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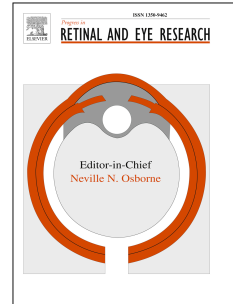

Posterior capsule opacification: What's in the bag?

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Posterior Capsule Opacification: What's in the Bag?

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Posterior Capsule Opacification: What's in the Bag?

Abstract

Cataract, a clouding of the lens, is the most common cause of blindness in the world. It has a marked impact on the wellbeing and productivity of individuals and has a major economic impact on healthcare providers. The only means of treating cataract is by surgical intervention. A modern cataract operation generates a capsular bag, which comprises a proportion of the anterior capsule and the entire posterior capsule. The bag remains in situ, partitions the aqueous and vitreous humours, and in the majority of cases, houses an intraocular lens (IOL). The production of a capsular bag following surgery permits a free passage of light along the visual axis through the transparent intraocular lens and thin acellular posterior capsule. Lens epithelial cells, however, remain attached to the anterior capsule, and in response to surgical trauma initiate a wound-healing response that ultimately leads to light scatter and a reduction in visual quality known as posterior capsule opacification (PCO). There are two commonly-described forms of PCO: fibrotic and regenerative. Fibrotic PCO follows classically defined fibrotic processes, namely hyperproliferation, matrix contraction, matrix deposition and epithelial cell trans-differentiation to a myofibroblast phenotype. Regenerative PCO is defined by lens fibre cell differentiation events that give rise to Soemmerring's ring and Elschnig's pearls and becomes evident at a later stage than the fibrotic form. Both fibrotic and regenerative forms of PCO contribute to a reduction in visual quality in patients. This review will highlight the wealth of tools available for PCO research, provide insight into our current knowledge of PCO and discuss putative management of PCO from IOL design to pharmacological interventions.

Key words: Posterior capsule opacification; Lens; cataract surgery; fibrosis; tissue regeneration; stem cells

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1. Introduction

Cataract - a clouding of the lens - is the most common cause of blindness in the world. It has a marked impact on the wellbeing and productivity of individuals and carries a major economic cost to healthcare providers (Brown et al., 2013; Resnikoff et al., 2004). Appropriate dietary intake and behavioural traits can help protect the lens and delay cataract formation, but ultimately cannot prevent the appearance of cataract if an individual is blessed with longevity. Recent reports in high profile journals have suggested agents could be used to treat nuclear cataract through disaggregation of crystallins (Cleary et al., 2010; Zhao et al., 2015), but this putative treatment still requires significant development and evaluation before these agents can be considered a reasonable therapeutic option. In the near future, the only means of treating cataract is by surgical intervention, which initially restores high visual quality. Unfortunately, a secondary loss of vision, termed posterior capsule opacification (PCO), develops in a significant proportion of patients post-surgery (Nibourg et al., 2015; Wormstone and Eldred, 2016). The text that follows aims to provide a background of events following cataract surgery, the study systems that can inform and lead to better understanding of the condition, the regulatory mechanisms involved and efforts directed to improve the management of PCO.

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1.1 Cataract surgery

A modern cataract operation involves making a small incision in the sclera or cornea to permit introduction of surgical tools with minimal physical disruption to the eye. An opening in the lens is made by continuous curvilinear capsulorhexis (capsular tear) in the anterior capsule. This is usually achieved with a capsulotome, however, femtosecond laser is also adopted by some surgeons, which in principle provides an anterior opening that is more consistent in size and position (Abouzeid and Ferrini,2014). This circular window in the

anterior capsule allows access to the central lens fibres, which are usually removed by phacoemulsification. In some cases, this process is assisted by femtosecond laser treatment and if required, traditional hydrodissection methods can be used. Residual fibre cells are removed by irrigation/aspiration. The product of cataract surgery is a capsular bag. This comprises a portion of the anterior and the entire posterior capsule. The bag remains in situ, partitions the aqueous and vitreous humours, and in the majority of cases, houses an intraocular lens (IOL). The production of a capsular bag following surgery permits a free passage of light along the visual axis through the transparent IOL and thin acellular posterior capsule (Figure 1A).

1.2 Post-surgical wound-healing and PCO

Despite the initial success of cataract surgery, it has long been known that nature fights back (Eldred et al., 2011; McDonnell et al., 1983; Nibourg et al., 2015; Saika, 2004; Wilhelmus and Emery, 1980; Wormstone and Eldred, 2016; Worst, 1977). Cataract surgery, however skilfully performed, is a controlled trauma to the eye. This disruption to the integrity of the eye invokes a raft of environmental and physical changes that instigate a wound-healing response. This fundamentally centres on an attempt to repair the damaged tissue, which in this case is the lens. Despite the physical stresses and strains placed upon the lens during cataract surgery, a proportion of the lens epithelium remain viable (Figure 1A). These surviving cells start to repopulate denuded regions of the anterior capsule, followed by growth over all available surfaces, including the IOL surface; the outer anterior capsule; and of primary importance the previously cell-free posterior capsule (Figure 1B). Cells continue to divide, begin to cover the posterior capsule and can ultimately encroach on the visual axis. A homogenous thin cover of cells is unlikely to affect the light path significantly, but subsequent changes to the matrix and cell organisation can give rise to light scatter. The most

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common changes with regard the all-important visual axis result from fibrosis: this is generally associated with matrix deposition, wrinkling/contraction of the posterior capsule, increased cell aggregation and increased presence of myofibroblasts (Figure 1B). This is referred to as the fibrotic form of PCO (Eldred et al., 2011). It is likely all patients undergo some level of fibrosis in response to surgery. In time, a number of patients will also present features attributed to a second category of the condition, known as regenerative PCO (Figure 1C). This is characterised by the formation of swollen globular cells known as Elschnig's pearls, which are believed to be the product of aberrant differentiation. These structures can encroach upon the visual axis and cause significant visual disruption (Findl et al., 2010; Kappelhof et al., 1987; Sveinsson, 1993; van Bree et al., 2011). In addition, lens fibre differentiation can occur in the peripheral capsular bag and is known as Soemmerring's ring (Findl et al., 2010; Kappelhof et al., 1987; van Bree et al., 2011). While both fibrotic and regenerative PCO contribute to visual disturbance, the regenerative form is the most severe (van Bree et al., 2011). Additional features can also contribute to visual deterioration, including cell growth on the IOL surface, anterior fibrosis and anterior capsular phimosis (Mullner-Eidenbock et al., 2001; Sciscio and Liu, 1999; Waheed et al., 2001). Ultimately, if these changes are sufficiently severe then visual impairment will ensue and corrective Nd:YAG laser capsulotomy surgery will be required.. Recent reports suggest the proportion of patients requiring laser capsulotomy three years after standard surgery is between 5 and 20% (Leydolt et al., 2020; Ursell et al., 2020). However, these figures are influenced by a number of factors, which include type of IOL implanted, age of patient and ocular co-morbidity.

2. Investigating PCO

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A multitude of approaches can be employed to gain an understanding of the regulatory processes that drive PCO and to evaluate and develop management strategies. These tools range from pure clinical data to simple cell cultures. Each approach has its benefits and limitations and it is important to consider these relative properties when predicting outcomes in patients, both human and animal. In the following section, a range of study tools will be introduced that will provide context for later sections that will describe our current understanding of PCO and the application of this information for PCO management.

2.1. Clinical data

Clinical trials performed to evaluate intraocular lenses provide indication of the influence different IOLs may have on PCO (Leydolt et al., 2020; Perez-Vives, 2018; Ursell et al., 2020). Many of these studies and retrospective analysis of such studies use Nd:YAG capsulotomy as an indication of PCO incidence. The majority of clinical studies have exclusion criteria to allow better comparison of data sets, therefore probably underrepresent the incidence of PCO across the general population. To aid assessment of PCO progression prior to Nd:YAG capsulotomy various imaging systems have been developed, with retroillumination the most commonly used for monitoring PCO in patients (Hollick et al., 2000; Leydolt et al., 2020). To better utilise the images and establish numerical assessment, analysis software such as EPCO and AQUA II (Kronschlager et al., 2019) have been developed. Typical clinical trials capture patient information at significant time-points, e.g. 1 month, 3 months, 6 months, 1 year, 2 years, 3 years which enables general patterns to be observed, including (but not limited to): PCO scale and type (fibrotic and/or regenerative PCO); IOL decentration; IOL tilt; visual axis opacification (Ursell et al., 2020, Leydolt et al., 2020 (Ale, 2011)). These studies provide general patterns and outcomes, but understanding the subtle stages of progression requires more frequent monitoring (Leydolt et al., 2020;

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Ursell et al., 2020). Projects have been performed to assess specific clinical events, such as Elschnig's pearl dynamics (Findl et al., 2010). Information such as this is invaluable and work of this nature should stimulate basic researchers to mimic such changes in their respective experimental models in order to establish the molecular and physiological basis for these phenomena.

2.2. Clinician and patient views

It is curious that little information is available that reports surgical opinion regarding PCO. In a recent survey of both human and veterinary ophthalmologists, there was common recognition that PCO was a significant problem associated with cataract surgery. Even with advances in IOL design, opinion supported the pursuit of additional management strategies using therapeutic agents or additional surgical approaches (Shihan et al., 2019). Clinicians play a key role in assessing PCO development within patients. They are the individuals providing direct care for patients and further engagement and knowledge exchange between clinicians, biologists and engineers is likely to enhance the provision of care in the future.

Surprisingly, an opinion often neglected(but one which would provide great insight) is that of the patient. There are very few studies that present the viewpoint of patients with respect to PCO and how it affects wellbeing (Addisu and Solomon, 2011; Salerno et al., 2017). PCO is often talked about in numerical terms, but for many patients it is debilitating and distressing. It would be extremely valuable to gather much more information from patients' pre- and post-cataract surgery experiences to understand the impact of PCO upon them.

2.3. Post-mortem analysis

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Material harvested post-mortem from cadavers provides an invaluable resource for the understanding of PCO morphology and identification of which molecules are present within the capsular bag as PCO progresses. Such molecules could be matrix components, cellular markers or growth factors. General features within the capsular bag of post-mortem specimens are often investigated using the Miyake-Apple method. This provides a posterior view of the capsular bag (Apple et al., 2001; Pereira et al., 2009). Techniques used to analyse post-mortem capsular bags include electron microscopy (Kappelhof et al., 1987; Marcantonio et al., 2000), immunochemistry (Marcantonio et al., 2000; Saika et al., 2002; Saika et al., 2000; Wormstone et al., 2002), Real-time polymerase chain reaction (qRT-PCR) (Wormstone et al., 2001; Wunderlich et al., 2000) and Enzyme-linked Immunosorbent Assay (ELISA) (Wormstone et al., 2001; Wormstone et al., 2002; Wormstone et al., 2000). While this type of investigation provides "scene of the crime" information it does not provide definitive proof that various molecular suspects are responsible for PCO development. This information does however, create a blueprint of clinical outcomes,which experimental models should try to emulate.

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2.4. Cell culture

Cell culture is the simplest method and the most common entry point into PCO research. This usually takes the form of primary cultures or cell lines. Although valuable, there are a number of difficulties in working with primary cultures (Wormstone and Eldred, 2016). In general, primary lens cells have limited longevity in culture and generally only survive for a few passages before they attempt differentiation or senesce, preventing long-term experiments and substantial data acquisition. One exception seems to be the chick lens, which has been used to establish dissociated cell–derived monolayer cultures of primary embryonic chick lens epithelial cells. These cells, grown on laminin-coated plates have been used to

investigate fibrotic and regenerative PCO (Boswell et al., 2017). In general, lens cell lines are more readily used than primary cultures. In the lens field, the three main lines used are HLE-B3 (Makley et al., 2015), SRA01\04 (Ibaraki et al., 1998; Weatherbee et al., 2019) and FHL124 (Dawes et al., 2007b; Reddan et al., 1999; Wormstone et al., 2004). They are all derived from foetal or neonate lenses. HLE-B3 and SRA01\04 were generated using viral immortalisation methods. FHL124 in contrast is a non-virally transformed cell line. Cell lines allow the study of PCO-related features that include migration, proliferation, matrix modification, matrix contraction and to a limited degree differentiation. It should be noted that cell lines demonstrate some level of transdifferentiation to myofibroblasts. This can limit understanding of changes directly linked to surgery/injury. However, expression of myofibroblasts increase with appropriate stimuli e.g. transforming growth factor β (TGFβ) and thus the human lens cell lines are valuable model systems to understand myofibroblast formation and fibrotic events. Moreover, they are readily available and amenable to molecular manipulation. As a result, cell line cultures are a workhorse to enable elucidation of the mechanisms governing human lens cell biology. However, the value of such work is greater when used in combination with more sophisticated experimental systems, such as in vivo animals and the capsular bag model.

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2.5. In vivo animal models

Animal models for PCO have utilised mouse and rat rodent systems and rabbit (Aliancy et al., 2018; Jo et al., 2014; Lois et al., 2003; Lois et al., 2005; Mamuya et al., 2014; Shihan et al., 2020; Wormstone and Eldred, 2016). The argument for the use of in vivo animal models is that they provide full replication of post-surgical events and access to appropriate stimuli, which arguably isn't replicated in vitro. However, this view requires caution, as it is unlikely

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that the inflammatory environment in these systems truly represents responses observed in human patients (Bito, 1984). Ongoing assessment of PCO progression in these systems is generally limited, with the majority of information obtained from detailed end-point examinations. Another note of caution that is applicable to any animal-based experimental system relates to differential expression of proteins within the lens. For example, receptor profiles can be quite different between species (Wormstone et al., 2006b). This discordance can hamper true discovery of the underlying mechanisms driving PCO in patients, but also poses complications when assessing the potential of pharmacological agents to treat PCO. When using animal systems it is therefore important to cross-reference findings with human data (when available) to ensure the findings from an animal system are relevant and can provide maximum benefit to the field.

Both the rat and mouse are commonly used experimental models. Due to the relative size of their lenses to humans, they cannot house a typical IOL. Nevertheless, models that allow investigation of PCO progression or PCO-like events are available. With respect to the rat, Lois et al (Lois et al., 2003) established a model that presented many of the characteristics of PCO seen in patients. These included cell growth on the central posterior capsule, fibroblastic-like cells with a spindle-shaped morphology and wrinkling of the capsule. Furthermore, Soemmerring's ring was observed, demonstrating that lens fibre differentiation had occurred. Despite the successful demonstration of the rat model, this approach is rarely used. In general, the mouse is the most commonly utilised in vivo system to investigate mechanisms of PCO. This is largely because the excellent genetic understanding of mice allows mechanistic testing in vivo using robust tools available for genetic manipulation. The ability to regulate gene and protein expression of specific targets is a valuable tool that enables mechanisms to be investigated and key targets in PCO formation identified (Mamuya

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et al., 2014; Shihan et al., 2020). Some groups have induced injury by performing a puncture wound in the anterior capsule (Saika et al., 2001), while others have created a linear tear of the anterior capsule to enable fibre cell removal (Call et al., 2004) . To aid fibre cell removal, pressure is applied to the eye cavity with forceps, which could cause additional trauma to the eye over generally applied clinical procedures in patients. Lois et al (Lois et al., 2005) advanced practice within the mouse model by performing simulated cataract surgery, which included continuous curvilinear capsulorhexis and lens fibre removal by hydroexpression. Using this approach, cell proliferation, migration, EMT and differentiation can be observed following surgery.

The rabbit in vivo system is a commonly used model in PCO research. It is however, seldom used as an investigative tool to identify mechanisms driving PCO. However, it has been employed as a tool to understanding mammalian lens regeneration (Gwon and Gruber, 2010). Generally, the rabbit is used to assess IOLs and their impact on PCO due to the comparable size of the rabbit and human lenses (Aliancy et al., 2018). This therefore requires less adaption from a standard cataract procedure to enable surgery on rabbits. As a consequence, surgical procedures used on rabbits have followed the trends observed in the clinic, such that procedures initially employed a linear capsulorhexis with simple extracapsular extraction (Behar-Cohen et al., 1995), but now employ continuous curvilinear capsulorhexis with phacoemulsification to replicate modern surgical procedures (Leishman et al., 2012). This model allows assessment of cell regrowth, matrix modification, EMT and differentiation. The rabbit undergoes a profound inflammatory response, which is sustained for much longer than is typical in human patients (Davidson et al., 1998; Pande et al., 1996)). The scale of this response is believed to accelerate PCO. The timescale of PCO formation and its severity in the rabbit model are major draws for its use as an evaluation tool for IOL testing.

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2.6. Tissue culture models

Tissue culture is a valuable tool to investigate PCO. This generally takes the form of lens epithelial explants (West-Mays et al., 2010) and capsular bag systems (Wormstone, 2020). In both cases the cells are maintained on their natural substrate, the lens capsule.

Lens epithelial explants have been established using lenses from a variety of species. Early efforts centred on the chick, but the bulk of studies to date have been performed using the rat lens explant system (West-Mays et al., 2010). Using this system growth, EMT and differentiation have been observed and thus epithelial explants are a tractable system for the study of PCO (Gordon-Thomson et al., 1998; Mansfield et al., 2004; McAvoy and Chamberlain, 1989). Explant cultures can also be established from human lenses (Maidment et al., 2004; Shu and Lovicu, 2017; Smith et al., 2019; Weatherbee et al., 2019). To date, growth, EMT, increased matrix production and contraction have been studied, but differentiation is yet to be observed. In terms of securing the explants to a culture dish, this is achieved either through the use of pressure from forceps or entomological pins (7-10mm long), which reduces the potential for detachment from the culture dish.

A further development on the explant system is the generation of capsular bag culture models. The aim here is to recapitulate the same spatial cell and tissue arrangement as observed clinically. Capsular bag models have several variants and have been utilised in a range of species including human (Liu et al., 1996; Nagamoto and Bissen-Miyajima, 1994; Wormstone et al., 1997), bovine (Saxby et al., 1998), canine (Davidson et al., 2000), rabbit (Duncan et al., 2007), chick (Walker et al., 2007) and porcine lenses (Jun et al., 2014).

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The majority of work using capsular bag models has used human donor tissue. A simulated cataract surgery is performed on donor eyes. In most cases, the cornea is removed for transplant purposes and to aid visibility, the iris is removed. A continuous curvilinear capsulorhexis creates an opening in the anterior capsule that enables removal of the fibre mass by hydroexpression or phacoemulsification. Remaining fibre material is removed by irrigation/aspiration. As with surgery in a clinic, the product of this operation is a capsular bag. If required, an IOL can be implanted and studied. The capsular bag can be isolated from the eye by cutting the zonules and transferred to a dish for culture. This enables pure populations of cells to be maintained under controlled culture regimes and is ideal for addition/inhibition studies to define roles of specific candidates. If the capsular bag is isolated, it is important to maintain general circularity. To achieve this, ring systems and pinning have been used (Dawes et al., 2012; Liu et al., 1996; Saxby et al., 1998). It has been suggested that both methods can compromise the bag and have a greater influence on findings; the ring because of its bulk and pinning because it is perceived as an extra stress, however in relation to the trauma of surgery this is likely to be negligible. Nevertheless, this system has proved to be of great value and has contributed to many significant findings in the field. A variant of the capsular bag model is to retain the zonules and transfer the ciliary/zonules/lens complex to a ring support (Cleary et al., 2010; Eldred et al., 2014; Eldred et al., 2019). The ciliary body is pinned to the ring and the capsular bag suspended over the lumen. This approach combined with improvements in culture conditions that better reflect surgical inflammation, maintains the capsular bag integrity and is ideal for studying IOLs in relation to PCO (Eldred et al., 2014; Eldred et al., 2019; Hillenmayer et al., 2020) (Figure 2).

After humans, the most common patients that undergo cataract surgery are canine. As with humans, canines undergo a wound-healing response following surgery that in turn causes

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PCO. In fact, the incidence of PCO in canines is 100% and similar to the rates observed in very young children. Canine models for PCO and the capsular bag in particular have direct relevance to a patient group and offer valuable insight into human PCO. The canine capsular bag model first described by Davidson et al (Davidson et al., 2000) built on the human model described by Liu (Liu et al., 1996). The model has been used to good effect to better understand canine PCO and develop better ways of managing the condition (Chandler et al., 2012)

Menko et al developed a modification of both the explant and capsular bag culture systems in chick (Walker et al., 2007). This model again involves simulated surgery on the chick lens. Fibre cells are removed by hydroexpression following disruption of the anterior capsule by incision. Four 'flaps' of the anterior epithelium are established by radial cuts. These flaps are folded back and secured to the culture dish, such that the preparation resembles a star. This method exposes the entire PC and thus both central and peripheral regions of this surface can be easily studied. Using this system, migration and growth across the posterior capsule and EMT have been investigated. (Walker et al., 2007). A similar approach has also been applied to the human capsular bag system to create a fully open test system (Eldred et al., 2016).

2.7. Stem cells

Following cataract surgery, there is evidence to suggest that the lens makes efforts to regenerate. In order to do so, fibre cell formation via cell differentiation is required. While clinical work has demonstrated that lens regeneration - in infants at least - is possible using modified surgical approaches (Lin et al., 2016), in most cases this is imperfect; the outcome more typically comprises the formation of Soemmerring's ring and Elschnig's pearls.

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Understanding the process of human lens cell differentiation is therefore important in terms of understanding how best to regenerate a lens post-surgery. It may also help us to understand how Soemmerring's ring and Elschnig's Pearl formation is initiated and regulated. The ability to establish lens organoids or 'lentoids' from stem cells provides an excellent system to achieve this and in turn advance our understanding of regenerative PCO.

The first method describing lentoid formation from human stem cells was published by Yang et al (Yang et al., 2010). This study used human embryonic stem cells (hESCs) and adopted a three-stage culture strategy that employed sequential inhibition and activation of Fibroblast growth factor (FGF), Transforming growth factor beta (TGFβ) and Wnt signalling to guide naïve stem cells to a three-dimensional lens-like organoid. In the first of the three stages, 100ng/ml Noggin (a bone morphogenic protein (BMP) ligand inhibitor), was added to the culture medium for six days to direct the naïve hESCs towards a lens placode phenotype. This initiation was followed by Stage 2, comprising a period of 12 days wherein the Noggin was replaced by 20ng/ml BMP4, 20ng/ml BMP7 and 100ng/ml basic FGF in the medium to facilitate the instigation of fibre cell differentiation. For Stage 3, the cultures were exposed to 20ng/ml Wnt3a and 100ng/ml basic FGF to encourage lens fibre cell maturation and elongation. During the early stages of culture, pluripotency markers NANOG and OCT3 were suppressed. Lens placode markers including Pax6, Sox2 and Six3 were up-regulated. Pax6 peaked at day 10, but gene expression was maintained throughout culture. Signals for Sox2 and Six3 both peaked at day 6 of culture and remained elevated at day 10, but were expressed at low levels beyond this time point. As the culture period extended, a progressive up-regulation in lens fibre cell markers CRYAA, CRYBB2, CRYGC and MIP was detected using quantitative real-time PCR (qRT-PCR), indicating lens fibre cell maturation and lentoid development. These molecular changes correlated chronologically with the physical

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formation of three-dimensional lentoid structures. Together, the morphological and molecular phenotype of the organoids generated was consistent with a human lens phenotype.

Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) made a significant breakthrough which demonstrated that induced pluripotent stem cells (iPSCs) could be generated directly from fibroblast cultures by the addition of Oct3/4, Sox2, c-Myc, and Klf4 (subsequently known as the 'Yamanaka factors'). The use of these factors to re-programme somatic cells back to 'stemness' provides major advantages experimentally over the use of embryonic stem cells; it removes some ethical challenges associated with the use of hESCs and increases the scope and amount of material available for research. Fu et al (Fu et al., 2017) generated induced pluripotent stem cells (iPSCs) from clinically isolated human urinary cells. They then adapted the three-stage protocol described by Yang et al (2010): following Stage one, differentiating cells formed structures that resembled fried eggs. They selected specific portions of the 'fried eggs' and transferred them to new culture vessels for continued culture in Stage 2 and 3 culture conditions. This adaption to the protocol aimed to select cells that were primed for lentoid formation, ultimately to increase the efficiency of the process and the lentoid yield. Changes in molecular markers followed a similar pattern to Yang et al (2010). The lentoids formed were relatively transparent and refractive. This study also provided ultrastucture detail of the lentoids.

Further adaptions to the established protocol were reported by Murphy et al (Murphy et al., 2018). In Stage 1, Noggin concentration was increased from 100ng/ml to 500 ng/ml; additionally, 10 nM SB431542, an Alk5 inhibitor, was added to the culture medium. In Stage 3 the FGF2 concentration was reduced from 100ng/ml to10 ng/ml. A major feature of this protocol was the use of receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a selection

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tool for lens purification during Stage 2 of the lentoid differentiation protocol using magnetic activated cell sorting (MACS). Following ROR1+ selection, cells were plated on Matrigel-coated dishes in M199 medium (Thermo Fisher Scientific) containing 10 ng/ml of FGF2. The mini lenses/lentoids generated using this protocol were shown to have some level of transparency and focussing power.

The work described above has made outstanding contributions to the field and has laid the foundation for future research. However, there is always scope for improvement and refinement. Here we present another advance that overcomes an unwanted variable associated with stem cell research.

A common feature of the protocols described above is the use of mouse sarcoma-derived Matrigel as a culture substrate (Fu et al., 2017; Murphy et al., 2018; Yang et al., 2010). Unfortunately, the composition of Matrigel varies batch-to-batch because it contains variable quantities of growth factors such as Transforming Growth Factor β (TGFβ) and Platelet-Derived Growth Factor (PDGF) that are commonly controlled and manipulated in developmental experiments. This inconsistency could influence the physiological outcomes of developmental experiments due to the Matrigel-derived growth factors contributing to the experimental conditions in undefined, variable ratios. We therefore aimed to generate lentoids from iPSCs using a defined replacement for Matrigel that was of standardised origin, thus moving the current protocol towards a better-defined system using fewer animal-derived components. We selected iMatrix-511 as our culture substrate, which is composed of recombinant human laminin 511-E8 fragments. iPSCs (Cellartis ChiPSC22) were seeded onto iMatrix obtained from Takara Bio (Cat. No T303) and subjected to the three-stage differentiation scheme as first detailed by Yang et al (Yang et al., 2010). This involved

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treating the cells in supplemented mTeSR1 (Stemcell Cat. No 85850) with the addition of specific growth factors at different stages: **Stage 1)** addition of 100 ng/ml Noggin from day 0 to 6, **Stage 2)** combination of 100 ng/ml bFGF, 20 ng/ml BMP4 and 20 ng/ml BMP7 from day 6 to 18, **Stage 3)** combination of 100 ng/ml FGF2 and 20 ng/ml Wnt-3a from day 18 onwards. Cells were seeded at a density of 10,000 cells per cm^2 and cultured in a humidified environment at 35^oC with 5% CO_2. Animal-free growth factors were used where available, thus moving the system towards a fully-humanised, xeno-free model representative of human development. Cultures were observed using a Nikon Eclipse Ti2 inverted light microscope and growth medium was changed every other day. Growth factors were obtained from Miltenyi Biotech and Peprotech and were prepared and stored according to the manufacturer's instructions. In order to validate the progression of iPSCs to lentoids, we chose to examine a set of genes using quantitative real-time PCR. The genes analysed were selected to best capture different stages of lens development. OCT4 was selected as a pluripotency marker; SSEA1 as a marker of differentiation; Pax6 as a marker of 'eye lineage' specification; PitX3 as a marker of fibre induction and lens maturation; αA-crystallin (CRYAA) as an indicator of crystallin accumulation in developing lentoids; FOXE3 as an indicator of lens cells; MIP/Aquaporin 0 to demonstrate the presence of mature fibre cells; and crystallins beta-2 (CRYBB2), and gamma-D (CRYGD) as markers of further maturation towards lens phenotype.

Following Stage 1 culture the cells formed a collection of distinct single-layer clusters (Figure 3A). Stage 2 culture induced progressive organisational changes that gave rise to structures resembling 'fried eggs' (Figure 3A), as first reported by Fu et al (Fu et al., 2017). These structures developed to form three-dimensional structures that possessed some refractive properties and had a defined outer margin. These structures continued to develop

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throughout Stage 2 culture to produce lentoids that were more consistent in form with a smooth surface and were clear and refractive in nature (Figure 3A). Stage 3 culture conditions seemed to merely continue this progression.

Gene expression analysis (Figure 3B) by qRT-PCR of RNA isolated from the cultures at specific time-points (Days 0, 6, 12 and 18) revealed that OCT4 decreased progressively over the course of the experiment, indicating loss of pluripotency of the cells; SSEA1 increased over the same time period, indicating differentiation of the cell population. Pax6 - the master 'eye' gene was strongly expressed from day 6 onwards. Pax6 peaked at day 12 then declined, indicating its role in defining an ocular cell phenotype. Key lens cell markers FOXE3 and αA-crystallin were significantly elevated at day 18 and in the case of αA-crystallin, levels were 32.65 ± 12.33-fold higher than day zero samples. This enrichment in αA-crystallin indicates the initiation and development of fibre cell differentiation, which is further supported by elevation of the lens markers PitX3 and crystallin beta-2 at the day 18 time-point. Gene expression of the mature fibre cell markers gamma-D crystallin and MIP was also elevated at day 18.

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In summary, we have progressed the current methodology for differentiation of human iPSCs into maturing lentoids. We adapted the previously-cited protocol by using iMatrix to replace Matrigel, thus reducing growth matrix-associated variability and removing one animal-derived component from the system. Despite this change, we achieved the same results as other groups who have employed this differentiation method, proving that our innovation is not detrimental to the overall process. With further refinement, we hope to use this method to examine the differences between normal and aberrant differentiation in relation to the

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formation of Soemmerring's ring and Elschnig's Pearls. This could help us gain new insights into the mechanisms that underpin regenerative PCO.

3. The biological regulation of PCO

Having introduced the range of experimental tools available for PCO research, we will now discuss the regulatory processes that govern PCO progression (Figure 4). To aid this discussion, we will describe the two major forms of PCO in relation to specific events and in order of involvement post-surgery. Fibrotic PCO is initiated by surgical injury, occurring in all patients to some degree. Fibrotic PCO follows classically defined fibrotic processes, namely hyper proliferation, matrix contraction, matrix deposition and cell transdifferentiation from an epithelial to myofibroblast phenotype (Eldred et al., 2011; Leask and Abraham, 2004). Regenerative PCO is characterised by lens fibre cell differentiation that gives rise to Soemmerring's ring and Elschnig's pearls and becomes evident at a later stage than the fibrotic form. Both fibrotic and regenerative forms of PCO contribute to a reduction in visual quality in patients, however it is reported that the regenerative form has a greater impact due to the refractive properties of Elschnig's pearls (van Bree et al., 2011).

3.1. Fibrotic PCO

In order to appreciate the changes that take place following cataract surgery that ultimately lead to posterior capsule opacification, it is important to appreciate the lens and its natural environment prior to surgery. The lens is an unusual tissue as it does not contain any blood vessels or nerves and gains all nutrients from the surrounding humours (Wormstone and Wride, 2011). It is a remarkable feat of biological engineering that the lens is capable of maintaining transparency for so long. It could be argued that the lens has developed a sense of independence and is capable of utilising the components of a relatively poor environment

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to manufacture proteins to aid cell survival and organ function. Indeed the bulk of survival factors, including transferrin, detected in the aqueous humour are produced by the lens (McGahan et al., 1995). Gene and protein expression patterns are well governed across the lens epithelium and fibre cell populations (Hawse et al., 2005; Hodgkinson et al., 2007; Maidment et al., 2004). However, disruption to the eye and perturbation of the lens through mechanical injury provokes significant change to the ocular environment, lens cells and the capsular matrix on which they reside (Figure 4).

The first objective of lens cells in the face of surgical insult is to survive. If the supply of growth and survival signals is insufficient, this will lead to cell death, presumably by apoptosis (Ishizaki et al., 1993). The intrinsic ability of lens cells to self-regulate their activity in what is naturally a harsh environment is likely to contribute to the long-term development of PCO. Human capsular bag studies have remarkably demonstrated that lens cells can survive in serum-free medium for over 1 year (Wormstone et al., 1997). Ishizaki et al (Ishizaki et al., 1993), in a study using rat lens cells, found that the underlying matrix and cell density were the major factors that enable lens cells to survive in a protein-free medium and avoid undergoing cell death by apoptosis. It has also been shown that human capsular bag cultures maintained in serum-free medium can release a number of cytokines and growth factors following cataract surgery, including interleukins, FGF and VEGF (Dawes et al., 2013; Eldred et al., 2016). A number of these factors play a role in proliferation, migration and cell transdifferentiation. A similar pattern of cytokine expression is also observed with the in vivo mouse model (Jiang et al., 2018).While endogenous production of PCO-promoting factors will undoubtedly play a long-term role in PCO formation, the influence of external stimulation immediately following surgery also has a marked impact on the events that follow.

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Following cataract surgery, there is a marked up-regulation of proteins within the aqueous humour. This is associated with an inflammatory response; coincidentally, fibrotic disorders are also often associated with inflammatory responses. The ultimate objective of an inflammatory response is to facilitate repair of the damaged tissue. Chemicals released from damaged tissue attract white blood cells, including lymphocyte T helper cells, which coordinate an immune response by releasing cytokines such as interleukins. These secreted proteins activate resident macrophages that further enhance the production of cytokines, chemokines and other inflammatory mediators as well as recruiting monocytes (Martinez et al., 2006), which can accelerate PCO development.

As surgical procedures have advanced to become less disruptive, a marked benefit has been the reduction in inflammatory response (Pande et al., 1996). The introduction of foldable lenses in conjunction with phacoemulsification to remove the fibre mass rather than hydroexpression is less traumatic to the eye due to the smaller incision required for this procedure. Interestingly, it has been shown in the capsular bag model that the method of fibre mass removal does not affect lens cell population or growth responses under comparable conditions (Quinlan et al., 1997), therefore potential reductions in PCO are likely to result from reduced inflammation. Assessment of protein flare in the aqueous humour following cataract surgery indicates a spike followed by a gradual return to baseline in a few weeks (Pande et al., 1996). The same general pattern was seen with phacoemulsification and hydroexpression cataract extraction methods; however, the scale of response was more severe with hydroexpression, presumably due to greater incision required to perform this surgery.

Breaching the blood aqueous barrier permits a number of blood proteins to enter the ocular environment, which can further enrich the local concentrations of cytokines and growth factors. These factors can directly stimulate lens cells or interact with the lens capsule, which can provide a long-term reservoir of molecules that can advance PCO over months and years (Wormstone et al., 2006a). A number of growth factors such as FGF, Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), interleukins and thrombin can promote survival, proliferation and migration (Eldred et al., 2016; Wormstone et al., 2001; Wormstone et al., 2004; Wormstone et al., 2000). Transforming growth factor β (TGFβ) is also elevated in the eye following surgery. TGFβ does not appear to be a key promoter of human lens cell growth (Wormstone et al., 2002; Wormstone et al., 2004) but is believed to play a critical role in many fibrotic events (Leask and Abraham, 2004). TGFβ is elevated in the eye to regulate the severity of inflammatory response through suppressing the actions of white blood cells (Streilein et al., 2002). This function of TGFβ affords overall protection to the eye, but active TGFβ enrichment of the aqueous humour can stimulate lens cells and drive fibrotic PCO.

A number of patients undergoing cataract surgery have uveitis, a chronic inflammation of the eye (Ozates et al., 2020). It has been reported that patients experiencing idiopathic anterior uveitis have higher levels of interleukins (IL), monocyte chemoattractant protein-1 (MCP-1) and interferon gamma (IFNγ) in the aqueous humour. It is therefore likely that cataract patients with uveitis will have a more severe and sustained inflammatory response that is likely to accelerate PCO progression (Ozates et al., 2020).

3.1.1. Cell proliferation and migration

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Proliferation, in association with migration, is an important aspect of wound healing and PCO. Some interesting data have been reported using the BrdU labelling and detection technique to identify dividing cells (Rakic et al., 1997; Wormstone et al., 1997; Rakic et al., 2000). In one study (Rakic et al., 1997), it was shown that simply performing a small capsulorhexis could up-regulate the rate of division in the lens relative to the unperturbed lens. Moreover, if the fibre cells were removed, the rate of cell division was stimulated even more. These experiments were repeated by Lin et al (Lin et al., 2016) who also reported an 11-fold increase in BrdU positive cells within the human capsular bag following fibre cell removal. Analysis of capsular bags following 3 days culture in serum-free medium showed increased numbers of BrdU positive cells (Wormstone et al., 1997). The distribution of these positive cells was predominately in the equatorial region, the natural site of division, however some cells were observed on the anterior capsule. Furthermore, if the capsular bags from aged donors (>60 years) were established in the presence of serum, then the dividing population was increased, but the distribution pattern remained the same. It is notable that lens cells growing on capsular bags maintained in serum-free medium from a wide age spectrum can effectively colonise the entire surface of the once cell-free posterior capsule. However, as expected, growth occurs at a greater rate in capsular bags from younger human donors than older donors (Wormstone et al., 1997). Supplementing the medium with 10% FCS dramatically increased the rate of cell growth on aged capsular bags (>60 years), but did not greatly enhance the cell growth rates on their younger (<40 years) counterparts (Wormstone et al., 1997; Wormstone et al., 1997). These data also reflect the increased requirement for secondary surgery to treat PCO in young patients (Moisseiev et al., 1989; Knight-Nanan et al., 1996). Furthermore, the findings suggest that powerful autocrine mechanisms in the young are active following surgical trauma. A number of growth factors have been identified as important regulators of lens cell growth and include basic FGF

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(McAvoy and Chamberlain, 1989; Wormstone et al., 2001), hepatocyte growth factor (HGF) (Choi et al., 2004; Wormstone et al., 2000), EGF (Hongo et al., 1993; Ibaraki et al., 1995; Majima, 1995) (Maidment et al., 2004; Wertheimer et al., 2015b), Thrombin (James et al., 2005), Wnt (Bao et al., 2012), interleukins (Dawes et al., 2013; Eldred et al., 2016) and VEGF (Eldred et al., 2016).

An interesting study employing young (<40 years) and aged (>60 years) capsular bag cultures assessed the levels of growth factors secreted into the medium within the first 2 days post-surgery and found significant decreases in IL-1β and IL-6 in the >60 years group (Dawes et al., 2013). In contrast, IL-10, IL-12, IL-13, and VEGF in the >60 years group were significantly increased compared with their younger counterparts. Capsular bags (cells and capsule) were also analysed and it was found that preparations from > 60year old donors had greater than or equal levels of FGF and HGF compared to their younger counterparts. In addition, aged capsular bags also presented higher rates of protein synthesis. This suggested that the established age-dependent differences in cell growth observed were not resulting from limited availability of growth factors. Attention then centred on signalling pathways. Inhibition of ERK, p38, and JNK signalling using specific inhibitors significantly suppressed cell coverage on the posterior capsule demonstrating the relative importance of each pathway. The activity status of these pathways was assessed by detection of pERK, p-c-jun, p-p38, and pJNK. A consistent pattern emerged such that phosphorylated signalling molecules were lower in aged cell populations; total signalling protein expression was unaffected by age. It would therefore seem that availability of stimulating factors is not a limiting factor as we age, but the ability to convert this resource into signalling activity is, which is likely to impede the rate of cell growth following surgery.

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A question that is often asked relates to the origin of cells leading to posterior capsule opacification. This is difficult to discern using in vivo models or clinical observation as the peripheral capsular bag is obscured. Information can, however, be extracted from capsular bag studies that provide some insight. When pinned to a dish, in the absence of an IOL, the anterior and posterior capsules of the capsular bag naturally lie on top of one another. In this configuration, cells start to colonise the central posterior capsule within the rhexis margin within the first week of culture (Eldred et al., 2016). When suspended cultures are established with an IOL implanted, the capsules do not initially form a close connection. With this arrangement, cell growth is observed on the posterior capsule and takes more than one week to reach the central posterior capsule (Eldred et al., 2019). This would suggest that equatorial cells can proliferate and migrate across the posterior capsule, but if the capsules come into close apposition, anterior epithelial cells can relocate to the posterior capsule and contribute to the coverage of the posterior capsule.

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3.1.2. Transforming growth factor β signalling

While arguably not a potent driver of lens cell growth, TGFβ is strongly implicated in fibrotic responses (Leask and Abraham, 2004). The major isoform synthesised within the eye is TGFβ2, although TGFβ1 and 3 can also reach the aqueous humour from the blood (Ohta et al., 2000; Schlotzer-Schrehardt et al., 2001). Post-mortem analysis of a capsular bag received from a donor one month following cataract surgery demonstrated increased levels of αSMA, a marker of myofibroblasts, and matrix contraction/wrinkling of the posterior capsule (Wormstone et al., 2002). This does not provide direct evidence that TGFβ can induce these changes, but many of these features are linked to TGFβ and fibrosis. A large body of evidence demonstrates the ability of active TGFβ to promote cellular responses that define fibrosis in PCO, namely: epithelial to myofibroblast transdifferentiation; increased

extracellular matrix production and deposition and matrix contraction (Eldred et al., 2011) (Figure 5).

In order to signal, active TGFβ binds to the extracellular domain of TGFβ receptor II (TGFβRII) dimer, which are autophosphorylated serine/threonine kinase receptors (Massague, 2012). A TGFβ receptor I (TGFβRI) dimer is recruited to form a hetero-tetrameric receptor complex with the TGFβ ligand. Due to conformational changes, TGFβRII transphosphorylates serine and threonine residues on the intracellular domain of TGFβRI. Activated TGFβRI can then regulate a number of signalling pathways. The best defined is the Smad signalling pathway, but Smad-independent pathways are also recognised and believed to play important roles in lens cell regulation (Derynck and Zhang, 2003).

A prerequisite for TGFβ involvement in the fibrotic response is availability. TGFβ is present in the aqueous humour of the eye: this is largely in a latent, inactive form (Ohta et al., 2000; Schlotzer-Schrehardt et al., 2001). The latent form of TGFβ is converted to the active form by proteolytic cleavage of pro-segments. In the normal eye, levels of active TGFβ are of low abundance and its activity can be further controlled by scavenger proteins in the ocular humours, for example α2-macroglobulin (Schulz et al., 1996). Mechanical injury resulting from surgery can significantly increase the abundance of active levels of TGFβ through increased production of TGFβ2 and influx of blood protein in concert with elevated proteases, such as plasmins, cathepsins and MMPs (Yu and Stamenkovic, 2000), which cleave latent TGFβ precursor protein. In addition, elevated numbers of reactive oxygen species can promote TGFβ activity (Chamberlain et al., 2009; Fatma et al., 2005).

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Analysis of post-mortem material provides evidence-linking TGFβ2 to PCO. Saika et al (Saika et al., 2002) analysed a number of post-mortem capsular bags obtained months and years following surgery to identify if active TGFβ signalling was evident. Samples examined for expression and distribution of Smad3 and Smad4, which are key proteins in the established TGFβ/Smad signalling pathway. Within this pathway, Smad2 and 3 are transcription factors, which are phosphorylated by ligand-activated TGFβRI. Both Smad2 and 3 have a high affinity for Smad4 and form a complex with it. This complex translocates to the nucleus allowing Smad2/3 to engage with co-activators or repressors and regulate gene transcription. All the post-mortem samples demonstrated Smad2/3 staining in the nuclei, which indicates an active signalling pathway (Saika et al., 2001). Interestingly, this study also examined Smad3 and 4 in native human lens tissue. In these preparations, Smad3 and 4 were distributed in the cytosol, with no evidence of translocation to the nucleus. A reason for this could be a low abundance of TGFβ receptors. If either of the receptors is lacking, ligand-induced signalling will be impaired. This also tallies well with a study of human lens cultures, which showed an inability of cultured human lenses to form fibrotic plaques (a feature of anterior subcapsular cataract, a lens condition that relates to PCO), unless they were severely challenged by chemical or mechanical injury (Marcantonio et al., 2003). Interestingly, TGFβ can induce anterior subcapsular cataract in a rat lens culture model (Hales et al., 1994) and in animals with a TGFβ overexpression system (Banh et al., 2006) implying differences in sensitivity and the natural configuration of TGFβ signalling between species. Work in our laboratory has utilised human lens epithelium to further test the sensitivity to TGFβ following injury. To isolate anterior lens epithelium the lens was first placed with the anterior surface down on a culture dish. A puncture was made in the posterior capsule and four radial incisions made towards the equator. The fibre mass was carefully removed and excess posterior capsule discarded. The remaining epithelium was quartered by

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a series of linear capsulorhexes. Each quarter was transferred to a separate culture dish and maintained in serum-free EMEM for 0, 6, 24 or 48 hours following dissection. Previous work has shown that a 2 hour exposure of human lens cells to 10ng/ml TGFβ will produce peak translocation of Smad2/3 to the nucleus, if a functioning TGFβ/Smad signalling pathway is present (Dawes et al., 2007a). Therefore, to assess when TGFβ/Smad signalling was active we exposed the cultured epithelium to 10ng/ml TGFβ for the final two hours of culture. At each respective endpoint, preparations were fixed for 30 mins in 4% formaldehyde. Smad2/3 visualisation was achieved using immunofluorescence methods and viewed with a Zeiss Axiovision fluorescence microscope. The data showed that the epithelium was unresponsive to TGFβ2 following 6 hours of culture, but exhibited Smad signalling in some cells at 24 hours while at 48 hours the majority of cells were actively signalling (Figure 6). This data shows that mechanical injury moves lens epithelial cells from being relatively unresponsive to TGFβ to being highly sensitive. TGFβ levels are likely to remain elevated in the ocular environment for days or weeks following surgery with adsorption of TGFβ to the lens capsule a likely source for long-term activity (Wormstone et al., 2006a). Therefore, once lens cells establish a receptor signalling apparatus, TGFβ is readily available to stimulate TGFβR-mediated signalling, which can induce long-lived fibrotic responses through Smad-dependent and Smad-independent signalling.

Smad signalling is the best-defined TGFβ signalling pathway and has been well studied in lens fibrotic disorders (Banh et al., 2006; Dawes et al., 2009; Saika et al., 2004; Saika et al., 2002). Experiments using murine lenses demonstrated increased myofibroblast expression in response to a puncture injury (Saika et al., 2001). If these animals received TGFβ neutralizing antibodies prior to puncture wound injury, Smad3 and 4 translocation to the nucleus was inhibited (Saika et al., 2001). The importance of Smad3 in lens fibrosis has been

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demonstrated using Smad3 knockout transgenic mice (Banh et al., 2006; Saika et al., 2004). Saika et al (Saika et al., 2004) reported that both TGFβ2-induced and injury-induced αSMA expression was inhibited in the lens epithelium of Smad3-knockout mice relative to wild type. Loss of Smad3 in mice also reduces the expression of trauma-induced type I collagen in the lens (Saika et al., 2004). A similar study was also performed on Smad3 knockout mice, which again used a puncture wound to the anterior epithelium and confirmed that Smad3 was required for expression of many fibrotic markers including myofibroblast markers such as αSMA (Meng et al., 2018). In addition, a Smad3 inhibitor, SIS3 suppressed TGFβ-induced expression of fibrotic markers in HLEB3 cells, suggesting an important role in a human model (Meng et al., 2018). Application of the same inhibitor also suppressed TGFβ-induced expression of myofibroblast markers in rat lens explants (Taiyab et al., 2019). In order to theoretically suppress the Smad signalling pathway, siRNA was used to prevent Smad4 expression in FHL124 cells (Dawes et al., 2009). Smad4 knockdown prevented TGFβ-induced αSMA and fibronectin expression, which suggests that TGFβ Smad3-Smad4 signalling is likely to control transdifferentiation of human lens epithelial cells. Curiously, this study found TGFβ-induced matrix contraction and Smad7 expression to be independent of Smad4 expression; suggesting that TGFβ-Smad independent pathways may also regulate lens fibrosis.

In addition to Smad-signalling, it has become increasingly apparent that TGFβ receptor activation can regulate a number of Smad-independent signalling pathways (Derynck and Zhang, 2003). Such pathways include Ras-Raf-MEK-ERK mitogen-activated protein kinase (MAPK) (Lee et al., 2007), the p38 MAPK pathway (Galliher and Schiemann, 2007), the JNK MAP kinase and Rho kinase signalling pathways (Moustakas and Heldin, 2009) and Wnt/β-catenin signalling (Crosby and Waters; Konigshoff and Eickelberg; Liu; Willis and

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Borok, 2007). The first demonstration that TGFβ could activate Smad-independent signalling pathways in lens cells was performed on FHL124 cells and showed that ERK and p38 MAP kinase signalling pathways were stimulated (Dawes et al., 2009). As FHL124 cells still promote matrix contraction in the absence of Smad signalling it is likely that matrix contraction is regulated by Smad-independent pathways. It is possible that the ERK MAPK signalling pathway could play a role in matrix contraction in PCO through its reported activation of myosin light chain kinase (MLCK), a key enzymatic regulator of contractile force (Klemke et al., 1997). It has been demonstrated that ERK1/2 signalling was activated in TGFβ2-induced EMT in the human lens cell line SRA01\04. Application of the MEK/ERK1/2 inhibitor, U0126, prevented the TGFβ2-induced upregulation of fibrotic markers, α-SMA, collagen type I, collagen type IV and fibronectin (Chen et al., 2014). Studies using the rat lens explant model also report that ERK activation is required for the initiation of TGFβ-induced EMT, but not its progression (Wojciechowski et al., 2017). Further investigations using the rat explant system have demonstrated that TGFβ treatment results in activation of Rho/ROCK and β-catenin/cyclic AMP-responsive-element-binding protein (CREB)–binding protein (CBP) signalling pathways; inhibition of either result in prevention of TGFβ-induced EMT in the lens (Korol et al., 2016; Taiyab et al., 2019; Taiyab et al., 2016). (Taiyib 2019; Taiyib 2016; Korol 2016). In addition, the p38 MAPK pathway has been found in chick lens cells to play a key role in TGFβ-induced transdifferentiation of epithelial cells to myofibroblasts through suppression of the pathway using the p38 specific inhibitor SB20358 (Boswell et al., 2017).

There is no doubt that TGFβ can induce both transdifferentiation of lens epithelial cells to myofibroblasts and promote matrix contraction. It is a common view that these two processes are linked, such that transdifferentiation to a myofibroblast is required for extracellular matrix

synthesis and contraction in fibrosis (Shu and Lovicu, 2017). Contraction of the extracellular matrix of the lens capsule is a key aspect of PCO as wrinkling of the capsule resulting from contraction can cause light scatter (van Bree et al., 2011). Human lens cell lines have been utilised to allow detailed investigations of the signalling pathways regulating fibrotic events in the lens. Using this experimental tool, assays have been developed to determine matrix production, transdifferentiation and matrix contraction (Dawes et al., 2008; Dawes et al., 2009; Wormstone et al., 2004). These studies suggest TGFβ regulates transdifferentiation and matrix contraction through independent pathways. The first indication to support this idea resulted from comparative studies of TGFβ1 and TGFβ2 using FHL124 cells (Dawes et al., 2008). This study suggested that TGFβ1 was a more potent inducer of matrix contraction than TGFβ2. In contrast, TGFβ2 was better able to induce αSMA expression. This triggered a series of inhibition studies to understand the pathways regulating TGFβ-induced fibrosis. Their findings revealed that αSMA expression and fibronectin/fibronectin receptors are not essential for TGFβ-induced matrix contraction to occur (Dawes et al., 2008).

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Together these findings demonstrate that TGFβ regulates a number of signalling pathways that are capable of interacting to create a signalling network. Careful dissemination of signalling pathway interplay will help further unravel the intimate role TGFβ plays in PCO formation.

3.1.2.1. Additional regulators/promoters of TGFβ signalling

While evidence is strong to support a role for TGFβ in fibrotic PCO formation, it is important to note that other factors can play critical support roles or could promote fibrotic events directly. For example, work carried out on the rat lens explant model has demonstrated that co-addition of EGF can enhance TGFβ-induced signalling and EMT (Shu and Lovicu, 2019).

In addition, treatment of explants with PD153035, an EGFR inhibitor, suppressed TGFβ-induced changes. It is important to note that EGF alone does not induce any fibrotic response (Shu and Lovicu, 2019). Basic FGF, Wnt, VEGF and CTGF have also been reported to mediate or amplify fibrotic responses (Cerra et al., 2003; Kubo et al., 2018; Ma et al., 2014; Mansfield et al., 2004; Taiyab et al., 2016). Recently, Gremlin has emerged as a potentially important pro-fibrotic molecule in PCO formation. Gremlin plays a role in a number of fibrotic conditions. Gremlin expression is significantly elevated in lens epithelial cells following cataract surgery on mice (Shihan et al., 2020). In a FN knockout mouse, gremlin levels were lower than wild type and this correlated with a reduced fibrotic response (Shihan et al., 2020). Exogenous administration of gremlin or TGFβ1 in FN knockout animals was able to restore the fibrotic response (Shihan et al., 2020). A study using a rat in vivo model also reports that gremlin inhibition is associated with reduced PCO scores (Ma et al., 2019). In general, pro-fibrotic actions of gremlin arise from inhibition of BMP signalling. BMP4 and 7 in particular are known to be anti-fibrotic. The ability of Gremlin to suppress BMP4 and 7 signalling is observed with HLE-B3 cells. Interestingly, addition of gremlin to HLE-B3 cells lead to increased αSMA, fibronectin and collagen I expression (Ma et al., 2019). In addition, 200μg/ml gremlin increased phosphorylation of Smad2 and 3 in a timeframe comparable to TGFβ (Ma et al., 2019), which supports the idea that gremlin can directly trigger TGFβ/Smad signalling (Sethi et al., 2011). In addition, Gremlin can also promote ERK and Akt signalling pathways (Ma et al., 2014).

A further cellular product that could contribute to TGFβ mediated fibrosis are reactive oxygen species. Using rat explants and whole lens cultures treatment with antioxidants could prevent TGFβ induced fibrosis (Chamberlain et al., 2009). In addition, work on mice has shown that glutathione depletion can upregulate EMT pathways (Wei et al., 2017; Whitson et al., 2017a; Whitson et al., 2017b). The basis for increased ROS by TGFβ was unknown, but a

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role for NOX4 in this process was proposed. NOX4 is elevated in FHL124 cells and rat explants by TGFβ (Das et al., 2016; Dawes et al., 2007b). Inhibition of NOX4 using VAS2870 suppressed TGFβ-induced fibrosis (Das et al., 2016). This was further investigated using NOX4 deficient mice (Das et al., 2020). These animals demonstrated a delay in fibrotic plaque formation with TGFβ over-expression compared to wild type. Fibrosis was therefore impaired, but not ablated with upregulation of NOX2 proposed as a compensatory mechanism (Das et al., 2020).

3.1.3. Extracellular matrix involvement and regulation

Extracellular matrix (ECM) plays a key role in the regulation of cell behaviour and increased synthesis and deposition of ECM proteins is a fundamental feature in the pathogenesis of fibrotic disorders (Leask and Abraham, 2004). The lens capsule is largely composed of collagen IV, along with laminin, heparin sulphate proteoglycans and tenascin (Cammarata et al., 1986; Danysh and Duncan, 2009). These ECM components provide a physical scaffold to ensure structural integrity of the lens capsule and permit lens cell attachment and migration. In lens fibrosis, cells over-synthesise and deposit new ECM components, which include fibronectin, vitronectin, collagen types I and III; these deposits contribute to the formation of fibrotic tissue.

Cell outgrowths onto vitronectin and fibronectin matrices from explant cultures have been reported (Taliana et al., 2006). Cells growing on these substrates had a more elongated/fibroblast-like cell appearance, increased αSMA staining and nuclear Smad expression. Interestingly these cellular changes were not observed with cell outgrowth from explants onto laminin, which retained a more typical epithelial cell phenotype (Taliana et al., 2006). The ED-A domain of fibronectin is believed to play a crucial role in the induction of

αSMA expression (Serini et al., 1998). Increased levels of fibronectin production have been observed in human lens capsular bag cultures treated with TGFβ2 (Eldred et al., 2019; Smith et al., 2019; Wormstone et al., 2002). A recent study (Shihan et al., 2020) examined the role of fibronectin in fibrotic PCO using a fibronectin conditional knockout mouse model. These animals developed a normal lens, but differences emerged when the capsule was ruptured and the fibre cells removed to model cataract surgery. The fibrotic response that developed following surgery was significantly attenuated beyond 3 days post-surgery and was associated with a reduction in cell proliferation, fibrotic extracellular matrix production and deposition. Using the Dissociated Chick-Derived Monolayers system (DCDML) it has also been shown that plasma-derived fibronectin upregulated TGFβ/Smad signalling and αSMA relative to cells plated on laminin (VanSlyke et al., 2018). The proteoglycan lumican has been identified in post-mortem capsular bag specimens (Saika et al., 2003). It has been shown in lumican knockout mice that following injury to the anterior lens capsule by a needle puncture, there was a delay in expression of αSMA and the appearance of transdifferentiated cells (Saika et al., 2003).

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Secreted protein acidic and rich in cysteine (SPARC) may play a role in lens fibrotic events by altering the expression of ECM proteins (Yan and Sage, 1999). Reports indicate that SPARC-null mice develop cataracts prematurely due to attenuated growth (Bassuk et al., 1999). Decreased laminin deposition is also observed in SPARC knockout mice relative to wild type lenses (Weaver et al., 2006). Additional studies investigated the effects of TGFβ in lens epithelial cells from wild type and SPARC null mice (Gotoh et al., 2007). TGFβ induced expression of both fibronectin and αSMA in both groups, but this was more marked in the SPARC null group, suggesting SPARC is involved in suppressing TGFβ-induced transdifferentiation. In addition, SPARC expression was promoted in lens cells by

dexamethasone, which correlated with a reduction in fibronectin and collagen type IV, which again links well with a role for SPARC and fibronectin in the regulation of transdifferentiation events.

3.1.3.1. Growth factor interactions with matrix

Many growth factors are regulated by matrix interactions. For example, heparin binding to FGF, HGF and VEGF can facilitate interaction with their associated receptors; as a result the distribution of heparin sulphate proteoglycans (and potentially other ECM components within the lens capsule) could play important roles in FGF, HGF and VEGF regulated fibrotic responses (Aviezer et al., 1994; Eldred et al., 2016; Yayon et al., 1991; Zarnegar and Michalopoulos, 1995). In a study by Cerra et al (Cerra et al., 2003), FGF alone could not initiate the formation of anterior subcapsular fibrotic plaques, however the severity of response observed with TGFβ was significantly increased when FGF was also added. TGFβ alone can also bind to several matrix components including decorin and collagen type IV (Paralkar et al., 1991). Matrix components within the lens capsule have the capacity to adsorb growth factors and thus provide an enriched environment that is likely to promote fibrotic events (Ishida et al., 2005; Wormstone et al., 2006a). Direct presentation of ligands to receptors, or through slow release resulting from the capsule by proteolytic cleavage (Tholozan et al., 2007), is therefore likely to provide long-term contributions to fibrotic change.

3.1.3.2. Integrins

Two-way communications between the cell and its underlying matrix is continuous and can have a significant influence on the behaviour of the cell. This is generally mediated by integrins, which are a group of distinct cell surface receptors composed of alpha and beta

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subunits. The interaction of specific integrins with their corresponding ECM ligands allows cells to adhere to and migrate across the ECM (Schwartz, 2001). In addition, integrins function as cell signalling hubs that permit signal transduction to and from the microenvironment (Schwartz, 2001). Dys-regulation of integrin expression or function can give rise to fibrotic changes.

Microarray analysis of the human lens epithelial cell line FHL124 was performed to identify which integrins are expressed in human lens epithelial cells and identify those of potential importance in lens fibrosis (Dawes et al., 2007b). Baseline expression analysis of β-integrin subunits in human lens epithelial cells identified integrin β1, β2, β3, β5 and β6 subunits. β1 integrin subunit was the most abundant. β1 integrin is the most promiscuous integrin subunit and is known to associate with 12 different α integrin chains (Elner and Elner, 1996); and is the most widely expressed integrin throughout the body (Elner and Elner, 1996). In the lens, it has been shown that β1-integrin regulates Erk1/2 and Akt phosphorylation and a loss of β1 integrin gives rise to elevated expression of early gene response 1 (Egr1), which results in apoptosis (Wang et al., 2017). With respect to the alpha sub-units, baseline expression in human lens epithelial cells showed α1, α2, α3, α4, α5, α6, α7, α10, α11, αE, αM, and αV were detectable (Dawes et al 2007). TGFβ exposure resulted in increased expression of α5, α11, αV and α5 integrin subunits.

α5β1 integrin expression is of particular relevance to fibrotic changes in the lens epithelium. α5β1 integrin together with its matrix ligand fibronectin, show enhanced expression following TGFβ exposure in both the human capsular bag and human lens epithelial cell line (Dawes et al., 2008; Dawes et al., 2007b; Grocott et al., 2007). It is a commonly held view that α5β1 integrin and fibronectin form a putative contractile apparatus with αSMA (Jester et

al., 1999; Jester et al., 1995). Data obtained from FHL124 lens cell experiments suggest that this may not be essential (Dawes et al., 2008; Dawes et al., 2009). Blocking the RGD binding site of α5 integrin using RGDS peptide revealed that the fibronectin/fibronectin receptor interaction was not essential to promote matrix contraction in response to TGFβ (Dawes et al., 2008). In addition, Marcantonio et al (Marcantonio and Reddan, 2004) also showed that expression and distribution of α5β1 in FHL124 cells altered in response to TGFβ exposure, such that a diffuse pattern was observed across the cell; membrane-bound α5β1 integrin did not appear to associate with actin filaments.

Integrin αVβ5 is reported to play an important role in fibrotic pathologies. This is mainly because it permits mechanotransduction in response to extracellular microenvironments (Kass et al., 2007) and is implicated in the transdifferentiation of cells to myofibroblasts (Lygoe et al., 2004). Exposure of lens epithelial cells to TGFβ gives rise to an increase in αVβ5 integrin expression (Dawes et al., 2007b; Grocott et al., 2007) in association with increased levels of transdifferentiation markers (Dawes et al., 2008; Wormstone et al., 2002). On the basis of these findings, it has been proposed that αVβ5 integrin can play a bidirectional role in lens fibrosis (Walker and Menko, 2009). Firstly, it could mediate TGFβ-induced transdifferentiation following trauma to the lens and secondly, act by facilitating signals from myofibroblasts back to the ECM, resulting in the activation of matrix-associated TGFβ. Disruption to lens integrity through injury can alter integrin expression in lens epithelial cells. Sponer et al (Sponer et al., 2005) showed that expression of αVβ6 integrin was up-regulated in cells residing in human capsular bags cultured in protein-free medium compared with cultured intact lenses. αVβ6 integrin is reported to play a role in TGFβ activation through its association with an RGD peptide in the latency associated peptide (Sheppard, 2004). Integrins αVβ6 and αVβ5 are likely to play important roles in regulating

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the levels of active TGFβ and the formation of microenvironments capable of driving fibrotic modifications of lens epithelial cells. αV integrin null mice were used to assess the impact of αV integrin on post-surgical fibrotic responses (Mamuya et al., 2014). This work revealed that cell proliferation was reduced and expression of fibrotic markers suppressed relative to wild type animals. Smad3 was not phosphorylated in null mice thus showing TGFβ/Smad signalling is suppressed (Mamuya et al., 2014).

Integrin-linked kinase (ILK)**,** a serine threonine kinase that binds to the cytoplasmic tail of alpha and beta integrin subunits, has been detected in lens cells of both murine and human origin (Weaver et al., 2007). ILK is a multidomain focal adhesion protein that regulates ECM adhesion and signal transduction. Evidence supports the notion that ILK is a regulator in myofibroblast formation (de Iongh et al., 2005; Li et al., 2003; Weaver et al., 2007). Expression of ILK in cultured mouse and human lens cells is associated with elevated expression of transdifferentiation markers, fibronectin and αSMA. In addition, the introduction of ILK-expressing constructs resulted in an altered morphology that was fibroblast-like in nature (de Iongh et al., 2005). Exposure of HLE-B3 cells to TGFβ leads to increased expression of ILK in association with a loss of the epithelial cell marker E-cadherin, and increased αSMA protein levels (Zhang and Huang, 2018). ILK siRNA knockdown or an ILK inhibitor (QLT0267) prevents these TGFβ-induced effects (Zhang and Huang, 2018). Moreover, it has been observed that ILK co-localises with α5β1 integrin; the presence of fibronectin further promotes this association (Weaver et al., 2007). ILK appears to be a potentially important protein in the regulation of transdifferentiation and is likely to involve association with integrins and matrix components classically linked with fibrosis. Elucidating the role of ILK in the grand scheme of lens fibrotic changes would be an interesting line of investigation.

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3.1.3.3. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) belong to a larger family of proteases known as the metzincin superfamily and are often associated with tissue fibrosis: evidence supports a key role in PCO formation (Eldred et al., 2012). There are 26 MMPs including six membrane-tethered (MT)-MMPs in addition to four natural MMP inhibitors, the tissue inhibitors of MMPs (TIMPs). The MMP and TIMP family members can act on multiple targets and perform a variety of functions, which maintain and remodel tissue architecture (Robert et al., 2016; Sivak and Fini, 2002). The involvement of MMPs in fibrosis seems counterintuitive, as the primary role of MMPs is to degrade matrix, while a hallmark of fibrosis is ECM accretion. However, MMPs are not limited in function to simply degrading matrix. Their role is far more complex. MMPs can aid the release of growth factors and cytokines such as FGF-2 and TGFβ, from the lens capsule and activate macrophages (Imai et al., 1997; Tholozan et al., 2007; Yu and Stamenkovic, 2000). MMPs have been reported to play a key role in anterior subcapsular cataract, such that MMP inhibitors could attenuate TGFβ-induced fibrotic plaque formation in whole rat lens cultures (Dwivedi et al., 2006). In human capsular bag cultures, TGFβ2 exposure increased the level of both MMP-2 and -9 in the media (Wormstone et al., 2002), which coincided with increased matrix contraction and increase myofibroblast detection. Interestingly, bathing media obtained from cultured ex vivo capsular bags (donor lenses from patients who have previously had cataract operations) in serum-free media, also revealed expression of MMP-2 and -9, indicating that ex vivo capsular bags secrete MMPs in a manner similar to TGFβ2-treated cultures (Wormstone et al., 2002). Furthermore, Wong et al. (Wong et al., 2004) found that application of 100µM Ilomostat (a broad-specrum MMP inhibitor) significantly reduced cell migration and capsular bag wrinkling. Additionally, radiolabel binding analysis demonstrated that the lens capsule is a

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store for TGFβ2 (Wormstone et al., 2006a) along with FGF (Tholozan et al., 2007). MMPs are reported to release growth factors from the ECM and it is therefore likely that MMPs can regulate the degree of matrix-bound and free levels of cytokines available to lens cells over extended periods.

Analysis of MMP expression has been performed on the native human lens, primary cultures and the FHL124 cell line (Eldred et al., 2012; Hodgkinson et al., 2007). The levels of MMPs in the native lens are relatively low with MMP14 the most abundant in the anterior epithelium (Hodgkinson et al., 2007). Primary cultured human lens explants demonstrated a general increase in expression of MMP family members, but the most notable increases were MMP2 and MMP14 (Hodgkinson 2008). Interestingly, gene profiling of FHL124 cells demonstrated that MMP2 and MMP14 gene expression was upregulated by TGFβ2 (Eldred et al., 2012). This pattern was also observed at the protein level. Inhibition strategies using siRNA did not suggest that MMP14 played a role in TGFβ-induced matrix contraction. However, siRNA and antibody neutralisation of MMP2 in both FHL124 and human capsular bags did indicate MMP2 was important in this process (Eldred et al., 2012). In addition, it has been suggested that MMP14 plays a role in MMP2 activation from its proform. However, MMP14 inhibition did not prevent TGFβ-induced activation of MMP2 (Eldred et al., 2012), suggesting other modes of activation are involved.

We have also conducted experiments to assess the potential role of MMPs in the activation of TGFβ1 from its latent form. To achieve this we applied commercially available inactive latent TGFβ1 to lens cells. To ensure that the application of this molecule was relevant to PCO, we first conducted match-paired capsular bag experiments with control cultures maintained in serum-free EMEM and the other treated with 10ng/ml latent TGFβ1. At day 28

end-point, matrix contraction/wrinkling was greater with latent TGFβ1 treatment relative to controls (Figure 7A). A similar pattern was also observed with αSMA expression (Figure 7A). Having demonstrated that TGFβ1 could be converted to its active form in the capsular bag we applied latent-TGFβ1 to the FHL124 cell line. Again, treatment promoted matrix contraction and enhanced αSMA expression (data not shown). We then focused our attention on Smad signalling as this provides a direct and rapid measure of ligand/receptor interaction. Latent-TGFβ1 was found to promote Smad2/3 translocation to the nucleus within 2 hours. To determine if MMPs played a role in this response the broad-spectrum MMP inhibitor GM6001 was applied at 12.5µM, 1 hour prior to latent-TGFβ1 addition. Pre-treatment with GM6001 inhibited Smad translocation (Figure 7B & C). A similar outcome was observed using MMP2 siRNA knockdown (data not shown). These findings suggest a key role for MMP2 in latent-TGFβ activation.

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3.1.3.4. Advanced glycation end-products

The proteins that compose the lens capsule, like many other basement membranes, are long-lived and subject to chemical modifications which accumulate over time. One of these modifications, advanced glycation end-products (AGE) (Stitt, 2001) has been investigated in relation to PCO. LC-MS/MS analysis of human capsule samples showed age-dependent increases in several AGEs (Raghavan et al., 2016). Moreover, many AGEs are increased in cataractous samples. Interestingly, cells cultured on AGE-modified basement membrane and human lens capsule compared with those on unmodified proteins demonstrated an upregulation of fibrotic markers in response to TGFβ. Human capsular bag cultures were also analysed for AGE content of capsule proteins (Raghavan et al., 2016). TGFβ2-induced αSMA expression increased with donor age. In addition, it has also been shown that receptors

for AGEs (RAGE) are present in lens cells and inhibition of RAGE can suppress TGFβ-induced αSMA expression (Raghavan and Nagaraj, 2016). This work infers that AGE modification of the lens capsule can enhance fibrosis and would suggest that aged cataract patients are likely to have a stronger fibrotic response than their younger counterparts who often have a strong regenerative response. This idea is supported (to some degree) by a study on explant samples obtained from paediatric cataract surgery (Wernecke et al., 2018). The samples showed greatest levels of outgrowth from neonates, which diminished with relative increases in age. However, αSMA was not significantly enhanced in these young cultures by TGFβ2 exposure.

3.2. Regenerative PCO

The key characteristics of regenerative PCO are the formation of Soemmerring's ring at the periphery of the capsular bag and the generation of Elschnig's pearls, which are refractive structures that can encroach behind the IOL optic and cause visual disturbance. It is known that regenerative PCO, and in particular Elschnig's pearls, contribute more to a loss in visual quality than the fibrotic PCO form (Lu et al., 2019; van Bree et al., 2011). Despite the importance of regenerative PCO in the clinic, our understanding of the biology that underpins its development is poor.

Analysis of post-mortem human clinical samples using transmission and scanning electron microscopy has shown evidence of normal and aberrant lens fibre cells within the peripheral lens capsular bag (Kappelhof et al., 1987). A similar pattern has been observed using histological techniques (Koch et al., 2019). The formation of Soemmerring's ring in the rabbit is also reported, which is generally more developed than in human and its configuration better reflects the organisation of the intact lens, including relatively normal

nucleated bow fibres both at the equatorial side and adjacent to the IOL (Kappelhof et al., 1985). In some cases, histological sections present a layer of cells on the posterior capsule, while in others it is absent (Kappelhof et al., 1987; Koch et al., 2019; Saika et al., 2002). It is unclear whether Soemmerring's ring formation is an extension of lens fibre differentiation originating from the lens equator or fibre cell differentiation from the cells lining the peripheral posterior capsule; the latter could reflect a pattern of development similar to embryological primary fibre cell formation.

Elschnig's pearls are clinically the most important component of regenerative PCO as they can sit within the visual axis and are both irregular and refractive. Elschnig's pearls are globular cells with an extended basal section (Sveinsson, 1993). They contain a nucleus, which can range from round to oval to lobulated and vary in general appearance from smooth to being covered in microvilli (Findl et al., 2010). It is also reported that pearls are connected by gap junction and desmosome-like structures and contain few organelles. Elschnig's pearls also share many characteristics associated with lens fibre cells (Sveinsson 1993). Clinical studies (Findl et al., 2010) have demonstrated that Elschnig's pearls can be dynamic. Findl et al (Findl et al., 2010) assessed over 6000 pearls in 85 eyes and found constant change over the period of observation. Initially the pearls were small, but continued to grow in volume, fragment or shrink.

It has been reported that cultured human lens capsular bags accrue material in the peripheral bag, which could indicate that some level of fibre cell differentiation is initiated; however, these preparations did not mature into well-defined Soemmerring's rings (Koch et al., 2019). Nevertheless continued development of the capsular bag system to better model regenerative PCO would be a worthwhile pursuit.

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Rat lens epithelial explants undergo fibre cell differentiation in response to high levels of FGF and vitreous humour (Chamberlain and McAvoy, 1989; West-Mays et al., 2010). A modification to the rat lens explant system produced very interesting data that is relevant to Soemmerring's ring formation (O'Connor and McAvoy, 2007). In this case, the explants were cultured as pairs, one on top of the other with the anterior epithelium of each explant adjacent to one another. The cultures were maintained in Medium 199 containing bovine vitreous humour, which is rich in FGF. These paired explants underwent fibre differentiation and formed convex lens structures that had light-focusing ability. The peripheral fibre cells presented markers for early stages of fibre differentiation, while the central cells contained markers of terminally differentiated fibres. Interestingly, following long-term culture, opacity developed in the central region of the lens-like structure. This study has great relevance to PCO as the anterior and posterior capsules of the capsular bag come in close contact. As cells populated the posterior capsule and became stable following the initial period of growth, the scenario was very similar to the paired explant system in the capsular bag and is likely to follow a similar developmental path.

In vivo PCO animal model studies report the presence of Soemmerring's ring (Lois et al., 2003; Zukin et al., 2019; Zukin et al., 2018). At present, these models have not been fully exploited to understand the mechanisms driving Soemmerring's ring formation, but a recent study has suggested that aldose reductase inhibition could promote its formation (Zukin et al., 2019). Moreover, in the rat, electrical fields play an important role in lens regeneration (Lois et al., 2010) following fibre cell removal. In the rabbit, the influence of biodegradable and non-biodegradable scaffolds to support lens regeneration has been assessed (Gwon and Gruber, 2010).

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Primary cell culture systems can also be used to generate lentoids (Boswell et al., 2017; Lin et al., 2016). Neonatal rabbit cultures can establish lentoids that have magnifying properties and express fibre cell markers, β and γ crystallin (Lin et al., 2016). This same group reported the presence of stem cells (Lin et al., 2016). Dissociated cell-derived monolayer chick lens cultures have been used to demonstrate that in addition to its ability to induce transdifferentiation of lens epithelial cells to myofibroblasts, TGFβ can also promote lens cell fibre differentiation. This response was blocked by rapamycin, which links mTOR signalling to induction of fibre cell differentiation by TGFβ (Boswell et al., 2017).

Lens regeneration has been well studied in urodeles, but has become increasingly relevant in mammals (Kumar and Reilly, 2020) with much of our basic understanding of lens fibre cell differentiation formed from developmental biology (Lovicu et al., 2011). A great deal of information can be drawn from developmental systems and translated to the study of human lens fibre cell differentiation in relation to regenerative PCO. A number of growth factors and cell signalling pathways have been identified to regulate fibre cell differentiation, which include BMPs, TGFβs, Notch, Wnts and FGFs (Lovicu et al., 2011). The central figures in fibre cell differentiation are the FGFs, which seem to be crucial for initiation of fibre cell differentiation in mammals (Lovicu et al., 2011). This information was utilised to great effect to establish lens organoid formation from embryonic stem cells through sequential inhibition and activation of FGF, TGFβ and Wnt signalling (Yang et al., 2010); this is a superb example of translating animal studies to a human platform. This also suggests that the major regulatory systems of fibre cell differentiation are relatively well conserved across mammalian species. Incremental refinement of these systems has increased the efficiency of lentoid generation and conditions have been improved to enable better manipulation of the

environment (Fu et al., 2017; Murphy et al., 2018) (Figure 3). Stem cell-derived lentoids could form a major study tool to characterise successful and aberrant fibre cell differentiation in relation to human PCO.

4. Management and prevention of PCO

4.1. Intraocular implants

It is important to remember that PCO is driven by wound-healing events, triggered by the trauma of cataract surgery. Nevertheless, an IOL can influence the path of PCO progression and serves as a valuable tool in the management of PCO. Over the past few decades, IOLs have emerged in many forms, constructed from a range of materials. At present the most commonly implanted IOLs are foldable square-edged IOLs with looped haptics. In the following text, we will discuss features associated with standard IOL designs and describe alternative design concepts for PCO prevention.

4.1.1. The barrier effect

The Alcon Acrysof™ IOL was the first commercial IOL to have a square-edge optic profile. While grand statements were made to suggest that the introduction of this IOL had eradicated PCO (Apple et al., 2001) proved unfounded, the notion that rates of PCO and Nd:YAG capsulotomy were reduced relative to other IOLs was justified (Perez-Vives, 2018). The improvement in patient outcome is attributed to the square-edge profile. A square-edge profile is now a common feature of commercial IOLs, however it is argued that the manufacture of the edge can differ such that some IOLs have a sharper edge than others, which can influence the outcome (Perez-Vives, 2018). A major topic of debate is the material of the IOL and its ability to enhance the interaction of the IOL and the lens capsule (Linnola,

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1997), while others argue that the benefits of a square-edge are independent of material (Nishi, 1999).

Linnola (Linnola, 1997) presented the Sandwich Theory of PCO to provide a mechanism by which a hydrophobic acrylic IOL with a square-edge optic (such as the Alcon Acrysof™) could reduce PCO progression. This theory presents the case that the IOL material can exhibit biocompatibility in a bioinert or bioactive manner. It is argued that hydrophobic acrylic material provides benefit through a bioactive principle. It is proposed that cells grow across the posterior capsule following surgery and encounter the optic edge. This provides some impedance to growth, but cells still progress behind the optic. If the cell population on the posterior capsule remains as a homogenous monolayer, its impact on light scatter is reduced. It is proposed that the bioactive material enables cells to adhere to both the IOL and the capsule, such that cells form a 'sandwich' between these layers. This tight adhesion and close apposition of the capsule to the IOL is believed to increase the barrier function and reduce further cell movement below the optic. The bioactive component identified to support this theory is the matrix component fibronectin, which is reported to be the key biological component that enables adhesion between the IOL, cells and capsule. The major evidence to support this idea is histological assessment of post-mortem capsular bag samples and IOLs removed from post-mortem capsular bags (Linnola et al., 2000a, b). Unfortunately, it is difficult to draw any meaningful conclusion from these data as samples were fixed prior to IOL removal. In such circumstances, fixation provides strong bonds between the biological material and the IOL. To assess if cell and matrix accumulation on the IOL has taken place the IOL needs to be removed prior to fixation and then undergo histological analysis. Recent work using the human capsular bags has illustrated that the major site of cell growth on hydrophobic acrylic IOLs is within the rhexis margin (Eldred et al., 2019). It would appear

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that if cells have a choice between the native capsule and an IOL they will choose the more complete natural substrate. This therefore suggests that IOL materials are unlikely to enhance the barrier effect through a bioactive biocompatibility mechanism. Work by Nishi (Nishi, 1999) demonstrated that a square-edge optic offered benefit regardless of material, which again suggests this is a physical phenomenon independent of material that could be enhanced by design features, such as haptic shape/angulation and the manufacturing process employed to generate the square-edge profile.

4.1.2. Open-bag IOLs

Standard IOL designs produce a closed bag system where the lens capsule is in close apposition to the IOL. In contrast, open bag devices partition the anterior and posterior capsules (Figure 8). This is proposed to allow irrigation of the capsular bag and diminish the impact of growth factors on PCO progression (Eldred et al., 2016; Eldred et al., 2014). Capsular ring devices implanted during cataract surgery have been tested in animal systems and human patients. These ring systems can house a standard IOL and show a marked reduction in PCO incidence (Alon et al., 2014; Hara et al., 2011; Hara et al., 1995). Recent reports have suggested that introduction of a square edge capsular ring and increasing apertures in the wall of the ring implant provide optimal outcomes when assessing PCO in a rabbit in vivo model (Slutzky and Kleinmann, 2018). The Anew Zephyr™ open-bag IOL is a disk-shaped 1-piece hydrophilic acrylic monofocal device that provides refractive correction and separation of the anterior and posterior capsules in a single device. Introduction of the Anew Zephyr™ IOL has been found to reduce PCO progression relative to Alcon Acrysof™ closed bag IOLS, in a suspended human lens capsular bag model (Eldred et al., 2019). Moreover, in the rabbit in vivo model, which exhibits aggressive PCO, PCO scores were low

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even 6 months following surgery, whereas control eyes implanted with Alcon Acrysof™ IOLs presented severe PCO in all cases (Aliancy et al., 2018).

4.1.3. The bag-in-the-lens IOL

In contrast to conventional surgery, which requires a single opening in the anterior capsule, the bag-in-the-lens (BIL) technique requires making an opening of the same size in the anterior and posterior lens capsules (De Groot et al., 2005; Tassignon et al., 2002). The edges of the capsule openings are slotted into a groove at the periphery of the IOL in a similar manner to a tyre being introduced to a wheel. According to this concept, if both capsules are placed in the groove and are well stretched around the optic of the IOL, any lens epithelial cells will be captured within the remaining space of the capsular bag and their growth will be limited to this space, so the visual axis can remain clear. This concept was first tested using the human capsular bag model (De Groot et al., 2005; Tassignon et al., 2002) and it was found that over a six week culture period cells did not proliferate and migrate across the IOL i.e. the cells were retained within the remaining capsular bag. A rabbit model was used for in vivo evaluation and again cells were retained within the residual capsule (De Groot et al., 2005; Tassignon et al., 2002). Significant numbers of BIL IOLs have been implanted and the outcomes are impressive. The introduction of BIL IOLs is particularly relevant to paediatric patients, who have rapid onset of PCO. Successful implantation of a BIL IOL prevented PCO, even in these extreme paediatric cases (Tassignon et al., 2007). The limiting factor in the uptake of this approach is the level of skill required by the surgeon to carry out this procedure. However, instructional courses are available along with an international panel of BIL instructors who can pass on their knowledge and demonstrate the technique. Currently, the majority of BIL surgeries are performed in Europe, but as surgical outcomes (short and

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long-term) continue to demonstrate excellent outcomes, particularly for children, this approach is likely to be adopted more readily by additional countries.

4.2. Alternative surgery

Previous work has shown that in animal systems lens regeneration can occur following cataract surgery, but this is reliant on retention of the anterior lens epithelium and capsule (Gwon and Gruber, 2010; Lois et al., 2010). Building on this work, Lin et al (Lin et al., 2016) employed a small, off-set opening in the anterior capsule and retention of the lens epithelial cells that could facilitate regeneration of a new lens from endogenous stem cells within the epithelial cell population. This work was carried out on infants who presented congenital cataract (present from birth). There was clear evidence that some level of lens regeneration occurred and provided some benefit to these patients. However, a number of issues need to be considered when contemplating this approach as an alternative for conventional surgery. The capacity to repair and regenerate is faster in the young and several months elapsed to see some level of visual improvement. In older patients (i.e. the vast majority of cataract patients), the ability to reform a lens will be significantly slower and less likely to be successful.

For this new form of surgery to be successful, the regenerated lens will need to closely reflect the normal healthy human lens and do so relatively quickly; otherwise, further care will be required. Lens regeneration surgery holds great promise, but at present has significant limitations and ethical concerns that prevents mass introduction of this technique for cataract patients (Liu et al., 2018; Solebo et al., 2018; Vavvas et al., 2018).

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4.3. Pharmacological treatment

Despite major advances in the design of IOLs and small incision surgery, PCO still remains a significant problem, both from a patient wellbeing perspective and as an economic burden to healthcare providers (Brown et al., 2013). It would therefore seem logical that pharmacological intervention in concert with optimal IOL design should offer the best opportunity to provide a sustained clinical benefit from cataract surgery. A recent study that surveyed both human and veterinary ophthalmologists revealed that the majority of practitioners were in favour of this approach (Shihan et al., 2019). A number of approaches can be adopted that target specific PCO-related events. The vast majority of proposed strategies address fibrotic PCO and are generally concerned with controlling the inflammatory response to reduce the initial stimulus provided by enrichment of the ocular environment; preventing proliferation and migration across the posterior capsule; prevention of myofibroblast transdifferentiation and matrix contraction, which usually involves targeting TGFβ signalling or pathways regulating this system.

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Non-steroidal and steroidal anti-inflammatory drugs are routinely used in surgery. However, in relation to PCO little benefit has been demonstrated (Laurell and Zetterstrom, 2002). Indeed, it was actually found that anti-inflammatory treatment of patients undergoing cataract removal surgery resulted in a higher number of patients developing fibrotic PCO 4 years post-surgery than subjects treated with a placebo. A recent study, however, has suggested that a combined application of non-steroidal and steroidal anti-inflammatory drugs could more effectively lower PCO incidence relative to single drug treatments (Hecht et al., 2020). Moreover, Symonds *et al* (Symonds et al., 2006) demonstrated that the steroid dexamethasone, which is routinely administered to patients undergoing cataract surgery, improves cell survival and increases collagen type I synthesis in a rat lens explant model of

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PCO. This study, therefore, suggests that lens cells are susceptible to the actions of anti-inflammatory molecules. Chandler et al (Chandler et al., 2007) tested the direct actions of cyclooxygenase 2 (COX-2) through application of COX-2 inhibitors rofecoxib or celecoxib, to canine capsular bags; both inhibitors suppressed PCO. A clinical trial was performed in dogs to compare celecoxib-impregnated IOLS against Bromofenac treatment (Brookshire et al., 2015). The Celecoxib-IOL showed better initial control of PCO (up to 12 weeks), while eyes that received bromfenac had better long-term control of PCO (56 weeks).

In terms of regulating cell growth across the posterior capsule, there are many candidates for drug targetting. The lens has a strong autocrine system that ensures baseline growth is relatively strong (Eldred et al., 2016). Inhibition studies in the human capsular bag have shown that inhibition of FGF, EGF and VEGF signalling (among others) can slow growth, but not completely prevent it (Eldred et al., 2016; Maidment et al., 2004; Wertheimer et al., 2015b; Wormstone et al., 2001). This suggests that multiple autocrine signalling pathways can contribute to PCO. Therefore, pathway inhibition may be more effective than targeting specific receptors, as it could suppress stimulation from multiple growth factors.

A number of the strategies proposed to prevent PCO concentrate on preventing TGFβ-induced fibrotic events (Eldred et al., 2011; Nibourg et al., 2015; Saika et al., 2008); in the majority of cases the major focus is on the ability of a given drug to prevent myofibroblast formation (Eldred et al., 2012; Nam et al., 2020; Smith et al., 2019; Zukin et al., 2018). It has also been reported that there is a sub-population of polyploid mesenchymal progenitors cells within the chick lens epithelium (Walker et al., 2010) and it is suggested that these cells could be a primary source of myofibroblasts following cataract surgery and therefore contribute to PCO development (Walker et al., 2010). These myogenic progenitor cells have also been

identified in the human lens epithelium (Gerhart et al., 2014), and antibody drug conjugates have been developed to specifically eliminate this population of cells (Gerhart et al., 2017; Gerhart et al., 2019). It is important to note that in relation to PCO the major light scattering element within fibrosis results from matrix deformation, which is generally referred to as matrix wrinkling (van Bree et al., 2011). It is generally assumed that transdifferentiation to a myofibroblasts is a pre-requisite for matrix contraction (Shu and Lovicu, 2017). This concept is based on the principle that myofibroblasts assemble an integrin, fibronectin and αSMA contractile apparatus, which leads to matrix contraction (Shu and Lovicu, 2017). This may be true in some systems, but work with FHL124 cells suggests this may not be essential in the lens (Dawes et al., 2008; Dawes et al., 2009). Therefore, it is important to consider the impact on matrix contraction, rather than solely concentrating on EMT, when developing and evaluating anti-TGFβ/fibrotic strategies to manage PCO. Furthermore, the ability of aldose reductase inhibition on PCO formation was recently assessed (Zukin et al., 2019; Zukin et al., 2018). In a mouse model of PCO, genetic and chemical inhibition of aldose reductase could suppress surgical induced events associated with fibrotic PCO (Zukin et al., 2018), however Soemmerring's ring formation was enhanced (Zukin et al., 2019). This is an important finding as it suggests suppression of fibrotic PCO or at least transdifferentiation to myofibroblasts could in turn promote the formation of regenerative PCO, which is likely to play a greater role in visual deterioration.

As an alternative, complete ablation of the lens cell population is a viable strategy (D'Antin et al., 2018; Duncan et al., 2007; Duncan et al., 1997; Liu et al., 2017; Zhang et al., 2019), but it is critical that non-lenticular tissues are unaffected by any putative treatment (Duncan et al., 2007; Rabsilber et al., 2007; Wertheimer et al., 2017). Another consideration is the stability

of an IOL in an acellular capsular bag (Spalton et al., 2014); however, modification of haptic designs to improve positioning within the bag may address these concerns.

Regardless of the approach adopted, it is important that an appropriate drug dose be delivered over a specific period to provide efficacy. Ultimately, a drug needs to reach the target cells at sufficient concentration to work. Utilising drug delivery strategies is an important consideration and area of development. The drug delivery possibilities include direct injection into the anterior chamber; modification of the irrigating medium; modification of the IOL/capsular tension ring; application using a closed-bag drug delivery device.

One mode of delivery is via the IOL. This approach has been considered over the past three decades and is becoming increasingly more sophisticated. Early drug modulation of the IOL included an FGF-saporin complex bound to a heparin surface modified IOL, which was tested in rabbits (Behar-Cohen et al., 1995). When in contact with the cells, FGF binds to the appropriate receptors on the epithelial cells and internalises the saporin, subsequently killing the cells. This particular system produced some side effects, including transient corneal oedema and iris depigmentation. Duncan et al (Duncan et al., 1997) showed, using a human capsular bag model, that thapsigargin (a specific inhibitor of the endoplasmic reticulum CaATPase) when directly coated onto a PMMA IOL can effectively kill the entire cell population within the bag. However, this was a simple coating procedure and does not account for additional stresses associated with in vivo surgery e.g. entry through the corneal/scleral tunnel. Therefore, a drug release system that is both physically robust and provides appropriate drug delivery is necessary. Strategies now include impregnating the IOL (Bouledjouidja et al., 2016; Brookshire et al., 2015; Wertheimer et al., 2015a). This has proved a useful way to provide long-term delivery of an agent. However, it is important that

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incorporation of any drug does not disrupt the light path through the IOL. A recent study (Ongkasin et al., 2020) reported that an impregnated IOL provided a sustained release of methotrexate for 80 days. The methotrexate-impregnated IOL was tested in the human capsular bag model and found to reduce αSMA expression, but did not significantly inhibit growth across the posterior capsule. Similar approaches have been developed for use in tension rings and one example reports a photodynamic regulated drug release from an IOL (Zhang et al., 2016).

An alternative therapeutic approach is to use a closed bag drug delivery system, such as the perfect capsule device, which permits high concentrations to be delivered to cells within the capsular bag for a designated period and then removed (Maloof et al., 2005). Duncan et al (Duncan et al., 2007) tested various agents, applied for a 2-minute period, in human capsular bags using this drug delivery system. Treatments included distilled water, 3M sodium chloride, 5-Fluorouracil (5-FU) (250ug/ml and 25mg/ml) and thapsigargin. Assessment of growth on the posterior capsule revealed greatest effect with thapsigargin and 5-FU, however in the case of 5-FU treated bags, cells were growing on the peripheral posterior capsule beneath the anterior capsule; no viable cells at all were observed in thapsigargin treated bags. In this in vitro study, distilled water was not found to significantly influence events in the capsular bag and interestingly when distilled water was applied in the clinic using the perfect capsule device this outcome was also observed (Rabsilber et al., 2007). A recent study also applied osmotic challenge to the cells within the capsular bag, but included a Na-K-Cl co-transporter inhibitor to prevent recovery; these results look promising (Zhang et al., 2019).

5. Conclusions

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PCO remains an important problem following cataract surgery. Both fibrosis and attempted lens regeneration can contribute to reduced visual quality in patients. However, the latter plays a greater role in visual disturbance. We have a wealth of resources and tools available that will aid advances in our understanding of PCO biology, which will enable better selection of putative agents to inhibit or prevent PCO formation. IOL designs continue to improve through greater appreciation of their influence on lens cell behaviour and PCO formation. A combination of improved IOL design and pharmacological treatment is likely to provide the best outcome for patients in the future and improved drug delivery strategies should play a key role in their success.

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Figure 1. PCO development following cataract surgery. A Schematic diagram illustrating (A) the capsular bag immediately post-surgery, (B) development of fibrotic PCO showing invasion of the posterior capsule and matrix deformation/capsular wrinkling and (C) the changes that result from a combination of fibrotic and regenerative PCO with formation of Elschnig's pearls and Soemmerring's ring.

Figure 2. The suspended human capsular bag model for IOL evaluation. Representative modified dark-field images of match-paired capsular bags implanted with a Hoya Vivinex or an Alcon Acrysof IOL captured on the day of simulated surgery. In both cases the capsulorhexis (arrowed) is fully seated on the IOL optic. (Eldred et al., 2019).

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Figure 3. Lentoid generation from iPSC cultures using iMatrix-511 as a culture substrate. (A) Images showing progression of naive iPSCs to lentoids. Images depict Days 0, 6, 9, 16 and 21 days of culture. All images were captured using a Nikon Ti2 inverted light microscope. Scale bar represents 100µm for Day 0 and 250 µm for all other days. (B) Quantitative graphs showing real-time PCR data obtained for key marker genes over the course of the experiments. The x-axis marks the sample time-points i.e. Days 0, 6, 12, 16 and 18 and the y-axis denotes the relative fold-change of each gene's RNA transcript over time. Note that the data has been adjusted such that the Day 0 values for each gene equals 1. All subsequent data points are thus relative and comparable to Day 0.

Figure 4. A schematic outline of (A) sequential events following cataract surgery that can ultimately give rise to PCO and (B) the relationship between selected growth factors and functional events associated with fibrotic and regenerative PCO. HGF – hepatocyte growth factor; FGF – fibroblast growth factor; VEGF – vascular endothelial growth factor; EGF –

epidermal growth factor; TGFβ – transforming growth factor β; BMP – bone morphogenic protein; EMT – epithelial to myofibroblast transdifferentiation; MET – myofibroblast to epithelial transdifferentiation.

Figure 5. Induction of fibrotic responses in a human capsular bag model. (A) Representative epifluorescence micrographs showing the distribution of the myofibroblast marker, α-smooth muscle actin and the matrix component, fibronectin associated with cells growing on the central posterior capsule of human lens capsular bag preparations maintained in SF or graded culture conditions. Images were captured at endpoint (day 28). (B) Pooled data showing quantitative αSMA and fibronectin expression in association with cells growing on the central PC of capsular bags maintained in serum-free or graded culture conditions. Data are presented as mean ± SD (n = 5). *Indicates a significant difference between groups (Student's t-test; $P \leq 0.05$). (Eldred et al., 2019).

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Figure 6. Time-dependent sensitivity to TGFβ in the human lens epithelium. Fluorescent micrographs showing Smad2/3 translocation in human lens epithelial explant cultures immediately following dissection or following culture periods of 6, 24 or 48 hours in serum-free medium with 10ng/ml added to the medium for the final 2 hours of culture. Scale bar represents 100µm.

Figure 7. Human lens cells can activate TGFβ. Latent-TGFβ1 can be activated by lens cells and is MMP dependent (A) Modified darkfield images captured following 28 days culture maintained in serum free medium or treated with 10ng/ml latent TGFβ1 showing matrix deformation of the posterior capsule and fluorescence micrographs showing an associated increase in SMA following treatment. AC - anterior capsule; PC –posterior capsule; arrows

indicate the capsulorhexis margin. (B) Fluorescent micrographs showing inhibition of MMPs prevents Smad2/3 nuclear translocation in FHL124 cells. FHL124 cells were either untreated (control), or pre-treated with the MMP inhibitor GM6001(12.5μM), followed by addition of latent TGFβ1 for 2 hours. (C) Nuclear Smad2/3 was quantified from fluorescent micrographs. *indicates significant difference, $p<0.05$, ANOVA with post hoc Tukey's test (n=3).

Figure 8. A schematic diagram illustrating the position of (A) a conventional square-edged IOL and (B) the open-bag Anew Zephyr IOL within the capsular bag and how their physical properties could provide a barrier to cell movement. (Eldred et al 2014).

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Figure 1

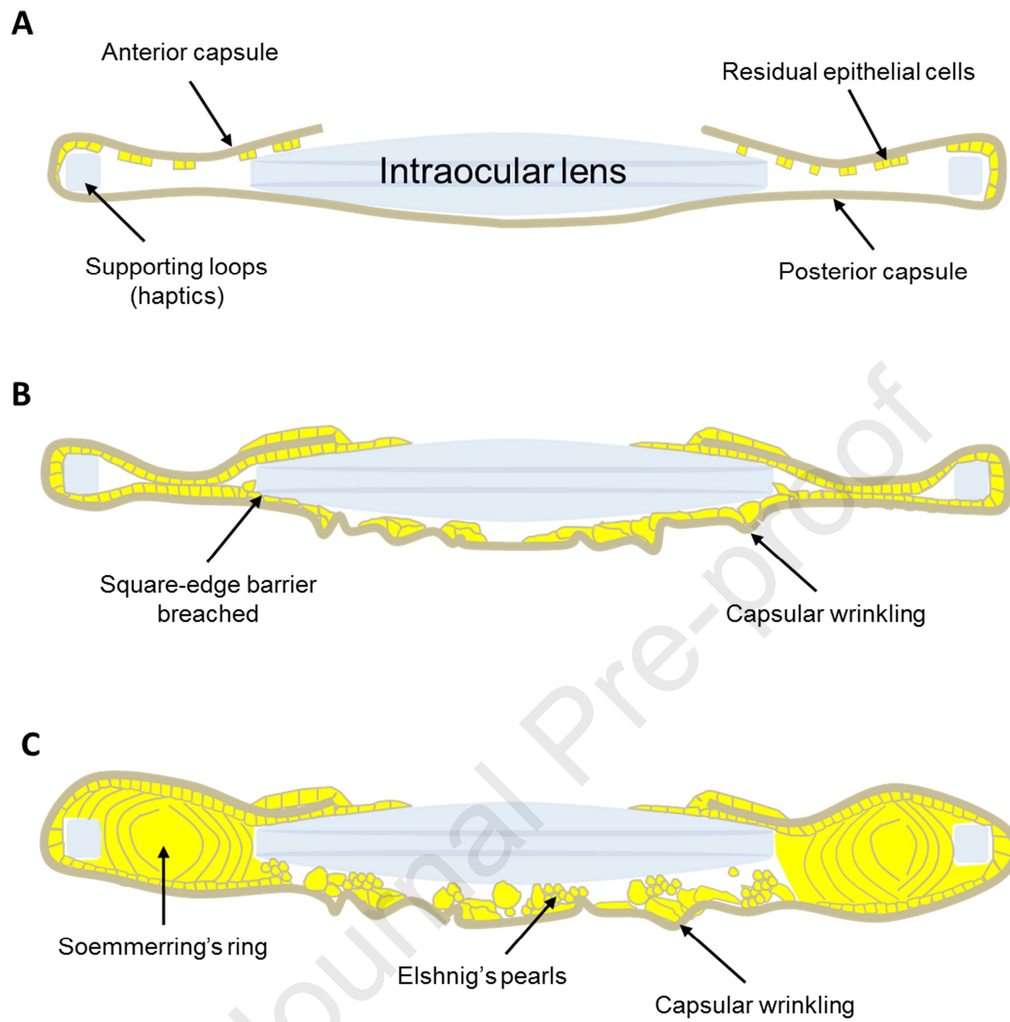

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Figure 2

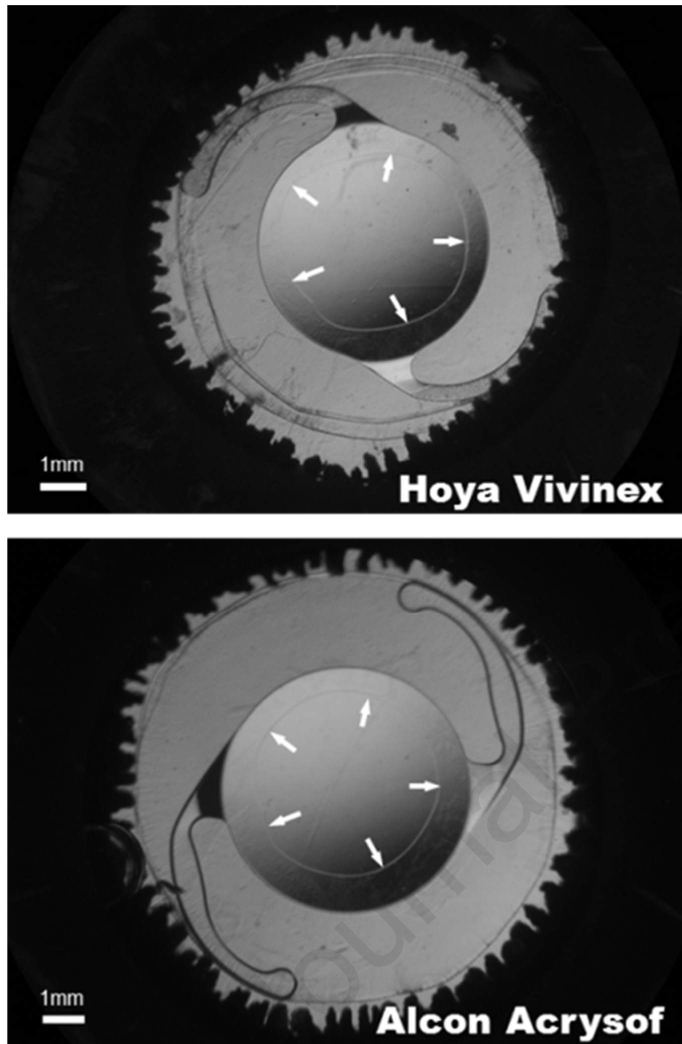

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Figure 3

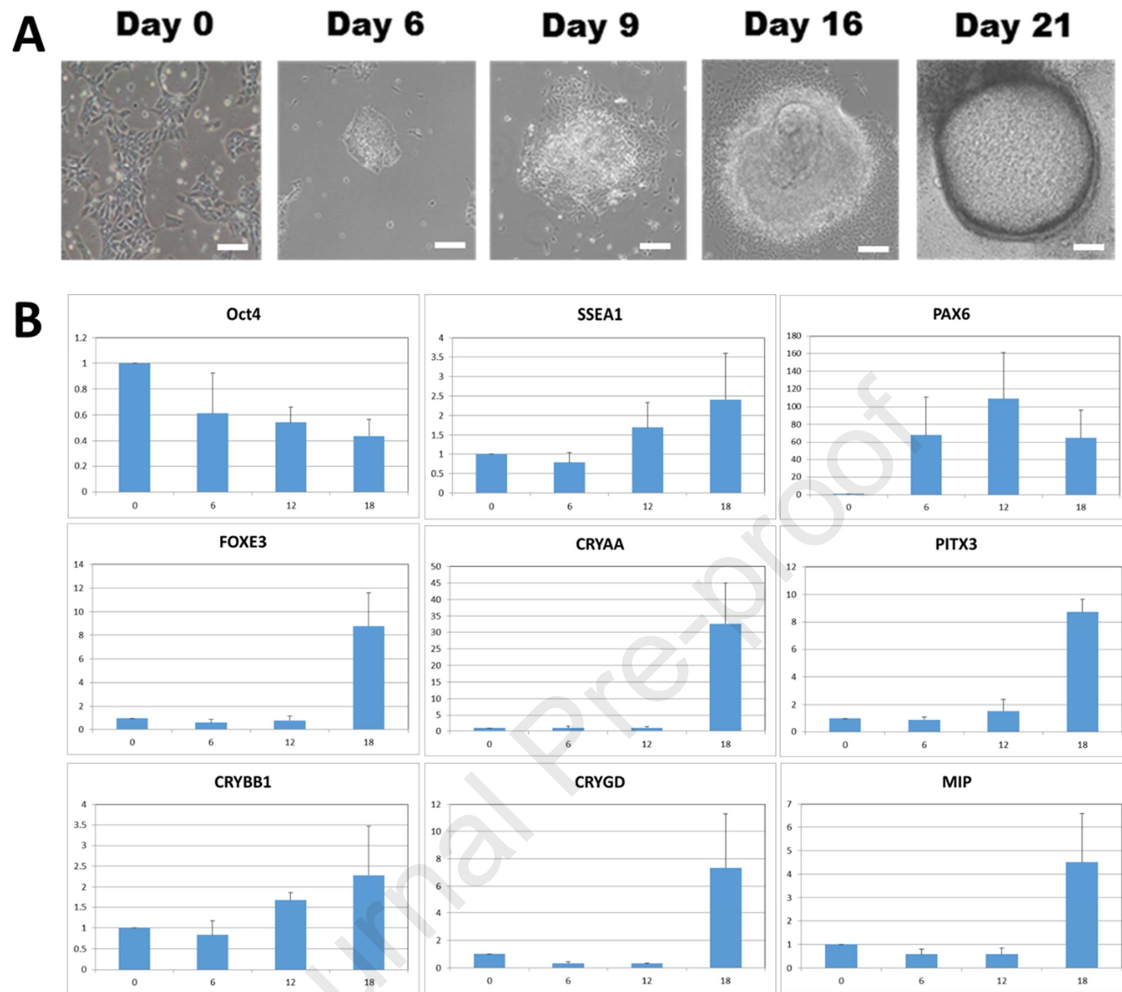

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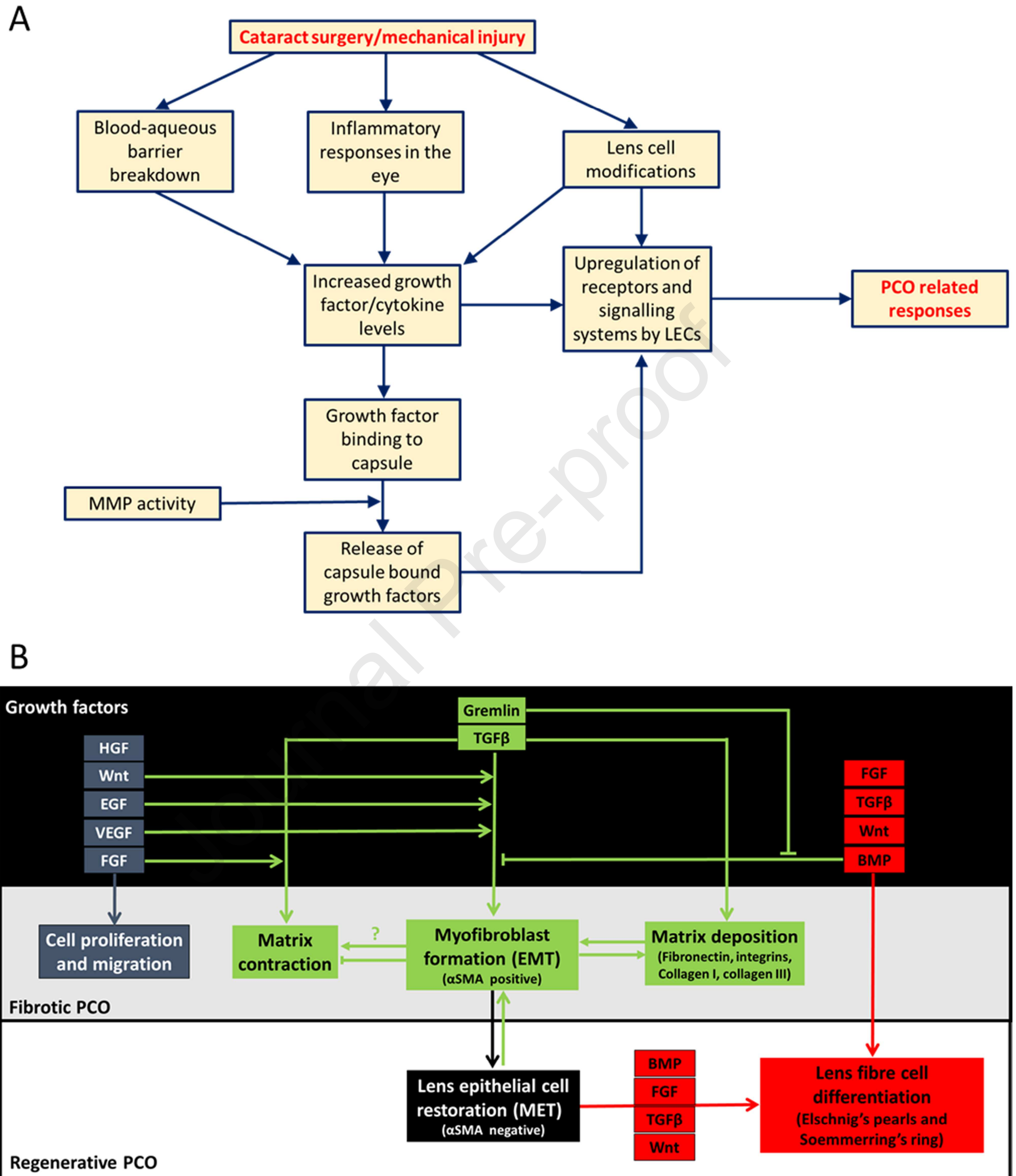
Figure 4



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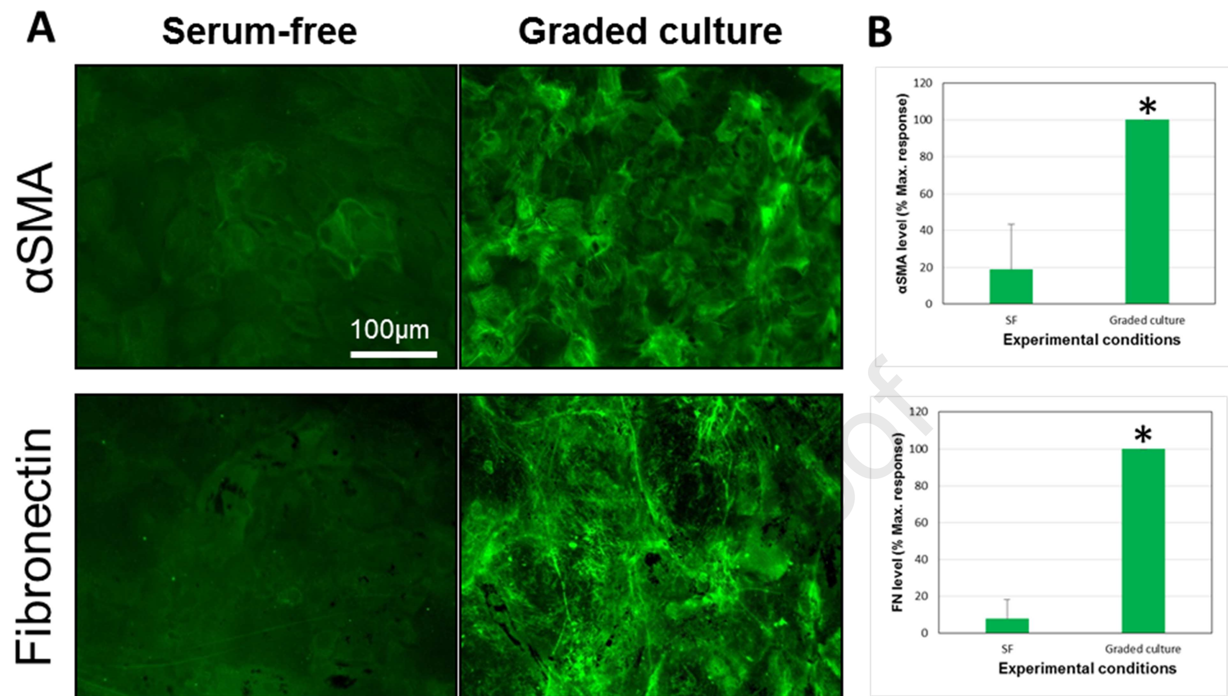

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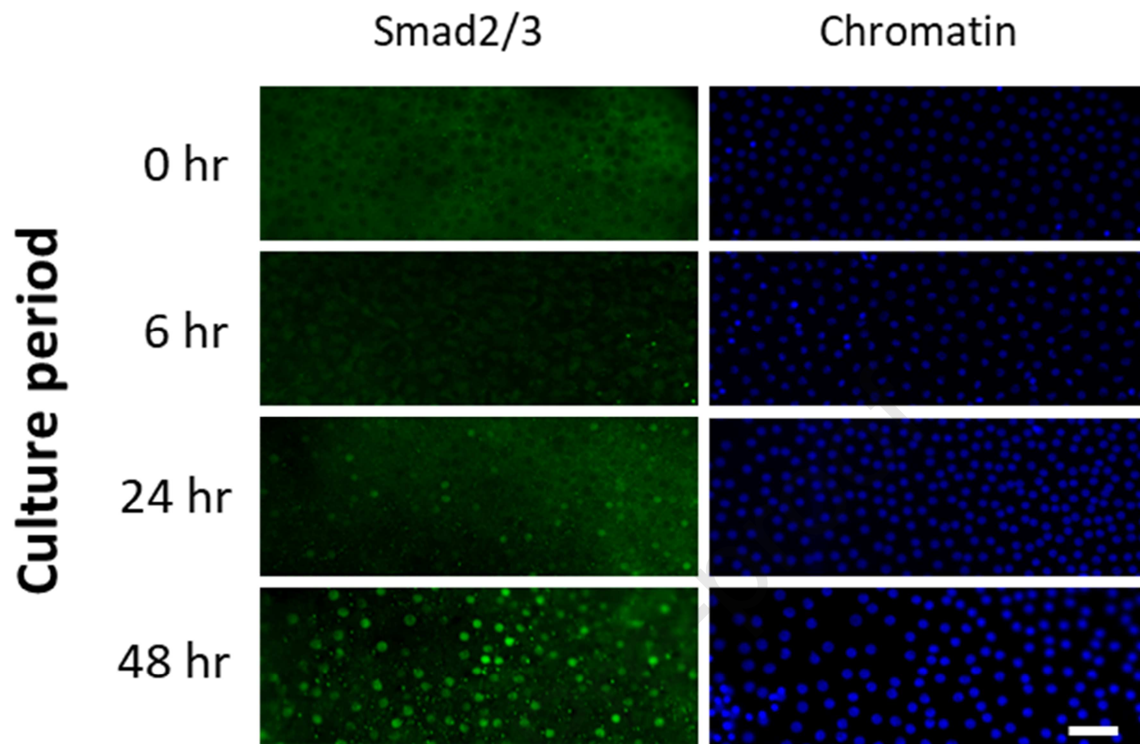

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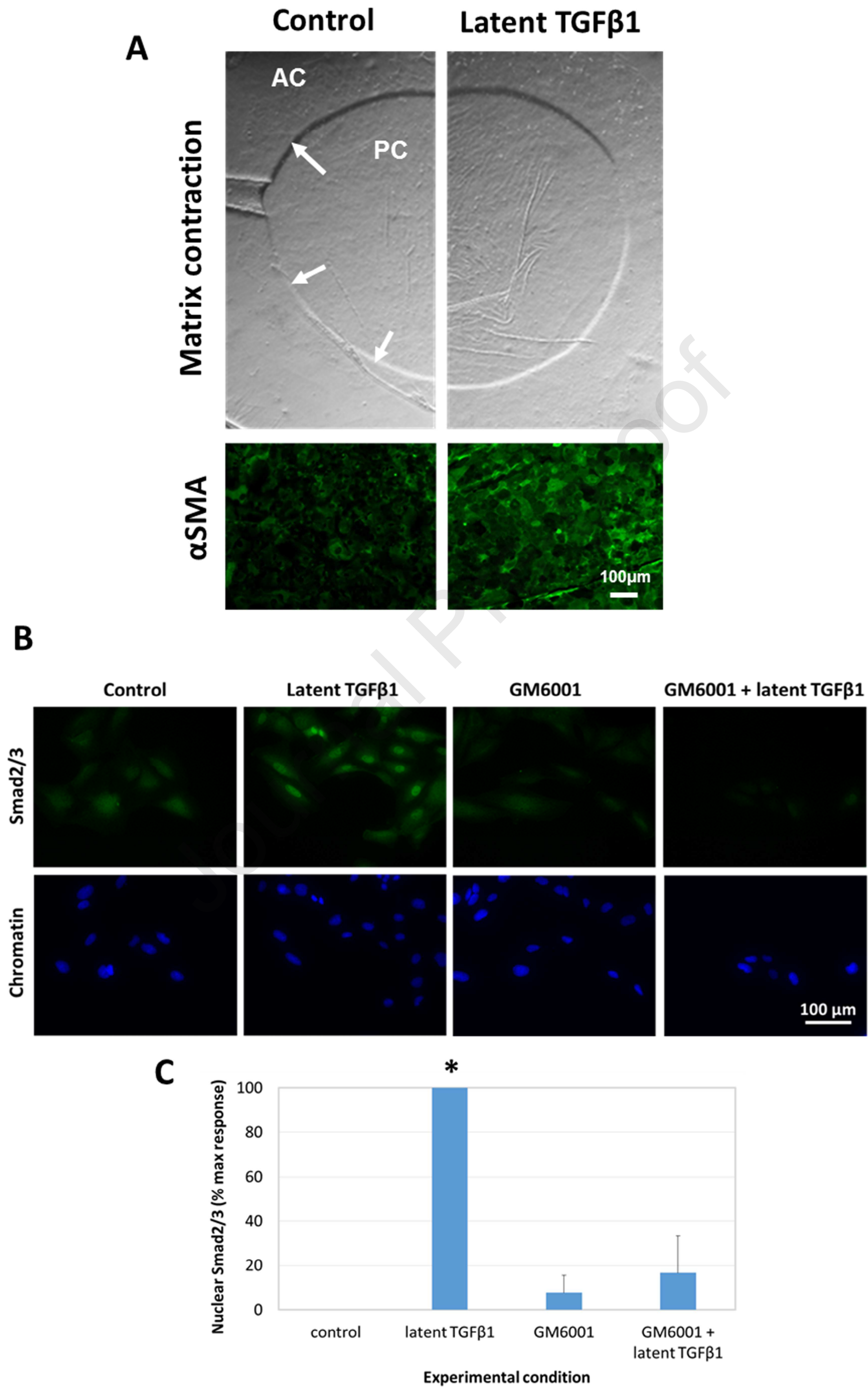

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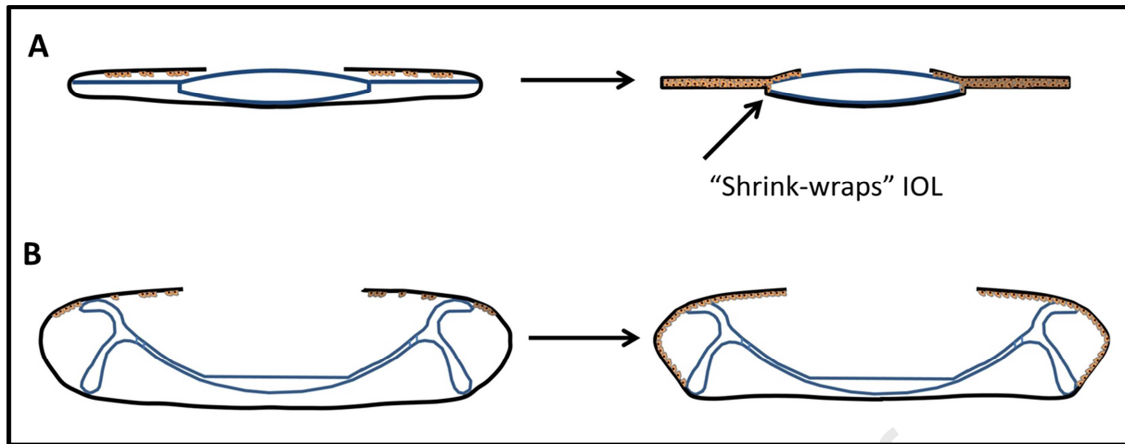

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Author statement

MW provided the first draft and YMW, AJOS and JAE contributed to manuscript development and editing. All authors conducted and conceived experiments that generated novel data included in the manuscript.

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