang, et al., 2015) that affect ECM deposition and morphology. Subsequently, once decellularized, CDMs may also

be modified via crosslinking (Kim et al., 2018), or functional blocking of active sites, or removing effects of certain macromolecules (e.g., removal of glycosaminoglycans) for mechanotransduction studies. All these enhance specificity and flexibility of CDMs making them suitable for different biological investigations specific to the question at hand.

However, generating CDMs that are robust require patience, adequate time, and a nuanced cell-specific approach, so they remain tethered to the slide/plate substrate or surface during removal of resident cells in culture. Moreover, though ECMs from various tissues generally consist of a similar core “matrisome,” each ECM has a distinct “matrisomal” signature (Naba, Clauser, Ding, & Whittaker, 2016; Naba et al., 2012). Thus, cell-specific optimizations for culturing and decellularization techniques are warranted to obtain physiologically relevant CDMs from different tissues/cells. Consequently, here, we provide a comprehensive protocol for obtaining robust CDMs from primary human trabecular meshwork (TM) cells isolated from diseased (i.e., glaucoma) or age-matched normal donor tissues. The TM is found at the iridocorneal angle in the anterior segment of the eye and is the principal tissue whose pathobiology results in ocular hypertension, associated with irreversible vision loss in glaucoma (Stamer & Acott, 2012). Additionally, we provide a protocol for obtaining pathomimetic CDMs either by treating TM cells in culture with glucocorticoids (for instance, dexamethasone) prior to ECM deposition (Raghunathan, Morgan, Park, et al., 2015) or crosslinking decellularized CDMs after ECM deposition for mechanotransduction investigations. Furthermore, we document some of our published findings where specific CDMs were used in ECM-cell interaction studies, causing disease phenotypes like endoplasmic reticulum stress, lysosomal stress, and stiffening of otherwise healthy TM cells (Raghunathan et al., 2018). Finally, because loss of TM cells contributes to TM tissue pathobiology in ocular hypertension (Alvarado, Murphy, & Juster, 1984; Zhu et al., 2017), we discuss the applicability of CDMs to regenerative outcomes. This encompasses CDM’s potential to provide optimal microenvironments for stem cell pluripotency and/or differentiation of TM cells.

2 Generating CDMs from trabecular meshwork cells obtained from age-matched normal or glaucomatous human donor tissues

2.1 Rationale

The TM is the primary egress for aqueous humor (Stamer & Acott, 2012), and increased resistance to aqueous drainage due to changes in TM ECM is considered a major player in ocular hypertension (Acott & Kelley, 2008; Vranka et al., 2015). *In vivo* animal models and *ex vivo* anterior segment organ perfusion cultures have provided important insights on how key isolated ECM components may contribute to dysregulated aqueous homeostasis and ocular hypertension (Haddadin et al., 2012; Keller, Bradley, Kelley, & Acott, 2008; Keller, Bradley, Vranka, & Acott, 2011; Keller, Sun, Yang, Bradley, & Acott, 2012; Raychaudhuri, Millar, & Clark, 2018; Swaminathan et al., 2014; Wang et al., 2018; Yang, Sun, Acott, & Keller, 2016). However, since increased aqueous resistance afforded by TM ECM changes is most likely a consequence of the complex interaction among its various component proteins and features (for example, fibrillary and non-fibrillary structural proteins, matricellular proteins, growth factors, glycoproteins, proteins responsible for ECM turnover, organization,

topography and stiffness), existing experimental models that focus on just one ECM component either fail to capture or entirely lose the complex association among multiple ECM factors. Furthermore, cells residing in the TM are potentially capable of altering the ECM to increase or decrease outflow during homeostasis (Acott et al., 2014; Lepple-Wienhues, Stahl, & Wiederholt, 1991). Similarly, changes in the TM ECM during pathology may subsequently contribute to reduced outflow facility and ocular hypertension through interaction with TM cells, compromising their function (Acott et al., 2014; Kasetti, Maddineni, Millar, Clark, & Zode, 2017). However, investigating the molecular mechanisms underlying bidirectional interactions, or dissecting a causal relationship between cells and their ECM in pathology, is challenging to study using animal models. On the other hand, CDMs provide a unique opportunity to perform such mechanistic studies (Cukierman, Pankov, Stevens, & Yamada, 2001). A particularly important facet of CDMs is that they possess critical protein components that serve to communicate with cells via receptor-mediated interactions (e.g., integrins, syndecans, CD44, Wnt and other canonical/non-canonical receptors) to direct cell behavior. For example, Diskin et al. proposed galectin-8 to modulate TM cell adhesion and spreading through interactions with α 2–3-sialylated glycans on β 1 integrins (Diskin, Cao, & Leffler, 2009). A number of studies have also demonstrated the role that integrins and integrin-linked kinase have on multiple TM cell functions including focal adhesion dynamics, phagocytosis and actin organization/contractility through direct interactions with multiple ECM proteins: vitronectin, fibronectin, collagen (Faralli, Filla, & Peters, 2019; Faralli, Gagen, Filla, Crotti, & Peters, 2013; Faralli, Newman, Sheibani, Dedhar, & Peters, 2011; Faralli, Schwinn, Gonzalez, Filla, & Peters, 2009; Gagen, Filla, Clark, Liton, & Peters, 2013; Zhong, Wang, & Luo, 2013). Some ECM proteins (e.g., fibronectin) are overexpressed in the TM in glaucoma and this may play a role in increased outflow resistance (Johnson et al., 1997). The extent to which overexpressed ECM may contribute to an altered morphology is poorly understood. However, it is feasible that elevated protein expression may result in a greater number of ligands being available with which cell surface receptors can subsequently interact. Mapping the distribution of ligands, or number of cell surface receptors, while difficult to ascertain *in vivo*, may be investigated *in vitro* using advanced imaging techniques such as atomic force microscopy. For example, using single cell/molecule force spectroscopy, it is possible to measure the distribution, number, and forces exerted during receptor-ligand binding on the CDMs. Thus, CDMs are a powerful tool for documenting cell-ECM interactions.

Hence, in this protocol, we describe an *in vitro* approach of how glaucomatous ECMs can be derived from cultured glaucomatous TM cells by removing the latter with an alkaline buffer and a non-ionic detergent, leaving the ECM behind. This decellularized ECM reflects several features of *in vivo* ECM with regards to interaction among its complex components and/or features. Thus, it enhances mechanistic studies involving ECM-cell interaction by seeding cells on decellularized matrices (Raghunathan et al., 2018) (see Fig. 1).

2.2 Materials, equipment and reagents

2.2.1 Materials and equipment

- T75 cell culture plastic flasks (Catalog number: 353133, Corning Life Sciences, Corning, NY)

- 15mL conical centrifuge tubes (Catalog number: 89039–668, VWR International, Radnor, PA)
- 50mL conical centrifuge tubes (Catalog number: 89039–656, VWR International, Radnor, PA)
- Centrifuge (5702, Eppendorf, Hauppauge, NY)
- Cell culture plates, 24-well (Catalog number: 3527, Corning Life Sciences, Corning, NY)
- Tissue culture treated dishes, 60mm diameter (Catalog number: 353004, Corning Life Sciences, Corning, NY)
- Round glass coverslips, 12mm diameter (Catalog number: 26023, Ted Pella, Inc., Redding, CA)
- Tweezers
- Fine-tipped sterile plastic transfer pipettes (Catalog number: 16001–192, VWR International, Radnor, PA)
- Autoclave
- Cell culture laminar flow hood
- Cell culture incubator (Forma Series 3 Water Jacketed CO₂ Incubator, ThermoFisher Scientific, Waltham, MA)
- Bright field microscope (EVOS FL Cell Imaging System, ThermoFisher Scientific, Waltham, MA)
- Hemocytometer (Catalog number: 0267110, ThermoFisher Scientific, Waltham, MA)
- Heratherm oven (ThermoFisher Scientific, Waltham, MA)

2.2.2 Reagents

- Primary human trabecular meshwork cells isolated from corneoscleral rims of normal and glaucomatous donor eyes and characterized following general consensus described elsewhere (Keller et al., 2018).
- Dulbecco's modified Eagle Medium/Nutrient mixture F-12 (DME/F-12, 1:1) (Catalog number: SH30023.02, GE Healthcare Life Sciences, Logan, UT).
- Penicillin/streptomycin/amphotericin-B (Catalog number: 15240062, Life Technologies, Carlsbad, CA, USA).
- Fetal bovine serum (FBS) (Catalog number: S11550, Atlanta Biologicals, Flowery, GA).
- Phosphate buffered saline (PBS, without calcium and magnesium) (Catalog number: SH30028.02, GE Healthcare Life Sciences, Logan, UT).

- 0.05% Trypsin-EDTA (Catalog number: 25300–054, ThermoFisher Scientific, Waltham, MA).
- Triton X-100 (Catalog number: 0694–1L, VWR International, Solon, OH).
- Ammonium hydroxide (Catalog number: AX1303–6, EMD Millipore Corporation, Darmstadt, Germany).
- DNase I (Catalog number: D3489–100MG, Spectrum Chemical, Gardena, CA).
- 3-Aminopropyl trimethoxysilane (Catalog number: 281778–100ML, Sigma-Aldrich, St. Louis, MO) or 3-aminopropyl triethoxysilane (Catalog number: A3648–100ML, Sigma-Aldrich, St. Louis, MO).

2.2.3 Reagents setup

- *Complete growth medium.* Isolated human trabecular meshwork tissues and primary cells are cultured in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DME/F-12, 1:1) with 2.5mM L-glutamine supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin/amphotericin-B. Culture media are generally stored at +4 °C.
- *Decellularization/Extraction buffer.* We normally prepare a total volume of 500mL decellularization buffer consisting of 20mM ammonium hydroxide (an alkali), 0.05% of Triton X-100 (a non-ionic detergent) in autoclaved deionized water. This is prepared by combining 1.25mL of ammonium hydroxide with 250μL of Triton X-100 in 498.50mL of autoclaved deionized water. Complete removal of cells via this decellularization buffer is achieved by effective solubilization of cellular cytoplasmic contents (by the alkali) and disruption of lipid-lipid and lipid-protein interactions (by the non-ionic detergent). To ensure sterility, this solution is prepared under the flow laminar hood. Alternatively, the buffer may be sterilized using a membrane filter. Decellularization buffer is normally stored at room temperature.
- *DNase I solution.* The final concentration of DNase I used to offset any effects of nuclear material from decellularized cell-derived matrices is 50U/mL. We normally arrive at this calculation using an average of the range of its enzymatic activity.

2.3 Protocols

2.3.1 Maintaining normal/glaucomatous human trabecular meshwork cell cultures and generating CDMs on 60mm diameter tissue culture plastics

1. Isolated and characterized normal/glaucomatous primary TM cells (method for isolation and consensus for TM cell characterization, described elsewhere; Keller et al., 2018) should be maintained in T75 tissue culture flasks in 9–10mL of complete growth medium until confluence is reached in a 37°C, 5% CO₂ incubator. Growth media changes are normally done anywhere from 2 to 3 days during this maintenance process.

2. Once confluence is reached, aspirate the growth media under the laminar flow hood, wash with 5mL of sterile PBS (without calcium and magnesium), and trypsinize cells using 3mL of 0.05% Trypsin-EDTA (Trypsinization typically takes 5–10min to complete) in a 37°C, 5% CO₂ incubator.
3. When trypsinization is complete, neutralize trypsin with approximately 7mL of complete growth media (bringing the total volume to 10mL). Transfer this mixture of cells into a 15mL conical centrifuge tube for centrifugation. After ensuring tube balance in the centrifuge, centrifuge for approximately 3.5min at a speed of 1500 rotations per minute (rpm).
4. Once centrifugation is complete, carefully aspirate the supernatant leaving the cell pellet behind. Resuspend the cells in 6mL 10% fetal bovine serum growth media, mix thoroughly using a 5mL serological pipette and a pipette gun, and count cells with any method of choice (automated or manual).
5. Seed 8000–10,000cells/cm² (approximately 150,000–200,000 cells per dish) in sterile 60 mm tissue culture treated plastic dishes under the laminar flow hood in 4mL of complete growth media. For robust and reproducible CDMs, cells of a lower passage are preferred; typically, not exceeding passage 4 for consistency. Though variation in growth rates of different cell strains may lead to different cell passages being used, the general consensus is not to exceed passage 7. Low passage cells normally have good growth rates and form confluent monolayers which are crucial for successful extracellular matrix (ECM) deposition. Sterile 60mm tissue culture treated plastics are what we normally use to obtain adequate material for real time quantitative polymerase chain reaction, proteomics, and Western blotting. See Section 2.3.3 for how CDMs are generated on 12mm round glass coverslips for immunocytochemistry and other purposes.
6. Cultured cells are then maintained in a 37°C, 5% CO₂ incubator with growth media changes on every other day. This is done for a total duration of 4 weeks to allow for sufficient and robust ECM deposition.

2.3.2 Decellularizing deposited ECM on 60mm diameter culture plastics to obtain CDMs

1. After 4 weeks of growth media changes in Section 2.3.1, step 6, aspirate approximately 3mL out of the total 4mL growth media, leaving approximately 1mL of growth media in the dish under a laminar flow hood. This is to ensure a smooth transition and exposure of the cellular environment to the decellularization buffer when added to avoid drying and prevent probable ECM detachment.
2. Denude the ECM of cells by pipetting a total volume of 10mL decellularization buffer (see Section 2.2.3) using a 10mL serological pipette, and slowly adding in steps of 3 by the edge of the 60 mm tissue culture dish in a circular fashion; add 2 mL first, then 4 mL and finally 4mL. At each step, wait for about 10 s, remove the buffer with a 1.5mL fine-tipped sterile plastic transfer pipette placed at the inner wall of the dish, and then wash with sterile PBS.

3. At the end of each of decellularization in step 2, wash with 2–3mL of sterile PBS (without calcium and magnesium) using a 10mL serological pipette by the edge of the dish, dispensing in a circular fashion. The circular fashion is to ensure mild, equal and simultaneous distribution of the PBS to prevent ECM detachment. Remove PBS using 1.5mL fine-tipped sterile plastic transfer pipette. Fine-tipped sterile transfer pipettes appear to produce the optimum suction pressure in avoidance of possible ECM detachment.
4. Repeat additional PBS washes twice more gently to ensure complete removal of the decellularization buffer and any remaining cellular debris.
5. Perform a quick inspection of the decellularized ECM under a brightfield microscope (see Fig. 2, *middle vertical panel*) under sterile conditions to ensure that all cells and debris have been removed. If complete decellularization is not achieved, repeat steps 2–4.
6. Add approximately 1.5–2mL of 50U/mL of DNase I solution at room temperature under the laminar flow hood for 1h to get rid of nuclear material.
7. Remove DNase I solution and wash with 3mL of sterile PBS (without calcium and magnesium).
8. For subsequent cell culture, prime the CDMs for 1h at 37°C with approximately 1.5–2mL of complete growth media.

2.3.3 Culturing TM cells and generating CDMs on circular 12mm diameter glass coverslips

2.3.3.1 Silanizing circular 12mm glass coverslips: Since cells innately adhere poorly on glass, glass coverslips/slides are silanized to ensure favorable culture conditions for initial cell adherence/attachment together with firm tethering of deposited ECM to glass substrates. Reagents like 3-aminopropyl trimethoxysilane (APTMS) or 3-aminopropyl triethoxysilane (APTES) can be used for silanization. There are two methods for silanization: vapor (under vacuum) and solvent based. Below is a protocol for how we silanize glass coverslips using the solvent approach prior to culturing cells on them for ECM deposition.

1. Plasma clean glass coverslips (in glass bottom petri dish) for 5min. To ensure minimal contamination, petridish should be covered once exposure is complete and subsequent procedures done under a fume hood.
2. Prepare fresh silanization solution containing 2.5% (v/v) APTES in 95% (v/v) ethanol in autoclaved water. Alternatively, APTMS can be used.
3. Add silanization solution to the glass coverslips in step 1, cover petridish and incubate for 45min in room temperature.
4. Carefully discard solution in glass organic waste bottle.
5. Rinse twice with 100% ethanol and remove ethanol.
6. Incubate at 65°C for 45min to dry. Petri dish should be covered while being taken to oven.

7. Silanized dishes may be sterilized in a biosafety cabinet by exposing to UV light for 30min.

2.3.3.2 Culturing cells on 12mm diameter glass coverslips for CDM generation

1. Similar to Section 2.3.1, step 5, seed 8000–10,000 cells/cm² on silanized 6sterile 12mm round glass coverslips in a 24-well plate under the laminar flow hood using 1.5mL of complete growth media per well.
2. Cultured cells are kept in a 37°C, 5% CO₂ incubator with growth media changes on every other day. Media changes are done for a total duration of 4 weeks to enhance robust ECM deposition.

2.3.3.3 Decellularizing deposited ECM on 12mm glass coverslips to generate CDMs

1. After 4 weeks of growth media changes in Section 2.3.3.2, step 2, remove about 1mL from the 1.5mL growth media, leaving approximately 0.5mL of growth media in each well under a laminar flow hood. The reason for this is same for Section 2.3.2, step 1.
2. Carefully transfer about six of the glass coverslips first and then their growth media into a 60mm diameter tissue culture plastic.
3. Subsequently, rid the ECM of cells following procedures described previously in Section 2.3.2, steps 2–4. Batch decellularization involving many glass coverslips in 60mm tissue culture plastics saves time and reagents compared to individual decellularizations in individual wells.
4. Once decellularization, sterile PBS washes and an inspection under a microscope are complete, transfer the circular 12mm diameter glass coverslips into a new 24-well plate.
5. Add approximately 300µL of 50U/mL of DNase I solution to each well at room temperature under the laminar flow hood for 1h to get rid of nuclear material.
6. Remove DNase I solution with 1.5mL sterile plastic transfer pipette and wash with 500µL sterile PBS (without calcium and magnesium).
7. For cells to be reseeded on the decellularized CDMs, prime with 300µL of complete growth media for 1h at 37°C.

2.3.3.4 Subsequent culture of human TM cells on generated CDMs

1. See Section 2.3.1, steps 1–3 for procedures on culturing, trypsinizing and centrifuging TM cells to obtain a pellet. Usage of low passage cells is preferred.
2. Once centrifugation is done, carefully remove the supernatant with an aspirating pipette and resuspend the cell pellet in approximately 6mL of the intended specific growth media. Mix cells thoroughly and obtain a cell count.

3. Remove the specific growth media that was used to prime generated CDMs in Sections 2.3.2 step 8 and 2.3.3.3 step 7.
4. Seed approximately 8000–10,000 cells/cm² on CDMs using the specific growth media.
5. Maintain cells in a 37 °C, 5% CO₂ incubator until specified experimental timepoints are reached for subsequent ECM-cell interaction assays (see Fig. 1, step 4).

3 Generating vehicle control- and glucocorticoid-induced CDMs from human TM cells in culture

3.1 Rationale

While non-glaucomatous eyes are relatively easy to procure, obtaining glaucomatous human donor eyes is challenging. However, pathology may be mimicked in normal cells by various means. For instance, because glucocorticoid (for example, dexamethasone) administration can lead to ocular hypertension in some individuals and can result in glaucoma if treatment is not stopped (Armaly & Becker, 1965; Lewis et al., 1988), dexamethasone-stimulated TM cells is generally recognized as an *in vitro* model of glaucoma (Clark & Wordinger, 2009). Additionally, many biochemical and biophysical TM changes underpinning ocular hypertension are similar for primary open angle glaucoma and glucocorticoid-induced glaucoma (Bollinger, Crabb, Yuan, Putliwala, & Clark, 2012; Johnson et al., 1997; Rohen, Futa, & Liitjen-drecoll, 1981; Wang et al., 2018). Thus, it is feasible to obtain pathomimetic CDMs by treating non-glaucomatous cells with dexamethasone chronically. In this section, we document a protocol for obtaining glucocorticoid-induced cell-derived matrices (GIMs) and their vehicle control cell-derived matrices (VehMs) from primary cultured TM cells isolated from normal human donor eyes (see Figs. 2 and 3). Interestingly, the elastic modulus of GIMs as determined by atomic force microscopy is similar to that of glaucomatous cell-derived matrices (GMs) (Raghunathan et al., 2018).

3.2 Materials, equipment and reagents

3.2.1 Equipment

- See Section 2.2.1

3.2.2 Reagents

- Refer to Section 2.2.2 in addition to dexamethasone (Catalog number: D4902–25MG, Sigma-Aldrich, St. Louis, MO) and its vehicle control, which is typically ethanol (Catalog number: 459844–500ML, Sigma-Aldrich, St. Louis, MO).

3.2.3 Reagents setup

- Same as Section 2.2.3.
- *Dexamethasone*. Prepare a stock solution of 1mM dexamethasone solution in 100% ethanol which serves as the vehicle control. For a final concentration of 100nM, add 1μL of 1mM dexamethasone to 10mL of growth media.

3.3 Protocols

3.3.1 Culture and treatment of human TM cells on 60mm tissue plastics with or without dexamethasone

1. After successful isolation of normal TM tissue and cells in culture (see Section 2.3.1, step 1), seed 8000–10,000cells/cm² in sterile 60mm tissue culture treated plastics under the laminar flow hood in 4mL of complete growth media per dish.
2. Keep cultured cells in a 37°C, 5% CO₂ incubator until cells are 70–80% confluent, which typically takes 3 days.
3. Remove growth media and treat cells to obtain a final concentration of 100nM dexamethasone or its vehicle control.
4. Change growth media on every other day, treating TM cells each time in the process with 100nM dexamethasone or its vehicle control for 4 weeks.
5. Periodically inspect cells under the microscope each time media is changed to monitor cell health.

3.3.2 Decellularizing deposited ECM on 60mm diameter culture plastics to obtain GIMs and VehMs—After 4 weeks of treatment, follow the same procedures previously described in Section 2.3.2, steps 1–7, to remove resident cells from their deposited ECM for characterization and subsequent cell culture. Removal of glucocorticoid-induced cells will generate GIMs whereas that of its vehicle control will yield VehMs. For subsequent cell culture on VehMs and GIMs, refer to Section 2.3.3.4, steps 1–5 (see Fig. 4).

3.3.3 Generating GIMs and VehMs on circular 12mm diameter glass coverslips

3.3.3.1 Silanizing circular 12mm glass coverslips

1. Follow procedures previously outlined in Section 2.3.3.1, steps 1–6.

3.3.3.2 Culturing cells on 12mm diameter glass coverslips for GIM and VehM generation

1. Similar to Section 2.3.3.2, step 6, plate 8000–10,000cells/cm² on silanized sterile 12mm circular glass coverslips in a 24-well plate under the laminar flow hood using 1.5mL of complete growth media per well.
2. Maintain cultured cells in a 37°C, 5% CO₂ incubator with growth media changes on every other day. On the third day, where cells are typically 70–80% confluent, aspirate growth media, and treat cells in the presence or absence of 100nM dexamethasone in complete growth media.
3. Change growth media on every other day, treating cells with dexamethasone or its vehicle control each time, for 4 weeks.

3.3.3.3 Decellularizing deposited ECM on 12mm glass coverslips to obtain VehMs and GIMs

1. When the 4-week duration in Section 3.3.3.2, step 3 is complete, follow procedures in Section 2.3.2, steps 2–4 to completely remove resident cells out of the deposited ECM and Section 2.3.3.3, steps 4–7 together with Sections 2.3.3.4, steps 1–5 for cell-ECM interaction studies.

4 Crosslinking of CDMs with genipin

4.1 Rationale

In glaucoma, the conventional outflow pathway, including the TM, is significantly stiffer than in normal age-matched tissues (Last et al., 2011; Wang et al., 2018). In the TM, stiffness may result from changes in its ECM, resident cells, both, or even in their interaction with each other (Raghunathan, Morgan, Park, et al., 2015; Schlunck et al., 2008). For instance, in TM cells, actin cytoskeletal changes may contribute to its stiffening (Clark et al., 1994; Duffy & Reilly, 2018; Hoare et al., 2009). Emergence of rho kinase inhibitors, which target actin cytoskeletal remodeling, as a new class of FDA-approved ocular hypotensive drug supports this hypothesis (Ren, Li, Le, Kopczynski, & Stamer, 2016; Williams, Novack, Van Haarlem, Kopczynski, & AR-12286 Phase 2A Study Group, 2011). In contrast, TM ECM stiffness has been attributed in part to crosslinking of its matrix components (Raghunathan et al., 2013), although direct evidence is yet to be shown. One of the means of biological crosslinking is directly by the action of specific ECM enzymes. For example, copper-dependent lysyl oxidase (LOX) (Maki, 2009; Rodriguez, Rodriguez-Sinovas, & Martínez-González, 2008) and calcium-dependent tissue transglutaminase 2 (TGM2) (Lorand, Graham, & Street, 2003) facilitate irreversible covalent cross-linking of structural ECM proteins like collagen, elastin and fibronectin. While LOX crosslinks collagen and elastin by forming highly reactive aldehydes on their lysine residues (Maki, 2009), TGM2 catalyzes the bonding reaction between the amino group of lysine and the carboxamide group of glutamine residues (Lorand et al., 2003). Consequently, these structural ECM proteins become highly resistant to proteolytic degradation/turnover leading to increased ECM deposition. Interestingly, both LOX and TGM2 are elevated in many fibrotic diseases (Cosgrove et al., 2018; López et al., 2010; Maki, 2009; Olsen, Sapinoro, Kottmann, Kulkarni, & Iismaa, 2011; Rodriguez et al., 2008; Sethi, Wordinger, & Clark, 2012; Shweke et al., 2008; Steppan et al., 2014; Vadasz et al., 2005), including glaucoma (Sethi, Mao, Wordinger, & Clark, 2011; Tovar-Vidales, Roque, Clark, & Wordinger, 2008) and is thought to contribute to tissue stiffening. For instance, single-nucleotide polymorphisms in LOX-like-1 (LOXL1), a LOX isoform, has significantly been implicated in exfoliation syndrome (Aung et al., 2017; Schlotzer-Schrehardt, 2009; Thorleifsson et al., 2007), a frequent cause of open angle glaucoma. Further, all five isoforms of LOX (LOX and LOXL1–4) are induced in TM cells by gremlin and transforming growth factor beta (TGFβ) (Sethi, Jain, Zode, Wordinger, & Clark, 2011; Sethi, Mao, et al., 2011), a profibrotic cytokine elevated in glaucoma (Tripathi, Li, Chan, & Tripathi, 1994). Moreover, induction and inhibition of ECM crosslinking with genipin and beta-aminopropionitrile (BAPN) increased and decreased outflow facility in *ex vivo* perfusion organ cultures, respectively (Yang et al., 2016). Similarly, overexpression of TGM2 in mouse eyes causes ocular hypertension by increasing

ECM crosslinking in the TM; its knock-out ameliorates ocular hypertension in TGF β 2-induced ocular hypertensive mice (Raychaudhuri et al., 2018; Raychaudhuri, Millar, & Clark, 2017). Therefore, targeting ECM crosslinking enzymes may present a viable therapeutic option to decrease ocular hypertension. However, the underlying downstream mechanisms of tissue stiffening are not fully understood and thus models to replicate such mechanical changes are essential. While CDMs with tunable stiffness have been used extensively in regenerative medicine (Kim et al., 2018), their utility in mechanistic mechanotransduction studies involving the conventional outflow pathways remains to be shown. Here, we document how generated CDMs can be cross-linked with a natural collagen crosslinker like genipin (see Figs. 5 and 6). Genipin crosslinks collagen by reacting with free amino groups on its lysine, hydroxylysine or arginine residues (Tsai, Huang, Sung, & Liang, 2000). Being a natural crosslinker derived from the Gardenia fruit, genipin is less toxic compared to glutaraldehyde, another potent crosslinking chemical (Sung, Huang, Huang, Tsai, & Chiu, 1998). Currently, we are exploring how subsequent TM cell culture on genipin-dependent crosslinked CDMs may shed light on downstream mechanistic pathways in cells.

4.2 Materials, equipment and reagents

4.2.1 Equipment

- Refer to Section 2.2.1.

4.2.2 Reagents

- Same as Section 2.2.2 in addition to genipin (Catalog number: 078–03021, Fujifilm Wako Chemicals U.S.A Corp., Richmond, VA).

4.2.3 Reagents setup

- See Section 2.2.3.
- *Genipin*. First prepare a 5% (*w/v*) stock solution of genipin, whose vehicle is typically dimethyl sulfoxide (DMSO) (Catalog number: 276855–1L, Sigma-Aldrich, St. Louis, MO) under sterile conditions. Then from the 5% stock solution, prepare 1% (*v/v*) genipin solution in sterile PBS (without calcium and magnesium) for 1 $\times$ CDM crosslinking.

4.3 Protocols

4.3.1 Generating 1 $\times$ crosslinked CDM in 60mm tissue culture plastics

1. After successfully generating pristine nuclear/cytoplasmic material-free CDMs from normal human TM cells in culture described in previous sections, add approximately 1.5mL of 1% genipin solution in PBS (see Section 4.2.3) for 5h under the laminar flow hood at room temperature.
2. Carefully remove the genipin solution with a 1.5mL fine-tipped sterile transfer pipette placed by the inner wall of the tissue culture plastic. Avoid touching the deposited matrix with the transfer pipette.

3. Wash with 3mL of sterile PBS using 5mL serological plastic pipettes placed at the edge of the culture plastic, dispensing in a circular fashion.
4. Remove PBS using a sterile transfer pipette positioned against the inner wall of the culture dish.
5. Repeat steps 3 and 4 five times.
6. Prime the generated 1× crosslinked CDM by adding about 1.5–2mL growth media for 1h at 37°C before subsequent cell culture (refer to Section 2.3.3.4, steps 1–5).

4.3.2 Generating 1× crosslinked CDMs on circular 12mm diameter glass coverslips

1. Follow exact procedures described in previous sections to obtain pristine CDMs on 12mm diameter glass coverslips.
2. For 1× crosslinked CDM, add 300µL of 1% genipin solution in PBS (see Section 4.2.3) for 5h.
3. Remove genipin solution with a fine-tipped sterile transfer pipette positioned by the inner wall of the culture plate and wash with 500µL of sterile PBS (without calcium or magnesium). Subsequently remove PBS with a transfer pipette.
4. Add about 300µL of growth media to the crosslinked CDM for 1h prior to subsequent cell culture.

5 Alternative methods/procedures

Here, using an alkali buffer with a non-ionic detergent, we present an optimized approach for removing resident cultured TM cells to generate TM-specific CDMs. This is one of the most widely used approaches thought to be harsher on cells but gentle on CDMs. However, there are a number of decellularization approaches optimized for different cell types or *ex vivo* perfusion cultures (Dang et al., 2017) and an excellent review has been reported elsewhere (Tello et al., 2016). These approaches broadly encompass using denaturing, hypotonic and chelating reagents.

6 Troubleshooting and optimization

Here, we provide some troubleshooting advice for the different kinds of CDMs whose respective protocols have been described in Table 1.

7 Application of CDMs in previous TM cell-ECM interaction studies

7.1 Previous studies involving glucocorticoid- and transforming growth factor β 3-induced CDMs

CDMs have most exclusively been generated as a means to elicit differentiation of stem cells or for tissue engineering approaches. To our knowledge there are only a handful of papers that have studied CDMs as models of disease (Amatangelo, Bassi, Klein-szanto, &

Cukierman, 2005; Castelló-cros, Khan, Simons, Valianou, & Cukierman, 2009; Van Bockstal, Lambein, Van Gele, & De Vlieghere, 2014). To our knowledge, we were the first to obtain CDMs from primary human TM (HTM) cells and use it for glaucoma investigations. Over the past few years, we've demonstrated that primary HTM cells robustly deposit anchored ECM that has been characterized by atomic force microscopy, immunocytochemistry, and proteomics (Raghunathan, Morgan, Chang, et al., 2015; Raghunathan, Morgan, Park, et al., 2015). We demonstrated that treatment of non-glaucomatous primary HTM cells with dexamethasone or transforming growth factor beta 3 (TGFβ3) results in the deposition of ECM that is more organized, stiffer, and is compositionally different than those deposited without any stimulus. A number of molecules that are upregulated in glaucoma were found to be overexpressed in the ECM of these treated cells. For example, myocilin, decorin, DKK2, fibrillin-1, fibrillin-2, thrombospondin 4 (THBS4), angiopoietin-related protein 4 (ANGPTL4), periostin and matrix-gla protein were upregulated sixfold or more in the ECM after dexamethasone treatment. We also observed elevated expression of microfibril-associated proteins (fibrillin-1, fibrillin-2, LTBP2, ADAMTSL4) in the ECM derived from dexamethasone-treated cells. Similarly, secreted frizzled related protein, periostin, gremlin, angiopoietin like protein, and plasminogen activator inhibitor protein 1 were all overexpressed twofold or greater in ECM deposited by TGFβ3-treated cells. Overexpressed in the ECM, both after dexamethasone and/or TGFβ3 treatment, periostin is a critical extracellular matrix protein whose expression levels strongly correlate with calcification, collagen deposition, collagen crosslinking and fibrillogenesis (Borra, 2009; Norris et al., 2007; Oka et al., 2007; Pohjolainen et al., 2012). Crosslinking of collagen in the ECM likely alters its biomechanical properties resulting in a stiffer ECM. Antagonists of the Wnt pathway dickkopf-related protein 1 (DKK1) (Fedi et al., 1999; Glinka et al., 1998), secreted frizzled related protein 1 (sFRP1) (Bhat, Stauffer, Komm, & Bodine, 2007; He et al., 2006), and Wnt5A (Topol et al., 2003) were also overexpressed in the extracellular milieu of dexamethasone-/TGFβ3-treated cells (Raghunathan, Morgan, Chang, et al., 2015; Raghunathan, Morgan, Park, et al., 2015). The presence of Wnt antagonists in the stiffer ECM after steroid treatment revealed a link between cytoskeletal remodeling, glucocorticoid response and ECM remodeling. Prolonged inhibition of Wnt has also been linked to loss of cell-cell communication resulting in apoptosis, reduced cellularity, or senescence (Bodine et al., 2005; Elzi, Song, Hakala, Weintraub, & Shio, 2012; Gess et al., 2008; Verma, Surabhi, Schmaltie, Becerra, & Gaynor, 2003; Zhang, Zhou, Wang, & Liang, 2013); all of which have been hypothesized to play a role in progression of glaucoma (Alvarado et al., 1984; Baleriola et al., 2008; Liton et al., 2005; Webber, Bermudez, Millar, Mao, & Clark, 2018).

Among proteins that were significantly downregulated, the most interesting one(s) were inter-α-trypsin inhibitor heavy chain 3 (ITIH3), tenascin-C, and melanotransferrin (MFI). ITIH3 is most recognized to interact with hyaluronan, a nonsulfated glycosaminoglycan, to stabilize the matrix (Zhuo, Hascall, & Kimata, 2004; Zhuo & Kimata, 2008). Hyaluronan is a major component of the TM ECM; but a role of ITIH in glaucoma, remodeling of the TM ECM has not been previously reported. Melanotransferrin (MFI), a glycosylated protein, is known to activate plasminogen activator (PA) and result in cell detachment (Michaudlevesque, Demeule, Bertrand, & Be, 2005; Rolland, Demeule, & B eliveau, 2006).

Inhibited expression of MFI combined with overexpression of plasminogen activator inhibitor 1 (PAI-1/SERPINE1) in ECM of TGF β 3 treated HTM cells suggests the likelihood of stronger cell attachment.

7.2 Previous studies involving glaucomatous and non-glaucomatous CDMs

More recently, we showed differences in CDMs between glaucomatous and non-glaucomatous donors (Raghunathan et al., 2018). Consequent to changes in ECM, we also demonstrate the latter's potent ability to modulate gene/protein expression, cellular mechanics, and resultant cellular function. One of the most significant effects was the marked stiffening of non-glaucomatous cells cultured on glaucomatous ECM. While it has been previously demonstrated by us and others in various cell types (Byfield, Reen, Shentu, Levitan, & Gooch, 2009; Engler et al., 2006; Raghunathan, Thomasy, et al., 2017; Solon, Levental, Sengupta, Georges, & Janmey, 2007) that substratum mechanical properties can modulate cell stiffness, for the first time, we showed that CDMs from diseased or normal tissues can differentially modulate cell mechanics. This was accompanied by significant overexpression of α -smooth muscle actin, a critical cytoskeletal protein implicated in fibrotic diseases (Kalluri, 2009; Kalluri, Neilson, Kalluri, & Neilson, 2003; Kalluri, Weinberg, Kalluri, & Weinberg, 2009), significant overexpression of myocilin and α B-crystallin in normal TM cells cultured on glaucomatous ECM; both genes have been associated with glaucoma and are also inducible by TGF β and dexamethasone (Welge-LuBen, May, Eichhorn, Bloemendal, & Lutjen-Drecoll, 1999; Zode, Kuehn, et al., 2011). α B-Crystallin is a small heat shock protein (HSPB5) that stabilizes polymerized actin contributing to formation of stress fibers and influences cell mechanics (Singh, Rao, Ramakrishna, Rangaraj, & Rao, 2007; Wang & Spector, 1996), and its expression is increased in glaucomatous eyes (Liitjen-drecoll et al., 1998). The role of myocilin in TM cells is unclear, although its intracellular accumulation leads to endoplasmic reticulum (ER) stress (Joe et al., 2003), apoptosis (Yam, Gaplovska-kysela, & Zuber, 2007), can result in modulation of Wnt pathway (Kwon, Lee, Ji, Rubin, & Tomarev, 2009; Shen, Ying, & Yue, 2012), and mutations in the gene can cause glaucoma (Fingert et al., 1999; Gobeil, Letartre, & Raymond, 2006; McDowell et al., 2012; Senatorov et al., 2006). We observed that on glaucomatous ECM, proteins involved in cell-cell adhesion, mRNA stability, or ER to Golgi vesicle-mediated transport were downregulated while those that regulate the ERK cascade, collagen and hyaluronan biosynthetic process, and Smad signal transduction were all upregulated in normal TM cells (Raghunathan et al., 2018). mRNA instability has been associated with a number of diseases including cancer, inflammatory disease, and Alzheimer's disease (Hollams, Giles, Thomson, & Leedman, 2002). Activation of Smad proteins is a critical part of the TGF β pathway that is implicated in TM dysfunction with aberrant expression of ECM proteins (Sethi, Jain, et al., 2011; Zode, Sethi, Chang, Clark, & Wordinger, 2011). These together suggested that normal TM cells were indeed shifting toward a "stressed" phenotype when cultured upon glaucomatous or pathomimetic matrices.

7.3 Manipulating CDMs from other cell types

CDMs have been used as substrates for cell culture *in vitro*. A number of studies have indeed reported CDM to elicit differentiation of progenitor cells. While some studies have used the unmodified CDM, others have modified the ECM enzymatically or with protein

adsorption to obtain more specific results. For example, Bhat et al. modulated the proteoglycan content of CDM enzymatically or treated the CDM with BMP2 or TGF β 1 and demonstrated that protein-binding to cell secreted ECM modulate osteoblast fate *in vitro* (Bhat, Boyadjiev, Senders, & Leach, 2011). In another study, decellularized matrices treated with chondroitinase resulted in loss of chondroitin sulfate chains in the ECM and subsequent changes to Wnt activity in mesenchymal stem cells (Hoshiba, Kawazoe, & Chen, 2014) demonstrating a bidirectional relation between intracellular Wnt activity and proteoglycans in the extracellular milieu. More recently, ubiquitous modification of CDM using click chemistry (azide-alkyne cycloaddition, Huisgen-reaction) (Ruff et al., 2017) was achieved through metabolic oligosaccharide engineering without altering the inherent molecular structure of the ECM. Employing such advanced methods would pave the way to obtain engineered constructs for studying the role of ECM proteins pathophysiology in a number of cell types.

8 CDMs as optimal substrates for regenerative outcomes in the conventional outflow pathways

In addition to ocular hypertension, a major hallmark of glaucoma is decreased TM cellularity (Alvarado et al., 1984). Though the mechanism(s) underlying TM cell loss is not fully elucidated, there is a strong relationship between cell loss, ocular hypertension, and glaucoma (Abu-Hassan, Li, Ryan, Acott, & Kelley, 2015; Yun et al., 2018; Zhu et al., 2016, 2017). Thus, a potential therapeutic strategy to restore TM's homeostasis in regulating aqueous drainage is to replace lost cells with functional ones (Castro & Du, 2019; Chamling, Sluch, & Zack, 2016; Du, Yun, Yang, & Schuman, 2013; Kelley et al., 2009; Yun, Zhou, Wills, & Du, 2016). Since the discovery of adult stem cells in the insert region of the TM almost four decades ago (Raviola, 1982), regenerative strategies to restore TM cells have only recently gained considerable focus/attention in the field. For instance, Abu-Hassan et al. (2015) demonstrated that repopulating an *ex vivo* cell depletion organ culture model with either fresh human TM cells or TM-like induced pluripotent stem cells (TM-like iPSC) restored TM's homeostatic function in regulating aqueous outflow. Using both young and old (with more extensive damage) glaucomatous transgenic mice with a human myocilin mutation, Zhu et al. (2016, 2017) showed that mice TM-like iPSC can restore aqueous outflow dynamics upon transplantation *in vivo*.

Before cell transplantation, these afore-mentioned studies characterized and differentiated stem cells into TM-like cells *in vitro* using plastic substrates and/or specialized culturing protocols/growth media with a mixture of growth factors that (i) could be expensive, (ii) are complex and time-consuming, (iii) may not be reminiscent of the native chemical or mechanical environment these cells are exposed to *in vivo*. *In vivo*, the ECM plays a vital role in maintaining stem cell pluripotency and differentiation via constant bidirectional interaction with resident cells (Kim et al., 2018). Any significant deviation from the nature and composition of this microenvironment will eventually lead to altered or compromised cellular structure and function (Ragelle et al., 2017). Consequently, although TM stem cells characterized and expanded *in vitro* on plastic substrates using specialized growth media might exhibit some aqueous homeostatic functions in the short-term once transplanted;

however, in the long-term, they may succumb to their diseased microenvironments and lose their function altogether or cause adverse effects.

Because TM CDMs are cell-type specific and possess the physical, biochemical and biomechanical factors that closely resemble the native microenvironment, they will potentially (i) engender an increased proliferative expansion of TM stem cells *in vitro* and *in vivo* upon transplantation, (ii) enhance the commitment of various sources of stem cells into TM cell lineage *in vitro* and *in vivo* and (iii) ensure that, once differentiated, TM cells retain their optimal function in the long-term *in vivo*. While the utility of CDMs as conductive substrates in regenerative medicine is well established in other tissues, their use in the TM is a relatively unexplored area. For example, an early study reported mesenchymal stem cell-derived matrices (MSCDMs) when co-administered with mesenchymal stem cells (MSCs), demonstrated better retention, facilitated the remodeling of critical sized bone defects in mice over 3 weeks (Zeitouni et al., 2012). Further, MSCDMs have been shown to enhance proliferation and maintain a better chondrocytic phenotype than cells differentiated on plastic substrates (Yang et al., 2018). Similarly, MSCDMs were shown to improve cardiac gene expression and resistance to oxidative stress (Ng, Ramasamy, Keong, & Hawa, 2019). Such examples of using CDMs to either stimulate differentiation or use in conjunction with stem cells are garnering an increased interest, thus setting a similar precedent for regenerative approaches to restore TM function.

9 Summary

In conclusion, we report here a comprehensively detailed method to generate glaucomatous CDMs from TM cells. Further, we describe how pathomimetic CDMs can be generated via pharmacological intervention or manipulated with crosslinking agents to alter their mechanical properties. We also discuss briefly a few recent studies that have manipulated CDMs by removing or quenching specific matrix proteins. Subsequently, we discuss how TM-specific CDMs have been characterized biomechanically and proteomically prior to demonstrating their feasibility as a substratum for mechanistic investigations. We conclude the chapter by envisioning that TM CDMs may be utilized in the future for regenerative applications to restore aqueous humor homeostasis.

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FIG. 1.

General workflow diagram. Summary of the three protocols documented herein for generating/characterizing CDMs together with potential mechanistic investigations. TM, Trabecular meshwork; ECM, Extracellular matrix; CDM, Cell-derived matrices; RT-qPCR, Real-time quantitative polymerase chain reaction.

FIG. 2.

Bright field images showing (left vertical panel) TM cells cultured for 4 weeks with vehicle control or dexamethasone, (middle vertical panel) ECMs decellularized with 20mM Ammonium hydroxide buffer to obtain (top) vehicle control- and (bottom) glucocorticoid-induced cell-derived matrices, (right vertical panel) generated cell-derived matrices subsequently recellularized for mechanistic investigations. Scale bar: 1000µM.

FIG. 3.

Representative immunolabeled cell-derived matrices showing major ECM proteins and complete removal of cellular (F-ACTIN) and nuclear material (DAPI). (Left vertical panel) Vehicle control- (VehM) and glucocorticoid-induced cell-derived matrices (GIM) immunolabeled for collagen IV (COLIV). (Right vertical panel) VehM and GIM immunolabeled for fibronectin (FN). Scale bar: 20 μ M.

FIG. 4.

Immunolabeled images of cell-derived matrices subsequently recellularized with TM cells for 3 days. (Left vertical panel) Vehicle control- and glucocorticoid-induced cell-derived matrices (VehM and GIM) immunolabeled for collagen IV (COLIV), filamentous actin (F-ACTIN) and DAPI. (Right vertical panel) VehM and GIM immunolabeled for fibronectin (FN), F-ACTIN AND DAPI. Scale bar: 20 μ M.

FIG. 5. Immunolabeled images of cell-derived matrices (CDM) before and after decellularization together with subsequent genipin-induced CDM crosslinking for 5h. 0% XCDM, CDM without genipin crosslinking. 1% XCDM, CDM crosslinked with 1% (v/v) genipin in phosphate buffered saline (PBS). 2% XCDM, CDM crosslinked with 2% (v/v) genipin in PBS. FN, Fibronectin; COLIV, Collagen IV. Scale bar: 20 μ M.

FIG. 6.

AFM-dependent characterization of CDMs with or without genipin crosslinking.

(A) AFM-derived images of CDMs with no crosslinking (0% XCDM), 1% genipin-induced crosslinking for 5h (1% XCDM) and 2% genipin-induced crosslinking for 5h (2% XCDM).

(B) Roughness of images in “A” quantified via root mean square (RMS). Four images were taken from random positions per sample and used for analysis. Columns and bars; means and SEM. ($N = 1$; 1-way ANOVA with Tukey multiple comparisons post hoc test; *** $P < 0.001$, for the group of interest versus control (0% XCDM); ## $P < 0.01$, for the group of interest versus “1% XCDM”).

Troubleshooting CDM generation.

Table 1

Problem	Reason	Solution
Trabecular meshwork cells die in the course of the 4-week duration of CDM deposition	Cells may be proliferating too quickly	Use cells that have a normal proliferation rate. Shorten growth media changes to every day or every other day. Increase the volume of growth media to 5mL for 60mm tissue culture plastics and 2mL for 24-well plates
Incomplete removal of cells from CDMs. Remnants of nuclear material seen as dark patches on CDMs	Duration, volume or number of decellularization buffer changes and/or DNase I may be too mild on cells	Increase duration, volume, and number of the extraction buffer changes and DNase I. Increase the number of PBS washes
CDMs detach from 60mm tissue culture plastics or circular 12mmglass coverslips	Cells were never 100% confluent prior to CDM generation	Use low-passage cells. Use cells that become 100% confluent with no spaces among them. Increase the initial seeding density of cells
	Cells did not die, but were stressed (appearance of stress vacuoles) during chronic cell culture	Increase the number and volume of growth media changes. Periodically inspect cells under a microscope during growth media changes
	Tilting, moving culture plastics and glass coverslips/plates excessively and touching CDMs with pipettes during the decellularization process	Reduce agitation of tissue culture plastic or glass coverslips to minimum during decellularization
	Using an aspirator connected to a vacuum pump whose suction pressure may be excessive for aspirating solutions during decellularization/washes. Using serological pipettes with inadequate control for aspirating or adding solutions	Use sterile plastic transfer pipettes to remove solutions when decellularizing. Alternatively, decrease the suction pressure if an aspirator connected to a vacuum pump must be used, and aspirate by tilting and removing off the sides of the dish. Avoid using serological pipettes with pipette tips for removing solutions
	Volume and number of buffer exchanges, PBS washes, or crosslinking reagent are excessive	Decrease the volume and number of buffer exchanges, PBS washes, or crosslinking reagent. However, volume of reagents should be sufficient to cover the entire surface of CDMs to avoid drying
	Adding too much liquid at once in one particular position of the culture plastic/glass coverslip during decellularization	Add liquid solutions gradually in graded steps in a circular fashion with pipettes placed at the edge of the culture plastic or well plate during cell removal
	Duration of crosslinking CDM may be too much	This may cause interactive forces among CDMs to be greater than their tethering to their substrate, hence detaching. Decrease duration of crosslinking CDM
	Tissue culture plastic was not ideal for CDM deposition. Glass coverslips/slides were either not silanized or silanization wasn't adequate	Use treated tissue culture plastics for CDM deposition (Though different treated tissue culture plastics of different diameters may be ideal for CDM generation, in our hands, the Easy-Grip 60mmtreated tissue culture plastic (Catalog number: 353004, Corning Life Sciences, Corning, NY) worked best). Alternatively, silanized rectangular glass slides in 4-well plates or glass bottom dishes can be used as a substrate for CDM deposition. Liquid silanization of glass coverslips is preferred over vapor deposition which generally is performed under inert gas in a glove box