Ex vivo 3D human corneal stroma model for Schnyder corneal dystrophy - role of autophagy in its pathogenesis and resolution

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Summary. Introduction. Multilamellar bodies (MLBs) are concentric cytoplasmic membranes which form through an autophagy-dependent mechanism. In the cornea, the presence of MLBs is associated with Schnyder corneal dystrophy (SCD). *Ex vivo* 3D modelling of the corneal stroma and SCD can help study pathogenesis and resolution of the disorder. Methods. Corneal stroma explants were isolated from cadavers and cultivated long-term for more than 3 months to achieve spontaneous 3D outgrowth of corneal stroma-derived mesenchymal stem-like cells (CSMSCs). The 3D tissues were then examined by transmission electron microscopy (TEM) for presence of MLBs, and by immunofluorescent labelling against markers for autophagy (p62, LC3). Autophagy was induced by classical serum starvation or rapamycin (RAP) treatment (50nM), and inhibited by the autophagy inhibitor 3-methyladenine (3-MA, 10 mM) for 24 hours. Results. CSMSCs can form spontaneously 3D outgrowths over a 3-4 weeks period, depositing their own extracellular matrix containing collagen I. TEM confirmed the presence of MLBs in the long-term (>3 months) 3D cultures, which became more abundant under starvation and RAP treatment, and decreased in number under autophagy inhibition with 3-MA. The presence of autophagy and its disappearance could be confirmed by an inversely related increase and decrease in the expression of LC3 and p62, respectively. Conclusions. MLB formation in long-standing CSMSC cultures could serve as a potential *ex vivo* model for studying corneal stroma diseases, including SCD. Inhibition of autophagy can decrease the formation of MLBs, which may lead to a novel treatment of the disease in the future.

Key words: Corneal stroma-derived mesenchymal stem-like cells (CSMSC), Schnyder corneal dystrophy, 3D model, Autophagy

Introduction

The cornea is the outermost layer of the eye, which serves as a protective barrier to infectious agents and provides two-thirds of its refractive power. It consists of 5 layers (from anterior to posterior): corneal epithelium, Bowman's layer, stroma, Descemet's membrane and corneal endothelium (Meek and Knupp, 2015). The epithelium is the outermost layer which undergoes heavy cell turnover (supported by the epithelial basement membrane); the endothelium is the innermost layer built from a monolayer of cells responsible for maintaining adequate hydration of the cornea (supported by the Descemet's membrane). The stroma represents 90% of corneal thickness. Under normal conditions, the stroma is transparent, however, when it comes in contact with liquids, it becomes edematous and hazy. The stromal

structure consists of collagen fibrills running parallel to the ocular surface - mainly collagen I and collagen V, which are connected or fastened together by proteoglycans. The specific arrangement of the lamellae contributes to the transparency of the cornea.

Between the lamellae are scattered flattened keratocytes/stromal cells, which are altered fibroblasts of mesenchymal origin, with long, thin cytoplasmic processes, creating a communicating 3D network. The main function of these cells in the normal stroma is to maintain its structure by producing new ECM components and secreting enzymes to degrade the old ECM (Michelacci, 2003). They also have an important role in wound healing and in keeping the cornea transparent.

Mesenchymal-derived, adult corneal stroma stem cells (CSMSCs) residing in the stroma may take part in the maintenance of the mesenchyme-derived parts of the cornea, in immune regulation of the surrounding microenvironment and in wound healing (Takacs et al., 2009; Vereb et al., 2016). They have the potential to differentiate into several different cell types, including keratocytes, chondrocytes, but also neural cells (Du et al., 2005).

Schnyder corneal dystrophy (SCD) is a rare hereditary disease characterized by bilateral abnormal deposition of intracellular and extracellular esterified and unesterified phospholipids and cholesterol in the stroma that causes subepithelial opacification posterior to Bowman's layer (Weller and Rodger, 1980). Cases can vary in clinical severity, but the loss of corneal transparency is progressive. To date, the only treatment option for SCD has been surgical, involving procedures such as phototherapeutic keratectomy (PTK), penetrating keratoplasty or deep anterior lamellar keratoplasty (Weiss, 2009; Woreta et al., 2015). SCD is often associated with hyperlipoproteinemia, but not all the patients have elevated lipoprotein levels in the serum. Transmission emission microscopy (TEM) shows multi lamellar bodies (MLBs) in the stroma, especially in the superficial layers. MLBs are round shaped cytoplasmic structures surrounded by membrane, containing multiple concentric electron dense lamellae rich in lipoprotein. Their diameter ranges from 100-2400 μm, and they are normally present in the lung in type II pneumocytes, where they take part in the storage and secretion of surfactant. They can also appear in different cell types under pathological conditions, most often in lysosomal storage diseases, including gangliosidosis, Tay-Sachs, Fabry and Niemann-Pick (NP). The formation of MLBs is autophagy-dependent (Hariri et al., 2000). Autophagy is a controlled degradation of sequestered cytoplasmic components by lysosomal hydrolytic enzymes. It is necessary to maintain cellular homeostasis under normal conditions and in response to stress. Increased autophagy serves a cell protective role. The lysosomal nature of autophagic vacuoles (AVs) and MLBs indicate a similar pathway in their formation. The development of MLBs

in long-term CSMSC cultures indicates that the cells undergo an intense catabolic degradation, while building and maintaining a 3D structure. To examine whether this could be an autophagy related mechanism, the cells in the 3D corneal stroma outgrowth were treated by 3-MA, which suppresses autophagy by blocking the synthesis of autophagosomes at the early stages of autophagy.

The present study establishes a spontaneously formed 3D model of human corneal stroma *ex vivo* in which long-term cultivation is used to induce MLBs in the CSMSCs, thus serving as a model for studying SCD. The effect autophagy has upon MLB formation and dissappearance is further studied by using autophagy-inducing agents (rapamycin (RAP) and starvation), as well as autophagy-inhibiting agent 3-MA. The findings can further serve the purpose of determining drug targets against SCD.

Materials and methods

Corneal stroma harvesting

All tissue collection complied with the Guidelines of the Helsinki Declaration and was approved by the Regional Ethical Committee at the University of Debrecen, Hungary (DEOEC RKEB/IKEB 3094/2010 and 14415/2013/EKU-183/2013). Hungary follows the EU Member States' Directive 2004/23/EC on presumed consent practice for tissue collection. Corneas were obtained from human cadavers within 24 hours of biological death. The bulbus and the cornea were prepared as previously described by our group (Szabo et al., 2015). Briefly, the bulbi were disinfected by 5% povidon iodide (Betadine, Egis, Budapest, Hungary) and after removing the conjunctiva, a corneal button was cut out. The epithelium with the Bowman's membrane and endothelium with the Descemet's membrane were removed by scraping. Small, approximately 6-7 mm diameter cubes were cut out from the central cornea. Isolation and culturing of CSMSC's was performed using three different donor eyes. Average age of the cultures was 239±130 days (n=3) and average ages of donors was 60±11.5 years.

Cell culturing and treatments

The central corneal grafts were seeded into 24-well cell culture plates, cultivated at 37°C, 5% CO_2, in low glucose DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) and 1% Antibiotic/Antimycotic Solution (PAA, Pasching, Austria). The cells were expanded in 1 mL of medium changed on alternating days and maintained without passaging.

To induce autophagy 50 nM RAP (Sigma-Aldrich) treatment and serum starvation (serum-free media) was used respectively for 24 hours. To inhibit autophagy the cultures were treated with 10 mM 3-MA (Sigma-

Aldrich), an inhibitor of autophagy, for 24 hours.

Immunofluorescent staining

Long-standing cultures of CSMSCs were collected and fixed in 4% paraformaldehyde after peeling off the cell culture plates using surgical forceps. The fixed 3D structures were dehydrated in ascending alcohol series and embedded in paraffin; 3-4 μm thick tissue sections were prepared using a rotary microtome, then mounted onto histological slides. After heat-induced antigen retrieval and blocking, immunofluorescent labelling was performed. The samples were stained for autophagy markers: microtubule-associated protein 1 light chain 3 (LC3), p62 protein also called sequestosome 1 (SQSTM1) and extracellular matrix proteins: type I collagen, type IV collagen, type V collagen. Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI) staining. (Table 1 summarizes the primary and secondary antibodies used for immunohistochemistry). Fluorescent images were taken by a ZEISS Axio Observer. Z1 microscope (ZEISS, Oberkochen, Germany) and an EVOS FL microscope (Advanced Microscopy Group, Bothell, WA). For image handling ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD) was used.

Transmission electronic microscopy

TEM was performed to analyze the ultrastructure of the 3D CSMSC cultures. After peeling the membrane, the tissue was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde with 0.2 M cacodylate buffer for 24 hours. The samples were then rinsed with PBS three times for 5 minutes each and kept at 4°C. Before Epon (Electron Microscopy Sciences, Hatfield, PA, USA)
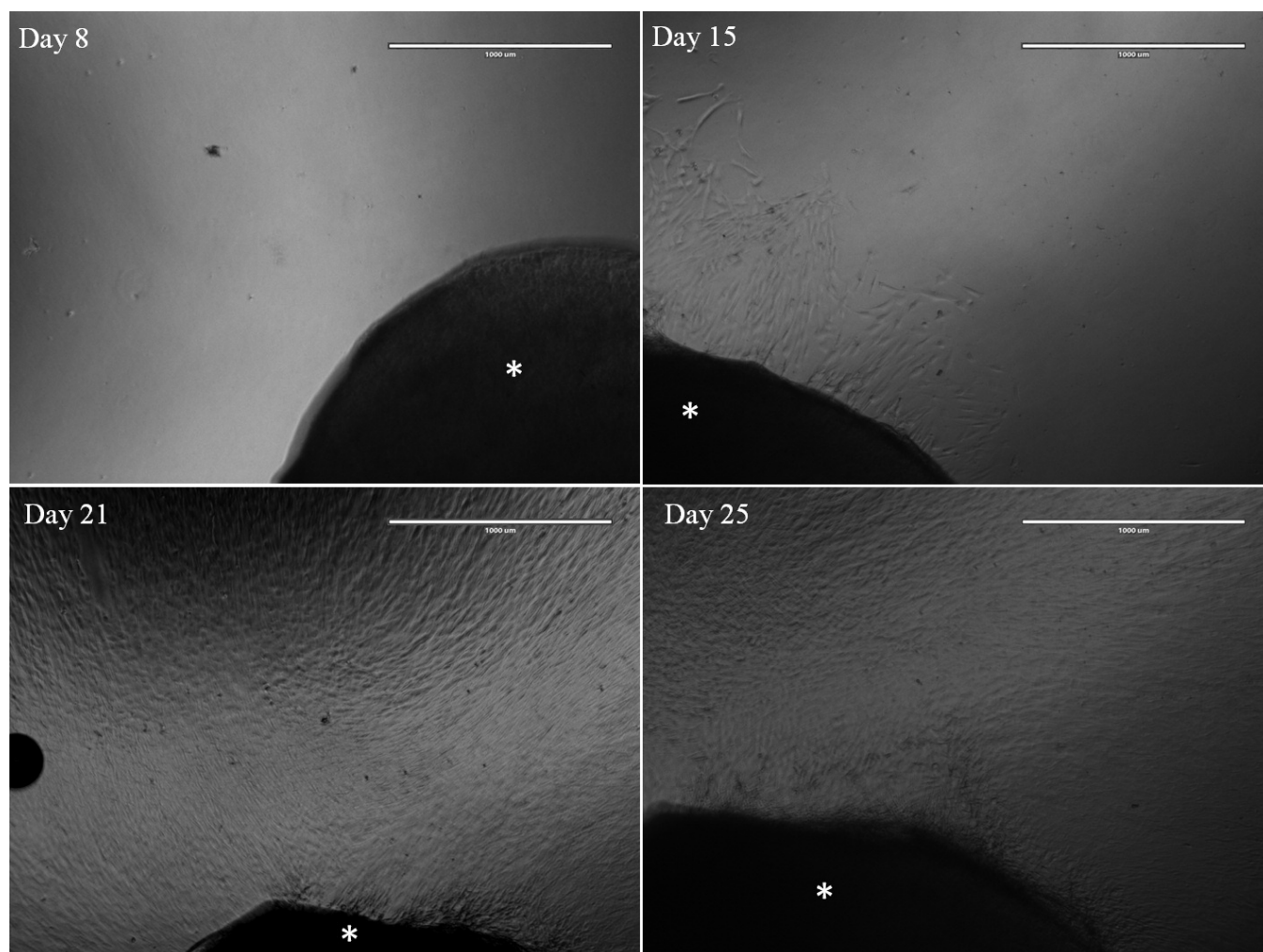
Fig. 1. Corneal stromal cell outgrowth from explants (*) cultivated *ex vivo*. Representative images of long-term cultivated cells are shown; 3D layering of the cells becomes noticable within 3-4 weeks (Day 25 in the images shown). Scale bars: 1000 μm.

embedding the samples were post-fixed in 1% osmium tetroxide, dehydrated in ascending alcohol series and briefly immersed in propylenoxide. A Leica Ultracut Ultramicrotome was used to cut ultra-thin (60-70 nm) sections which were stained with uranyl acetate and lead cytrate. The images were taken by a Tecnai 12 transmission electron microscope (Phillips, Amsterdam, the Netherlands).

Results

Phase contrast images of *ex vivo* cultivated corneal stroma grafts showed cell outgrowth from the explant assuming fibrosblastic morphology within 2 weeks of cultivation, and continuous monolayer formation during the weeks 2-3. Upon reaching confluence, the cultures underwent stratification and formed a 3D structure after 4-5 weeks (Fig. 1).

Immunofluorescent staining of long-term 3D stroma showed *de novo* ECM deposition abundant in multilamellar collagen type I, similar to the tissue of the native corneal stroma (Fig. 2). In contrast, type IV and V collagen did not show positivity in the samples (data not shown).

TEM results show heavy MLB formation, with AVs being seen in the untreated long-term cultivated corneal stroma. The effect of RAP treatment and serum starvation enhanced the expression of MLBs and AVs, while the 3-MA treatment attenuated it. The mitochondria seem to be intact under 3-MA treatment also (Fig. 3).

Similarly, immunohistochemical analysis of typical autophagy markers LC3 and p62 showed that before treatment, the 3D corneal stroma tissues contained positive AVs. Further induction of autophagy by RAP treatment and serum starvation enhanced the amount of AVs, while treatment by 3-MA resulted in attenuated LC3 and p62 expression, and therefore presence of decreased number of AVs (Fig. 4).

Discussion

The corneal stroma is a specially organized tissue that serves as a clear, refractive medium. Anything

Table 1. Summary of antibodies used for immunohistochemistry.

| Antibody	Catalog No.	Company	Clonality	Antigen	Dilution	Secondary antibody	
						species	dilution
LC3	NB100-2220	Novus Biologicals	Rabbit polyclonal	MAP1LC3B	1:200	Goat	1:1000
p62 (SQSTM1 (D-3))	Sc-28359	Santa Cruz	Mouse monoclonal	amino acids 151-440 of SQSTM1 of human origin	1:200	Donkey	1:1000
Collagen I.	Ab34710	Abcam	Rabbit polyclonal	Human collagen I. AA 1-1464	1:200	Donkey	1:1000
Collagen IV	#MS-747-S	Thermo Scientific	Mouse monoclonal	Human glomeruli	1:50	Donkey	1:1000
Collagen V	Ab7046	Abcam	Rabbit polyclonal	Full length native protein (purified) corresponding to collagen V. AA 1-1745	1:500	Donkey	1:1000
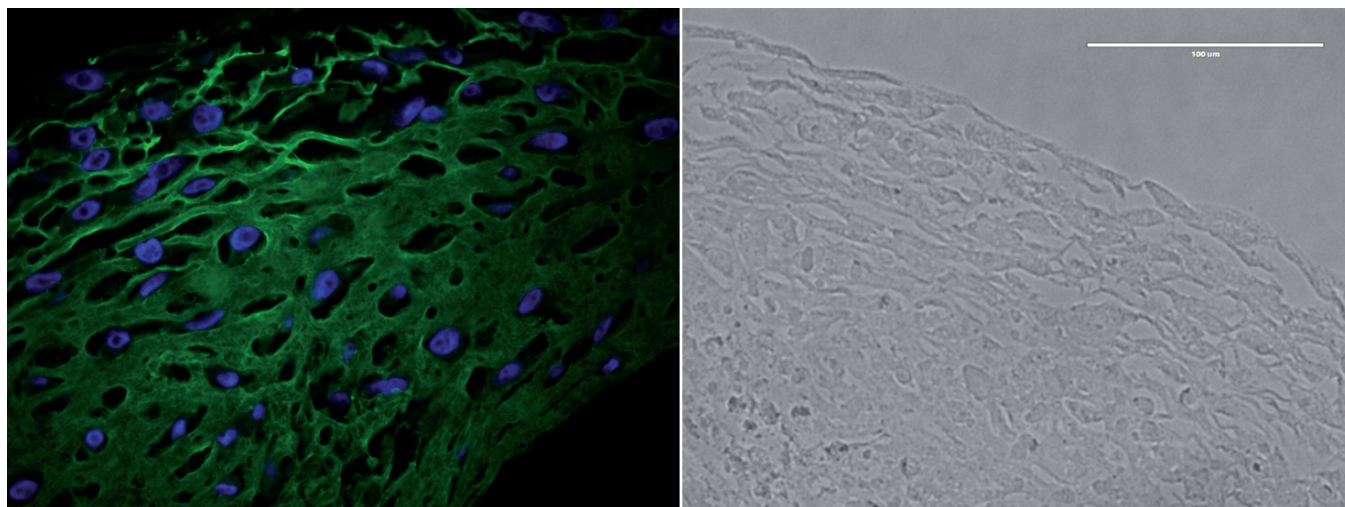
Fig. 2. *De novo* ECM deposition of corneal stroma cells after long–term cultivation. Immunofluorescent staining for collagen I (left, antibody dilution: 1:200, secondary antibody: anti-rabbit, wavelength: 488 nm) and phase contract image (right) are shown accordingly. Left, x 63; right, x 40.

disturbing its anatomy is prone to causing reduced transparency and therefore, impaired vision. The transparency of the cornea is a consequence of the detailed ultrastructure of the tissue and depends upon the organization of type I collagen fibrils which, in the cornea, are narrower than in many other connective tissues. This arrangement is sustained by the association of type I collagen with other ECM molecules such as type V collagen, fibril-associated collagens with interrupted triple helices (FACITs) and small leucine-rich proteoglycans (SLRPs). Beside these, other cellular components such as the crystallins are also important for
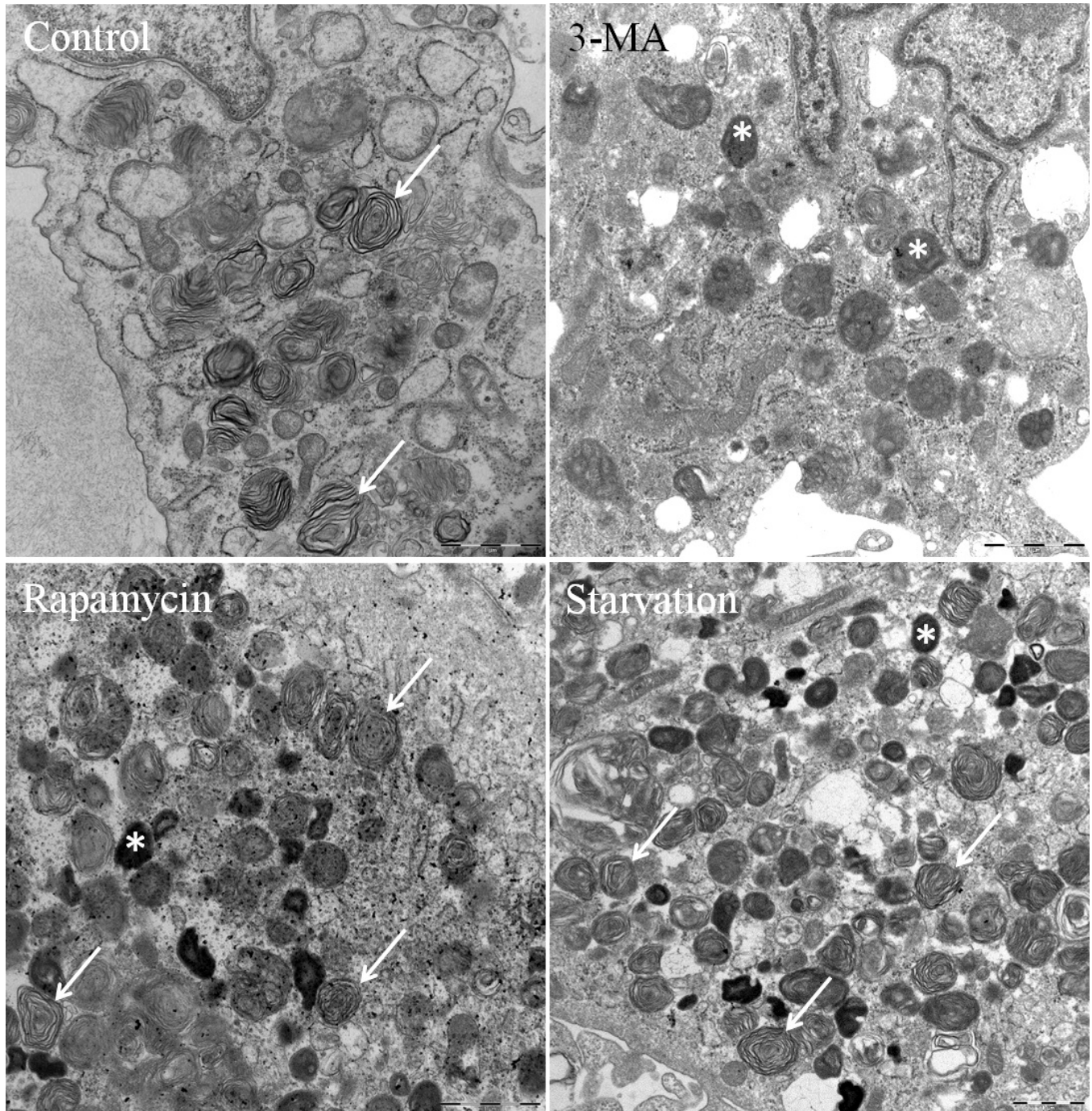
Fig. 3. TEM of 3D long-term cultures of human corneal stroma and presence of MLBs (arrows) and autophagic vacuoles (*). Scale bars: 1 μm.
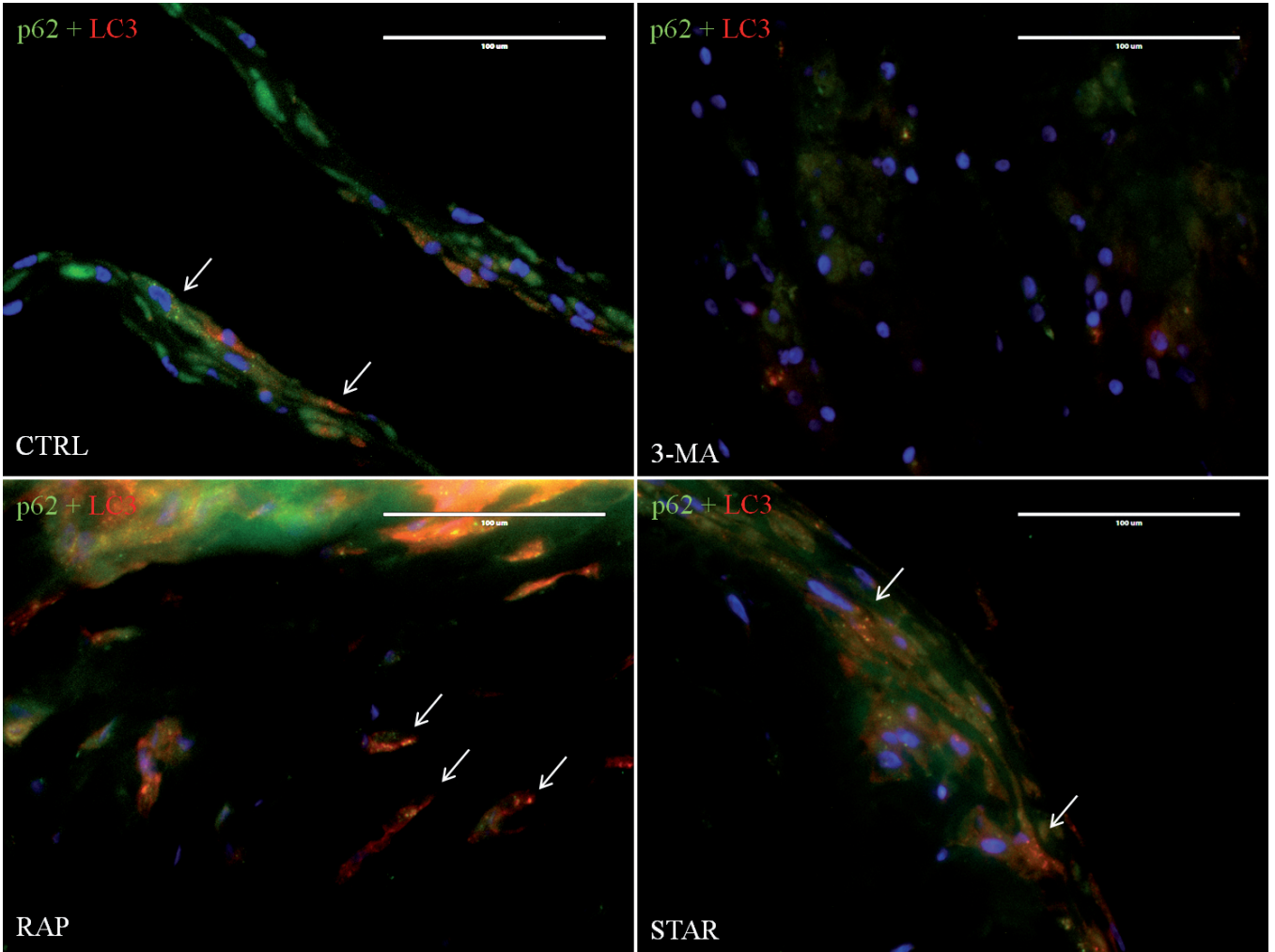
Fig. 4. Immunohistochemical analysis of the 3D corneal stroma tissue for presence of autophagy. Scale bars: 100 μm.
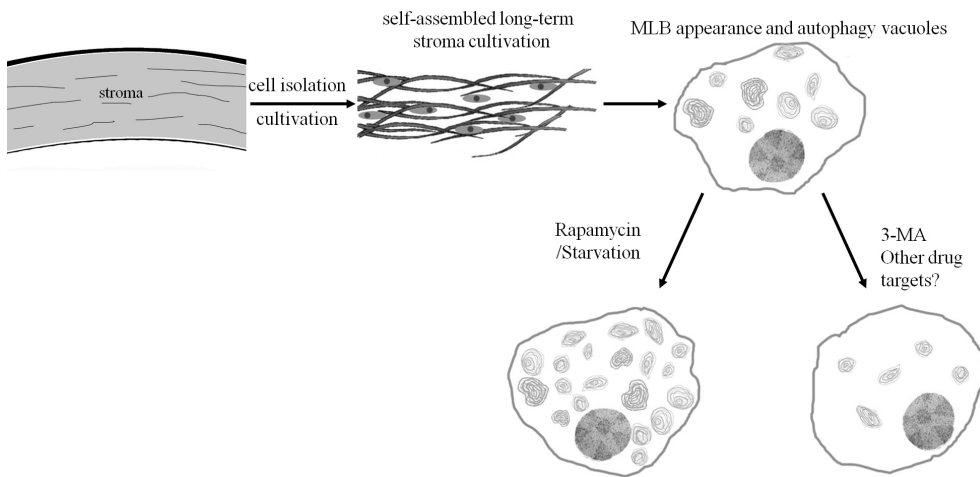

Fig. 5. Proposed mechanism of Schnyder corneal dystrophy formation and cleareance of MLBs – potential implication for drug development.

maintaining corneal transparency, as they confer transparency to the stromal resident cells – the keratocytes. Disruption of the corneal stroma type I collagen organization can lead to corneal transparency complications such as corneal scarring (Meek and Fullwood, 2001; Meek and Boote, 2004; Massoudi et al., 2015; Meek and Knupp, 2015).

It has been known that cultivation of keratocytes in serum-containing media generates differentiation into fibroblasts and partly myofibroblasts (Jester et al., 1996). Corneal fibroblasts can create a self-assembled 3D structured tissue consisting of collagen type I (in serum and ascorbic acid supplemented media) that shows close histological resemblance to human native cornea stroma (Proulx et al., 2010). These 3D human fibroblast cultures grown over long-term on a disorganized collagen substrate have been found to have proteoglycan deposition and sulfation throughout the produced multilayered structure (Ren et al., 2008). TEM images of such cultures after 8 weeks reported no intracellular changes or presence of MLBs or any other storage or clearance pathways' activation.

Recently, CSMSCs isolated from the cornea limbal stroma and cultivated in selective stem cell media have been shown to be able to secrete a well-organized ECM rich in collagen type I, V and VI, which had greater resemblance to native stroma tissue than keratocyte-derived corneal fibroblasts expanded in serum containing media (Wu et al., 2014). Similarly, we recently characterized cultured CSMSCs isolated from the central part of the corneal stroma and showed their surface marker profile regarding mesenchymal- and stemness related markers (CD73, CD90, CD105, CD140b), and lack of hematopoietic markers. The keratocyte marker CD34 appeared to be negative for over 10 passages in these cells (Vereb et al., 2016).

An intact and well tangible 3D collagen structure synthesized by the central corneal stroma-derived CSMSCs could be maintained over long periods of time with intracellular changes becoming apparent in the morphology of CSMSC, which is similar to that seen in SCD, as large amounts of MLBs accumulate in the cells' cytoplasm. Likely, the long-term cultures undergo spontaneous intracellular changes resemblant of MLBs and that of SCD - a rare autosomal dominant corneal dystrophy characterized by progressive, abnormal cholesterol and phospholipid depositions in the cornea. The diagnosis of SCD is based on observation of crystals and/or stromal haze, in older patients accompanied by arcus lipoides. The condition is slowly progressive and leads to severe reduction of visual acuity in most cases aged 50 and more. Gradual loss of corneal sensation may also be present. About 50% of the cases show corneal crystals, while in the acrystalline form of the dystrophy these deposits are missing, which can cause difficulties and delay in the diagnosis (Weiss, 2009).

Genetic studies show that SCD is caused by mutations in the *UBIAD1* gene located on chromosome 1p36. This gene encodes a protein linked to lipid metabolism (Orr et al., 2007; Weiss et al., 2007). The structure of MLBs suggests a local lipid metabolic disturbance in the pathogenesis of SCD. To date, no treatment modality is available for stopping or slowing down SCD progression, nor is there a way to rid the cells off the MLBs.

MLBs are actually lysosomal organelles expressed under various physiological and pathological conditions, which contain multiple concentric membrane layers that are found in various cell types, where their main function is storage and secretion of lipids. The role of autophagy is crucial in MLB biogenesis under normal cellular function, and it has been shown that inhibition of autophagy with 3-MA can block MLB expression in the lung in type II alveolar Mv1Lu cells transfected with Mgat5 (Hariri et al., 2000). Stimulation of autophagy by serum starvation can result in an increased size of MLBs in NP type C-mimicking drug U18666A-induced cholesterol-rich lysosomal vacuoles (Lajoie et al., 2005). All cell types found in the eye rely upon replenishment or regeneration through removal of destroyed/unused proteins and cell organelles. Autophagy has an essential role in maintaining the physiological state and balance of the eye (Petrovski et al., 2012). Autophagy is responsible for the maturation of lens fiber cells, removal of shed outer segments from photoreceptors in the retinal pigment epithelium (RPE); moreover, it is involved in mechanisms described in the pathogenesis of dystrophies affecting the corneal endothelium (Frost et al., 2014).

The TEM results demostrate that autophagy inhibition reduces the number of MLBs and leads to formation of dense vacuoles that are processed or degraded through the MLB pathway. In accordance with the previous findings, when cells were treated with RAP or exposed to serum starvation, the rate of autophagy increased in the 3D corneal stroma outgrowing cells, resulting in greater number and slightly enlarged MLBs. The TEM results were further supported by immuno-fluorescent staining for the markers of autophagy (LC3 and p62), showing punctated appearance of the autophagic vacuoles under RAP and starvation treatment, while the decreased immunopositivity of the autophagy markers indicates the presence of moderate autophagic activity.

Defects in the autophagy clearance systems have been known to cause pathogenic changes in certain diseases; in type 2 granular corneal dystrophy (GCD2), accumulation of mutant TGFβ-induced protein (TGFβIp) causes hampered autophagic clearance and ultimately vision impairment (Choi et al., 2012). It was later reported that corneal fibroblasts from homozygous GCD2 patients exhibit a significantly higher LC3-II and p62 expression compared to wild type fibroblasts (Choi and Kim, 2015). When wounded, quiescent corneal stromal keratocytes become activated and undergo transdifferentiation into myo/fibroblasts through activation of the TGFβ signalling. Scar tissue formation can lead to disturbed ECM formation, and eventually

hazy cornea (Hassell and Birk, 2010).

Our long-term cultivated CSMSCs for over 3 months and without any passaging, have been hereby considered as nearly as close a model for studying corneal dystrophy as the *in vivo* conditions. The process of producing and self-assembling the corneal stroma *ex vivo*, with strong expression of collagen type I, and the evidence for presence and inhibition of autophagy in this 3D structure, thus decreasing the formation of MBLs (summarized in Fig. 5), can serve the role of slowing down the progressive loss of stromal transparency in SCD or any other condition involving MLBs and/or autophagy degradation pathway involvement.

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