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Keratocytes are induced to produce collagen type II: A new strategy for in vivo corneal matrix regeneration

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Abstract

The stroma, the middle layer of the cornea, is a connective tissue making up most of the corneal thickness. The stromal extracellular matrix (ECM) consists of highly organised lamellae which are made up of tightly packed fibrils primarily composed of collagens type I and V. This layer is interspersed with keratocytes, mesenchymal cells of neural crest origin. We have previously shown that adult corneal keratocytes exhibit phenotypic plasticity and can be induced into a neuronal phenotype. In the current study we evaluated the potential of keratocytes to produce collagen type II via phenotypic reprogramming with exogenous chondrogenic factors. The cornea presents a challenge to tissue engineers owing to its high level of organization and the phenotypic instability of keratocytes. Traditional approaches based on a scar model do not support the engineering of functional stromal tissue. Type II collagen is not found in the adult cornea but is reported to be expressed during corneal development, raising the possibility of using such an approach to regenerate the corneal ECM. Keratocytes in culture and within intact normal and diseased tissue were induced to produce collagen type II upon treatment with transforming growth factor Beta3 ($TGF\beta_3$) and dexamethasone. In vivo treatment of rat corneas also resulted in collagen type II deposition and a threefold increase in corneal hardness and elasticity. Furthermore, the treatment of

corneas and subsequent deposition of collagen type II did not cause opacity, fibrosis or scarring. The induction of keratocytes with specific exogenous factors and resulting deposition of type II collagen in the stroma can potentially be controlled by withdrawal of the factors. This might be a promising new approach for *in vivo* corneal regeneration strategies aimed at increasing corneal integrity in diseases associated with weakened ectatic corneal tissue such as keratoconus.

Keywords: Cornea, stroma, keratocytes, growth factors, Transforming growth factor beta3, keratoconus

1 Introduction

It has become increasingly important to develop new therapeutic interventions for tissue engineering and regeneration as current tissue regeneration technology is yet to deliver in the clinical setting. The introduction of cell reprogramming via the production of induced pluripotent stem cells (Takahashi & Yamanaka, 2006) has heralded a new revolution and has driven the search for the ultimate tissue regeneration treatment. However, before genetically induced cell reprogramming based methods can be successfully translated into therapeutic treatments, problems such as the low efficiency and safety issues associated with such technology first need to be dealt with.

The stromal layer of the cornea is composed of highly organised lamellae which are made up of tightly packed collagen fibrils of collagen types I and V (Marshall, Konstas, & Lee, 1993) interspersed with keratocytes, mesenchymal cells of neural crest origin. Keratocytes are quiescent, their main function being the repair of the cornea. This is possible as they can transform into an activated phenotype in response to growth factors and cytokines at the site of injury. The activated keratocytes have morphological characteristics of fibroblasts and

myofibroblast (Fini, 1999; Jester, Rodrigues, & Herman, 1987). The biosynthetic activity of these repair phenotypes is mostly limited to the production of fibrotic repair extracellular matrix different in composition to that produced by the normal keratocytes and the persistence of myofibroblasts results in scarring and loss of transparency of the cornea (Desmouliere, Chaponnier, & Gabbiani, 2005; Ross & Benditt, 1961). Populations of stem cells have been identified in many mesenchymal tissues and in the corneal stroma stem cells are thought to reside in the limbal region, the transition zone between the cornea and the sclera (Du, Funderburgh, Mann, SundarRaj, & Funderburgh, 2005). Specialised features of the limbal microenvironment such as the presence of a dense blood supply is thought to help maintain the stem/progenitor character of corneal stromal stem cells *in vivo* (Pinnamaneni & Funderburgh, 2012). However there are no definitive markers for the stem cell population within the stroma. In previous studies we have shown that all keratocytes from adult human and rat corneas differentiate into a neuronal phenotype when treated *ex vivo* and *in vivo* with neuronal lineage specifying growth factors (Greene et al., 2013). During this phenotype switch genes and proteins linked to multipotency and neuronal differentiation were up-regulated. We have shown that, in the cornea, stemness is not limited to a sub-population of cells found near the corneal limbus. Although a limbal niche might provide the required environment for cells to be maintained in a particular differentiation state, all cells in the cornea are amenable to phenotype reprogramming.

The cornea, in particular, presents a challenge to tissue engineers. After cataracts, corneal damage and diseases are the largest cause of vision loss, affecting more than 10 million people worldwide (Whitcher, Srinivasan, & Upadhyay, 2001). Currently, the most successful treatment for corneal blindness is replacement of damaged corneal tissue with human donor corneal tissue. Apart from drawbacks such as complications arising from immune rejection, donor-derived infection, and expensive pretransplant screening, the major problem with this

approach is the severe shortage of donor tissue. Significant progress has been made in both the generation of artificial corneas and stem cell therapy (Levis et al., 2015). However, corneal transplantation using cadaveric donor corneas remains irreplaceable. Keratoconus, an ectatic corneal dystrophy, affects approximately 1 in 2000 individuals worldwide. The progressive thinning of the corneal stroma typically occurs over decades and results in a conical shaped cornea that then impairs vision due to irregular astigmatism and myopia (Krachmer, Feder, & Belin, 1984). The corneal thinning is a result of the loss of corneal stromal extracellular components such as collagen (Klintworth & Damms, 1995; Rabinowitz, 1998; Sherwin & Brookes, 2004). Currently there are no treatments which stop corneal thinning. Interventions are mainly focussed on stiffening a weakened cornea or improving the visual acuity rather than stopping disease progression.

The goal of this study was to investigate the potential of keratocytes to switch to a phenotype that secretes collagen type II. The reason being that type II collagen has been reported to be expressed during cornea embryonic development (Linsenmayer, Fitch, & Birk, 1990) and its re-expression might be induced for corneal repair. The most suitable driving factors for differentiation were first identified in literature. These factors were then used to drive keratocytes to differentiate into a cell phenotype that secretes collagen type II thereby leading to deposition *in vitro* in cell culture and *ex vivo* in organotypic slice cultures. When choosing growth factors, cytokines and chemicals that might bring about collagen deposition in the corneal stroma it was important to consider known effects of exogenous growth factors. For example, the TGFβ family of growth factors are known to be the most potent inducers of chondrogenic differentiation (Heng, Cao, & Lee, 2004). TGFβ proteins stimulate the synthesis of collagens and fibronectin (Ignotz, Endo, & Massague, 1987). However, $TGF\beta_1$ and $TGF\beta_2$ are known to cause ECM deposition associated with scarring, possibly due to conversion of keratocytes into the myofibroblast phenotype (Funderburgh, Mann,

Funderburgh, Corpuz, & Roth, 2001). For this study, TGFβ_3 was chosen as previous studies have shown that ECM deposition elicited by it does not cause fibrosis or deposition of scar tissue (Karamichos, Hutcheon, & Zieske, 2011). A combination of TGFβ and dexamethasone has been routinely used to induce progenitor cells to differentiate into chondrocytes in vitro (Diekman, Rowland, Lennon, Caplan, & Guilak, 2009; Johnstone, Hering, Caplan, Goldberg, & Yoo, 1998; Winter et al., 2003). Therefore a combination of TGFβ_3 and dexamethasone was used to drive the differentiation of keratocytes towards a chondrocyte phenotype.

The induction of collagen type II could potentially be used to strengthen a weakened keratoconic cornea and for this research to be of therapeutic value it would first needed to be tested on diseased keratoconic corneas. An important aim, therefore, was to determine whether keratocytes in keratoconic tissue were amenable to this method of cell reprogramming and subsequent production of collagen type II rich ECM. The effect of type II collagen deposition on the biomechanical properties of the *in vivo* and *ex vivo* treated corneas was evaluated by using nanoindentation testing, a bioengineering approach that enables analysis of hardness and elastic modulus. The final aim was to establish whether collagen type II deposition can be induced *in vivo* in the corneas of rats without an adverse effect on the optical properties of the corneas, and whether it also led to corneal stiffening.

2 Methods and materials

2.1 Tissue collection

Cadaveric whole human corneas and keratoconic corneas obtained at the time of transplant surgery were obtained from donors sourced through the New Zealand National Eye Bank

(Auckland, New Zealand). Prior to the use of tissue, research ethics approval and consent was obtained from the Northern X Regional Human Ethics Committee. Ethics approval for animal studies was obtained from the University of Auckland Animal Ethics Committee. Eyes from 6-8 week old adult male Wistar rats were obtained and the corneas were carefully dissected out using surgical scissors with the aid of a dissecting microscope.

2.2 Isolation and culture of corneal keratocytes and organotypic culture of intact cornea

The corneal epithelium and endothelium were gently scraped off with a keratome and discarded. The stromal tissue was digested in 0.4% type II collagenase (Sigma-Aldrich), in Hanks Balanced Salt Solution (GIBCO, Life Technologies) at 37°C with gentle mixing on an orbital shaker. A variety of digestion times were tried with 5 hours being the time required for optimal tissue digestion and cell viability. Isolated keratocytes were cultured in either 12 or 24 well cluster plates (Falcon) on glass coverslips in 2- 3 ml of cell culture media. Cells were kept in a humidified incubator at 37 °C with 5% CO_2. Culture media was changed after 24 hours and then every two days subsequently or more frequently if required. For cell pellet culture, corneal fibroblasts were pelleted by centrifuging at 300g for 7 minutes at 20 ˚C in a plastic conical tube. Culture media was added to the tubes. After 24 hours of incubation at 37ºC, the cells had contracted and formed a pellet which did not adhere to the walls of the tube. For *ex vivo* experiments, corneas were placed in an organotypic air-liquid interphase culture system. Briefly, the explants of healthy tissue were cultured on 0.4µm pore size cell culture inserts (Millicell) at the interface between culture medium and a CO_2 rich environment. The tissue was placed epithelium side up on cell culture plate inserts with 3 ml of culture medium. The culture media was changed every other day.

2.3 Chondrogenic differentiation of keratocytes

Chondrogenic differentiation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10ng/ml TGFβ_3, 10^{-7}M Dexamethasone, 1% Glutamax 1% Anti-Anti. Fibroblast induction medium consisted of DMEM supplemented with 10% foetal bovine serum (FBS), 1%Anti-anti, 1% Glutamax. In the control medium the addition of FBS was omitted. For obtaining a monolayer of cells, keratocytes were seeded on glass coverslips at a density of $15x10^4$ per cm^2. Cultures were maintained for up to 3 weeks. For *in vivo* treatment of rat corneas, eye drops were formulated using Gellan gum, a water soluble polysaccharide produced by the bacterium, Pseudomonas elodea. The use of gel base formulation allows a prolonged corneal residence time and increased ocular bioavailability of the therapeutic agent. A 0.5% solution was prepared and growth factors were added to the gel with constant stirring. A ten times higher concentration of growth factors than that used in the culture medium was used to make up for the drug lost through nasolacrimal drainage and blinking.

2.4 Immunohistochemistry and mRNA expression of collagen types I and II.

All samples were fixed in 4% paraformaldehyde. Tissue Samples were embedded in Optimal Cutting Temperature compound (Tissue-Tek) before being snap frozen in liquid nitrogen and cut into 15 μm thick sections. For immunohistochemical analysis the cells and sections were labelled with primary antibodies against collagen I (ab63080, Abcam), collagen II (MAB8887, Millipore), collagen III (2150-0081, Biogenesis), nestin (MAB5326, Millipore), keratocan (sc-66941, Santa Cruz Biotechnology), Vimentin (V6630, Sigma) and alpha smooth muscle actin (NCL-SMA, Novocastra), followed by the secondary antibodies of Alexa fluor dye conjugated goat anti-mouse and anti-rabbit IgG (Molecular probes). An Olympus FV-1000

confocal laser scanning microscope (405nm, 473nm and 559nm wavelength lasers) and Leica DMRA fluorescence microscope were used for imaging.

The mRNA extraction from samples was carried out using the PureLink RNA MicroKit (Invitrogen). 100 ng of RNA was converted to cDNA using the Superscript® Vilo cDNA Synthesis Kit (Invitrogen, Life Technologies). Custom TaqMan® Gene Expression Assays were obtained and real- time PCR was performed with the 7900HT Fast Real-Time PCR System (Life technologies). Results were analysed using RQ Manager Version 2.2 (Applied Biosystems). Taqman gene assays used: Col1a1-Rn01463848_m1, Col2a1- Rn01637087_m1.

2.5 Optical and Biomechanical testing

The Phoenix Micron IV Rodent eye Imaging System (Phoenix Research Labs) was used to examine the corneas of treated rats. The slit-lamp attachment was used to examine the layers of the cornea in detail and check corneal integrity and transparency. For biomechanical testing, nanoindentation was carried out on treated and untreated human keratoconic corneas and rat corneas. Custom-made rigs were built for testing the samples. The indent load used for the human samples was 50 μN. For the rat globes a range of loads between 3 and 5 μN were used. The indentation process was automated by the Hysitron Triboindenter. The hardness of the sample is determined by the area of residual indentation (Ar) after the tip is unloaded.

$$Hardness = \frac{Maximum\ Load\ (P)}{Area\ of\ residual\ indentation\ (Ar)}$$

Where P is the maximum indentation load and Area is the contact area of the conospherical tip with the sample. The reduced elastic modulus is a representation of the elastic modulus in both the sample and the indenter tip as shown by the following equation:

$$\left(\frac{1}{Er}\right) = \left(\frac{1-\nu i^2}{Ei}\right) + \left(\frac{1-\nu m^2}{Em}\right)$$

Where ν refers to Poisson's ration, *i* referrers to the indenter and m refers to the sample material. The reduced elastic modulus tells us how elastic a sample is. Because the same indenter tip is used for each test the reduced elastic modulus can be used to compare the elasticity in each sample being tested.Results

2.6 Corneal keratocytes are induced to secrete cartilage specific collagen type II upon treatment with chondrogenic factors

Corneal keratocytes from adult human corneas were seeded in either the chondrogenic differentiation medium or fibroblast proliferation medium. Within two to three days the keratocytes seeded in the chondrogenic differentiation media formed spherical cell aggregations approximately 50-100 μm in diameter (Figure 1A). The spheres labelled for the chondrocyte specific collagen type II in the central portion and nestin around the periphery (Figure 1B). Once the spheres were placed in the fibroblast proliferation media cells from the spheres started spreading outwards to populate the culture dish thereby forming a cell monolayer which did not label positively for collagen type II (Figure 1C-D). In contrast, keratocytes seeded in the fibroblast proliferating medium formed an even monolayer of fibroblast-like cells which did not label for either nestin or collagen type II (Figure 1E-F). Keratocytes seeded into fibroblast proliferation medium failed to form the necessary cell aggregations. In order to form fibroblast clusters, the confluent fibroblasts were dissociated from the culture dish, pelleted and grown as a pellet culture in chondrogenic differentiation medium for a further three weeks. Whilst the cell pellets labelled positive for the corneal

stroma specific ECM protein keratocan (Figure 1G), they did not label for type II collagen (Figure 1F).

2.7 Keratocytes in intact corneal tissue secrete collagen type II upon treatment with chondrogenic factors

Slices of adult human cornea that were placed in organotypic slice culture in either control medium or chondrogenic differentiation medium for one week did not result in any visible deposition of type II collagen in the stromal ECM (Figure 2B). However tissue slices incubated in chondrogenic differentiation medium for two weeks labelled for the chondrocyte specific collagen type II (Figure 2C and Figure **3**B). The newly produced type II collagen was deposited evenly throughout the ECM without forming large aggregates. The labelling was seen strongly along the pre-existing collagen framework of the corneal stroma and was distributed across the entire thickness of the stromal layer. Treatment for one week did not result in any visible deposition of type II collagen in the stromal ECM (Figure 2B). The amount and pattern of the native collagen type I appeared to be slightly altered in the treated corneas (Figure 2D-F). In general, the intensity of the labelling was similar but the distribution was more extensive and the amount of labelling was higher in the corneas placed in the control culture condition (Figure 2D) possibly due to activation of keratocytes into fibroblasts. The *in vitro* human corneal tissue experiment was then extended to an *in vivo* rodent study. After two weeks the rats were euthanized and the corneas processed for immunohistochemistry. Only the treated corneas labelled positive for collagen type II with a higher degree of deposition observed in the anterior part of the cornea (Figure **3**D).

2.8 Induction of collagen type II deposition in keratoconic corneas

In order for this method of *in vivo* reprogramming to successfully translate into a possible treatment for keratoconus it was necessary to affirm that keratocytes in keratoconic corneas are also amenable to the induction of collagen type II deposition. Keratoconic corneal buttons obtained after corneal transplant surgery were placed into organotypic tissue culture. Half of each button was put into control medium and the other half placed in chondrogenic differentiation medium and maintained for two weeks. After two weeks the tissue was processed for immunohistochemistry. The stromal ECM of the treated half of the cornea was positive for type II collagen (Figure **3**F). Although the intensity of the labelling was lower in keratoconic tissue when compared to healthy corneal tissue, the labelling pattern was similar and followed an ordered arrangement along the backbone of pre-existing collagen lamellae. Vimentin labelling revealed stark differences between keratocytes in the untreated and treated keratoconic corneas. In general the keratocyte density was lower in the untreated corneas with a scarcity of cells in the posterior part of the cornea (Figure **3**G-H). Also, the keratocytes in the chondrogenic factor treated corneas appeared healthier, had a more filamentous and complete morphology when compared to keratocytes in untreated corneas (Figure **3** I-J). Keratocytes in chondrogenic factor treated corneas were longer and had a larger number of cell processes which labelled strongly for vimentin when compared to the keratocytes in the untreated corneas.

2.9 TGFβ3 and dexamethasone treatment does not induce deposition of fibrotic proteins or cause corneal opacity

Human corneas cultured in the chondrogenic differentiation medium for up to three weeks were labelled for collagen αSMA (Figure 4A-B) and collagen type III (Figure 4C-D) which

are associated with fibrosis and scarring. There was no evidence of any fibrotic matrix deposition upon treatment with TGF beta 3 and dexamethasone. These results confirm previous findings that, unlike TGFβ_1 and TGFβ_2, TGFβ_3 does not induce the differentiation of corneal keratocytes into myofibroblasts.

Slit lamp examination was performed on the live rats throughout the study period. Upon examination, treated and untreated corneas were indistinguishable with no signs of scarring or opacity. Back of the eye imaging to reveal the blood vessels showed clear corneas which did not obstruct the passage of light and *in vivo* cross section imaging (Figure 4 E-F) of the rat revealed transparent corneas through which light easily passed. There was no sign of any corneal opacity or cloudiness which would lead to the obstruction of light passing through the cornea.

2.10 Change in mRNA expression of collagen type II and type I upon treatment in vivo

Rat corneas which were treated in vivo for one week, and three weeks followed by a non-treatment period of four weeks were subjected to quantitative gene expression analysis. The aim was to determine whether type II collagen expression decreases again and/or permanently ceases after growth factor treatment is withdrawn. The effect of the treatment on native corneal collagen type I was also investigated. The one week treated corneas expressed collagen type II and expression levels dropped considerably upon withdrawal of the treatment (Figure 5A). For type I collagen expression, the one week and seven week corneas were compared to the untreated corneas. It was found that there was an initial spike in type I collagen expression after one week treatment but by week seven type I collagen expression was significantly lower and comparable to its expression in the untreated cornea (Figure 5B).

2.11 Collagen type II deposition enhances biomechanical properties of corneas

It was hypothesised that the laying down of type II collagen would affect the stiffness and elasticity of the corneas. In order to evaluate these changes the *in vivo* rat corneas and *ex vivo* treated human corneas and their matching controls were subjected to nanoindentation testing.

A matched pair of corneas from a keratoglobus patient was cultured ex vivo in either the control medium or the chondrogenic differentiation medium for eight weeks and tested. Keratoglobus is a condition similar to keratoconus and results in an extremely thin cornea with a large globular shape when compared to a normal cornea with a gradual curve. For the purpose of this study a matched pair with this condition would provide the most reliable results as corneas from a single patient with the same condition in both eyes were being compared. Testing revealed a significant increase in hardness and elastic modulus in the treated cornea (Figure C).

For the *in vivo* treated rat corneas, corneas treated for one week did not have a significant increase in either hardness or elasticity (Figure A). This result matches our immunohistochemistry data which revealed that one week was not sufficient time for deposition of collagen II (Figure 2B). However, in the three week *in vivo* treated corneas there was a clear difference between the treated and control eye (Figure B). In the right eye exposed to the growth factor treatment both the hardness and reduced elastic modulus were markedly higher.

3 Discussion

The TGFβ family of growth factors are known to be the most potent inducers of chondrogenic differentiation (Heng et al., 2004). A combination of TGFβ family of proteins and dexamethasone has been routinely used to induce progenitor cells to differentiate into chondrocytes *in vitro* (Diekman et al., 2009; Johnstone et al., 1998; Kolambkar, Peister, Soker, Atala, & Guldberg, 2007; Winter et al., 2003). It has previously been reported that scleral cells after four weeks in a chondrogenic differentiation medium containing $TGF\beta_1$ and BMP2 expressed cartilage markers including aggrecan, and collagen type II. Furthermore, human scleral cells retained their chondrogenic potential in vivo after being transplanted into a rat cartilage defect (Seko et al., 2008).

The corneal stromal extracellular matrix is composed of tightly packed heterotypic collagen fibrils primarily made up of collagen types I and V. Similar to corneal fibrils, cartilage fibrils are heterotypic fibrils made up of types II and XI and have a uniform diameter of 25 nm making them slightly smaller than the 36nm corneal fibrils (Mendler, Eich-Bender, Vaughan, Winterhalter, & Bruckner, 1989). Collagen II is the major fibril component of cartilage and is similar to collagen I in that the molecule essentially consists of a single uninterrupted helical domain 300 nm in length. Owing to their similarities, collagens II and XI are considered to be the cartilage analogues of collagens I and V in other tissues. Furthermore, collagen type II is expressed during development of the chick corneal stroma and is only later replaced by type I in the mature chick stroma (Linsenmayer et al., 1990). We know from literature that collagen types IX and XI co-localise with type II collagen in cartilage. There is some evidence that type IX collagen expression has been shown during very early stages of primary avian cornea formation. From literature, unlike in cartilage, where collagen types II and IX expression are coupled, the expression of collagen type IX in cornea appears to be genetically uncoupled

from that of collagen type II. mRNA for collagen type IX shows a transient presence in the primary stroma, whereas the expression of collagen type II persists (Quantock & Young, 2008) . The function that collagen type XI performs in cartilage appears to be analogous to collagen type V's function in the cornea. Traditionally collagen type II is thought to be exclusively associated with cartilage; however there is evidence for the role of this collagen type that is independent of chondrogenesis and therefore the production of type II collagen by keratocytes does not necessarily associate with other cartilage matrix molecules (Kosher & Solursh, 1989). We suspect that this is the case in the cornea and future studies will aim to dissect this further. We would therefore expect type II collagen to form a fibril in combination with type V collagen however this is speculative. There is the possibility of heterotypic type I/type II fibril formation however we do not know if this is the case in the present study.

Keratocytes seeded in culture medium containing TGFβ_3 and dexamethasone and in the absence of serum spontaneously formed cell spheroids within two to three days by cell aggregation and by three weeks these cell clusters labelled positive for type II collagen. When the medium was changed to a control medium containing fetal calf serum the cell clusters dispersed into a monolayer of cells. Cells growing in the monolayer no longer expressed type II collagen. These results suggest that cell aggregation is important for collagen type II induction. Studies on chondrogenic differentiation of stem cells have also shown that three-dimensional or pellet cultures are necessary for chondrogenic differentiation (Winter et al., 2003). Keratocytes which were first proliferated as fibroblasts in serum containing medium and then grown in pellet culture with chondrogenic differentiation medium did not secrete collagen type II. This suggests that once proliferated as fibroblasts the cells lose the ability to differentiate along a chondrogenic pathway and that the quiescent

keratocyte phenotype along with cell aggregation is crucial to chondrogenic differentiation of corneal keratocytes.

Ex vivo culture of normal and keratoconic corneas in chondrogenic differentiation media resulted in uniform deposition of type II collagen along the stromal lamellae. Collagen type II labelling was not restricted to a particular location or subpopulation of cells suggesting that the reprogramming was not as a result of proliferation of a side population of progenitor cells. *In vivo* treatment of corneas in rats caused the deposition of type II collagen in a manner similar to that seen in *ex vivo* culture. However, stronger immunolabelling of type II collagen was seen in the anterior part of the cornea when treated *in vivo*, reflecting easier diffusion of growth factors into the anterior layers of the stroma from the ocular surface.

Studies looking at differences in keratocyte density in keratoconic corneas have reported an overall decrease in cell density. Our results also confirm this. However, unlike other studies which have reported a marked decrease in cell density in the anterior part of the stroma (Hollingsworth, Efron, & Tullo, 2005; Ku, Niederer, Patel, Sherwin, & McGhee, 2008; Mencucci et al., 2010; Niederer, Perumal, Sherwin, & McGhee, 2008), we saw a marked decrease in keratocyte density in the posterior part of the stroma of the untreated keratoconic cornea. In this study, the treated half of the keratoconic cornea which was cultured in the chondrogenic medium had an increased keratocyte density when compared to the control tissue. Furthermore, the posterior region of the stroma appeared to be repopulated by keratocytes. The keratocytes in the treated half also had a better morphology with large prominent nuclei and several cell processes. This indicates that the treatment with the two factors have possibly caused keratocytes to proliferate and repopulate the stroma, in particular the posterior part which was devoid of keratocytes. In this study Collagen type II was deposited in the keratoconic tissue in vitro. The cell cultures experiments indicate that

corneal keratocytes expanded as fibroblasts in vitro in the presence of serum are not amenable to collagen type II induction. However, the activated keratocytes found in vivo are wound fibroblasts, a cell type that might be different from in vitro serum-induced fibroblasts. We know, from literature that wound fibroblasts are not terminally differentiated and can return to a keratocyte-like phenotype. This, therefore, might make wound fibroblasts in vivo amenable to collagen type II induction.

In this study, even upon long term *in vitro* and *in vivo* treatment there was no evidence of corneal opacity. This is probably due to the deposition of the collagen II in uniform layers along the pre-existing collagen lamellae. Deposition of collagen type III which is associated with fibrosis and alpha-smooth muscle actin which produced during myofibroblast formation leads to opacity and scarring. Expression of these are seen during corneal wounding, but neither of these proteins was expressed in the treated corneas suggesting that wound healing cascades which bring about scarring were not triggered.

Quantitative measurement of collagen type II mRNA expression showed that its expression was lowered upon withdrawal of $TGF\beta_3$ and dexamethasone. This suggests that the deposition of type II collagen in the ECM can be controlled. This is important if a treatment based on this approach is to be developed; it would not be desirable to induce irrepressible extracellular matrix deposition. Initially, upon treatment, type I collagen RNA levels were also increased. The initial up regulation of the native type I collagen could be due to the activation of the relatively quiescent keratocytes into an activated phenotype. However, Immunohistochemical labelling of type I collagen did not reveal any significant increase in its deposition at the protein level upon treatment with the two factors.

The "fibrillar" types of collagen such as types I and II self-assemble and crosslink to form highly crystalline fibres that have a high stiffness, low extensibility and a remarkable elastic

energy storage capacity (Wells, 2003). It is the crosslinking which contributes towards the stiffness and tensile strength of the fibres. Nanoindentation has been employed in the assessment of postoperative therapeutic methods such as crosslinking for keratoconus (a corneal dystrophy) and post-LASIK ectasia in the eye. In one study done on human cadaver corneas it was found that collagen crosslinking caused a two-fold increase in the elastic modulus in the anterior corneal stroma while the posterior stroma was unaffected by the treatment (Dias, Diakonis, Kankariya, Yoo, & Ziebarth, 2013). In our study only anterior corneal elasticity was measured. It would be interesting to evaluate posterior stromal elasticity in the future. The failure of sufficient growth factor to reach the posterior part of the stroma might be a limiting factor. However, histological data from this study does indicate that posterior stroma keratocyte density is altered upon TGFβ_3 and dexamethasone treatment.

While nanoindentation does not measure the properties of the individual collagen fibrils it can measure the changes in the inherent elastic property of the cornea which will be altered upon collagen II deposition with a subsequent increase in collagen crosslinking. Structural differences within the stroma will be reflected in the corresponding differences in biomechanical properties. The results here show that there was a three-fold increase in elastic modulus and hardness in the reprogramming factor treated rat corneas. The elastic modulus is a measure of a substance's resistance to being deformed elastically. A higher elastic modulus indicates that a material is more difficult to deform. In this study an increase in hardness and elastic modulus in three week treated corneas when compared to one week treated corneas is consistent with the immunohistochemical labelling results that show at least two to three weeks of treatment is required for the laying down of a sufficient density of type II collagen.

The replacement of damaged tissue with engineered tissue is a feasible method for the thinner epithelial and endothelial layers of the cornea (Ide et al., 2006; Pellegrini et al., 1997). The corneal stroma presents a challenge due to the fact that it is a much thicker tissue layer and

has a highly organised microstructure. Traditional tissue engineering methods encompass *in vitro* techniques that use cells and extracellular matrix scaffolds to produce substitutes that can replace wounded or diseased tissues. Engineering clinically viable corneas has not been possible mainly because classical tissue engineering methods are based on a scar model. Such an approach does not support the assembly of a highly organised tissue such as that produced during normal stromal development. Efforts focussed at defining the physiologically relevant *in vitro* microenvironments and using a developmental approach for building stromal tissue will no doubt yield better results. Apart from the traditional tissue engineering approaches there has also been some progress made towards recognising the effect of microenvironment conditions on cells and their capacity to self-organise and produce useful proteins in these altered conditions. *In situ* induction of cells can be employed to drive tissue repair; however, such a strategy when used for corneal regeneration would lead to the activation of corneal keratocytes into the undesirable wound healing fibroblast and myofibroblast phenotypes. This would then lead to the production of a fibrotic scar rather than organised tissue. Therefore, for such an approach to be viable, it would be necessary to bypasses the wound healing pathway. We propose an *in vivo* approach to corneal regeneration thereby avoiding problems arising from in vitro manipulation of corneal keratocytes. With regard to the treatment time period, we know that collagen type II is deposited in 3 weeks and we would expect it to remain in the matrix unless it is degraded. Therefore an initial 3 week treatment with TGFbeta3 and dexamethasone would be sufficient to stabilise a weak cornea. However we do not know for how long this initial collagen II deposition will be sufficient. This requires long term in vivo studies. We envisage a treatment period of 3 weeks initially and based on more long term in vivo data similar follow up treatment periods annually or biannually might be sufficient. However at this stage we cannot be sure.

Modulating the cells using in situ phenotype reprogramming to bring about extracellular matrix deposition in a controllable manner offers a new approach for the regeneration of the corneal stroma. This could be a viable regenerative therapeutic for stopping the progression of corneal thinning and might provide a long term solution when used in combination with physical methods for correcting corneal ectasia.

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Figure 1: Keratocytes cultured for 3 weeks in chondrogenic differentiation medium containing TGFβ_3 and dexamethasone formed spheres (A) which labelled for nestin (red) around the periphery of the spheres and collagen type II (green) within the core (B). The culture medium was then switched to serum containing fibroblast proliferation medium for 1 week (C). This change in culture conditions caused cells from the spheres to spread out and populate the dish. Cells in monolayer were negative for type II collagen (D).
Keratocytes cultured in control fibroblast proliferation medium for 3 weeks (E) were negative for nestin and collagen Type II (F). After 3 weeks in pellet culture the cell pellet was sectioned and labelled negative for the chondrocyte specific type II collagen (G) and positive for the keratocyte marker Keratocan (H).

Figure 2: Human corneal slices cultured for 2 weeks in control medium (A and D) were negative for type II collagen and positive for type I collagen respectively. Human corneal slices cultured for 1 week (B and E) and 2 weeks (C and F) in chondrogenic differentiation medium and labelled for collagen type II (B and C) and type I (E and F). Strong labelling for type II collagen was seen in corneal slices treated for 2 weeks (C) whereas slices treated for only 1 week were negative for type II collagen (B). Slices cultured in chondrogenic differentiation medium for both the time periods, although less strongly labelled when compared to the control treated slices, were positive for the native corneal collagen type I (E and F).

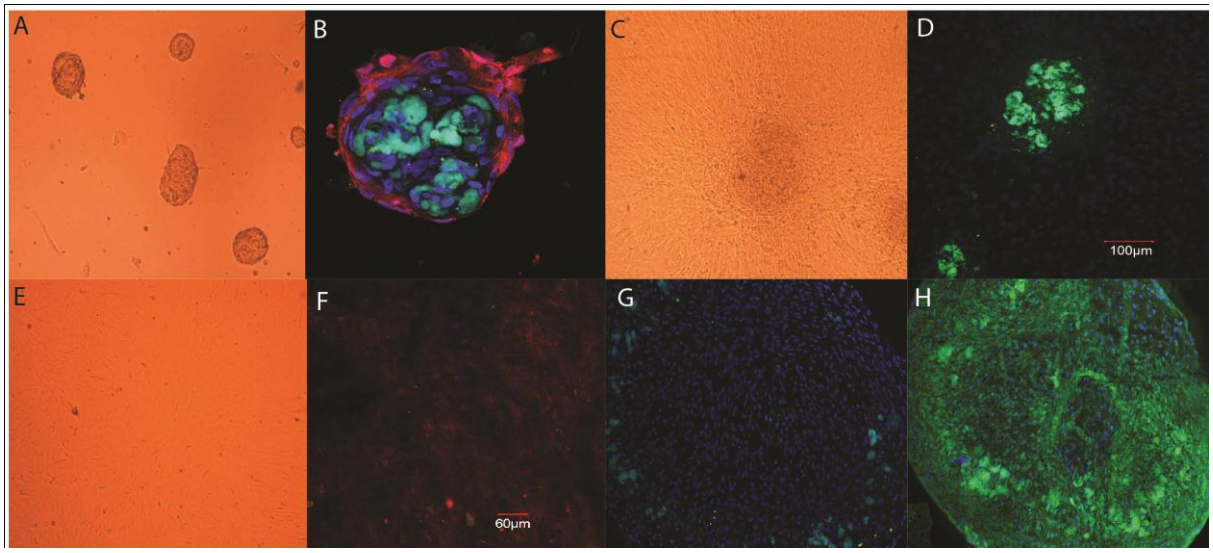
Figure 3: Human corneal slices cultured for 2 weeks in control medium (A) and chondrogenic differentiation medium (B) and labelled for collagen Type II. In vivo untreated (C) and treated (D) corneas with a widespread labelling of type II collagen in the TGFβ3 and dexamethasone treated corneas of rats treated for 3 weeks.

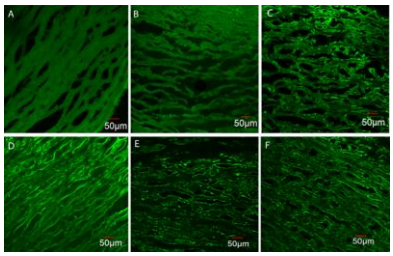
Keratoconic corneal button cultured *in vitro* in control medium (E, G and I) and chondrogenic differentiation medium (F, H and J) for 2 weeks and labelled for collagen Type II (E and F) and Vimentin (G-J) respectively. The fibroblast population in the treated half of the keratoconic button (H and J) is increased in number and the keratocytes appear to look healthier and intact with multiple, long cell processes (J) when compared to the untreated half of the keratoconic cornea (G and I).

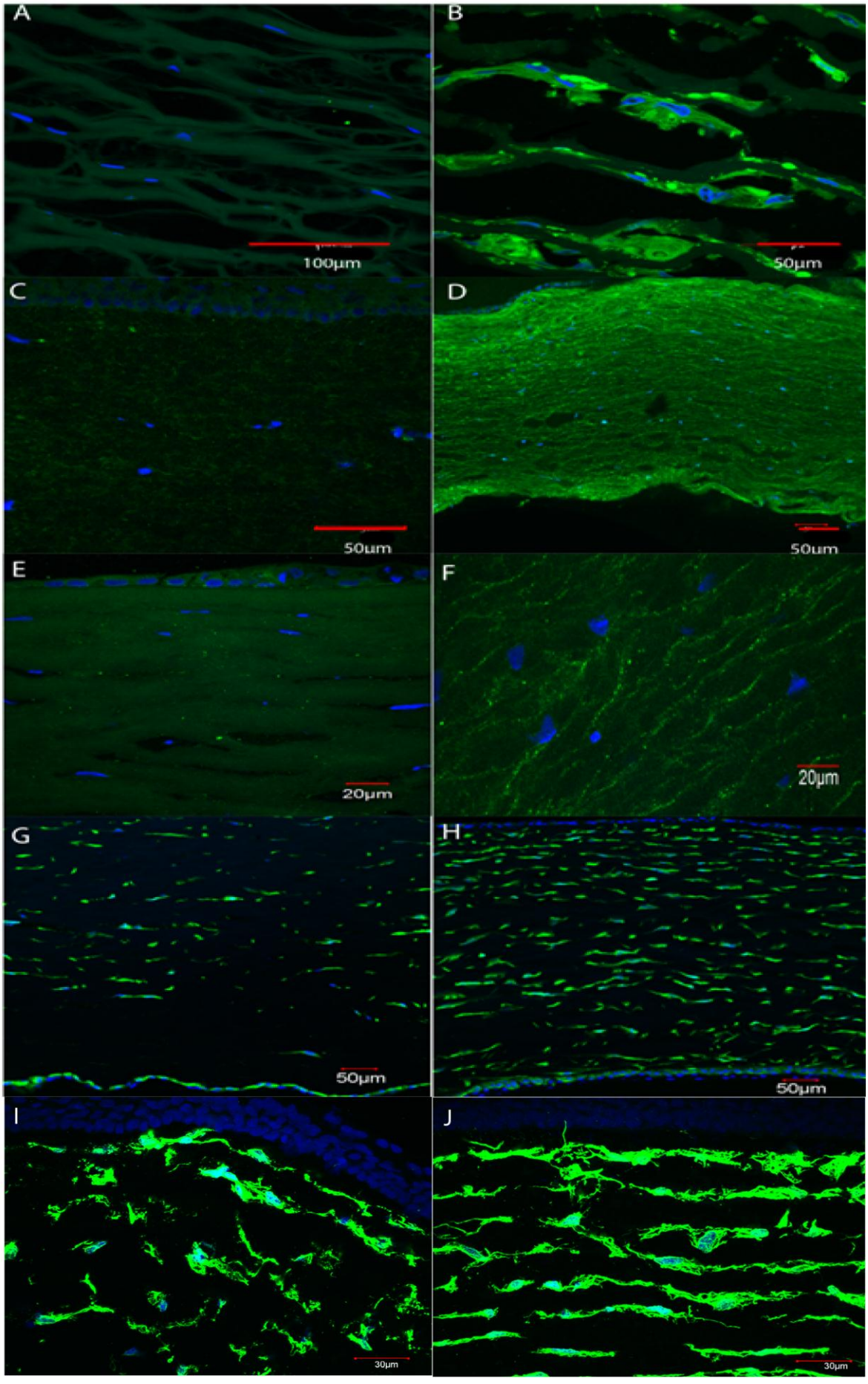
Figure 4: Ex vivo cultured human keratoconic cornea cultured for 3 weeks in control medium (A and C) and chondrogenic differentiation media (B and D) and labelled for Alpha smooth muscle actin (αSMA)(A and B) and type III collagen (C and D). There was stronger labelling for αSMA in stromal layer of corneas cultured in control medium (A) when compared to corneas cultured in chondrogenic differentiation medium. There was no apparent difference in collagen type III labelling. The in vivo imaging of the cross section of the cornea treated for 3 weeks (E) and untreated (F) reveals a clear, transparent cornea through which light easily passes. There was no sign of corneal opacity or scarring.

Figure 5: Quantitative gene expression of A: collagen type II and B: collagen type I in in vivo treated corneas. Upon withdrawal of the treatment there is a marked decrease in type II collagen expression (A). Native corneal collagen type I expression was also initially upregulated, however upon long term treatment (up to 7 weeks) its expression was comparable to the control untreated cornea (B).

Figure 6: Reduced elastic modulus (Er) and hardness (H) of A: 1 week treated and control rat corneas and B: 3 week treated and control rat corneas reveals a marked increase in both parameters in the 3 week treated corneas. C: Comparison for 8 week ex vivo treated and untreated corneas clearly shows an increase in elastic modulus (Er) and hardness (H) in the treated cornea.



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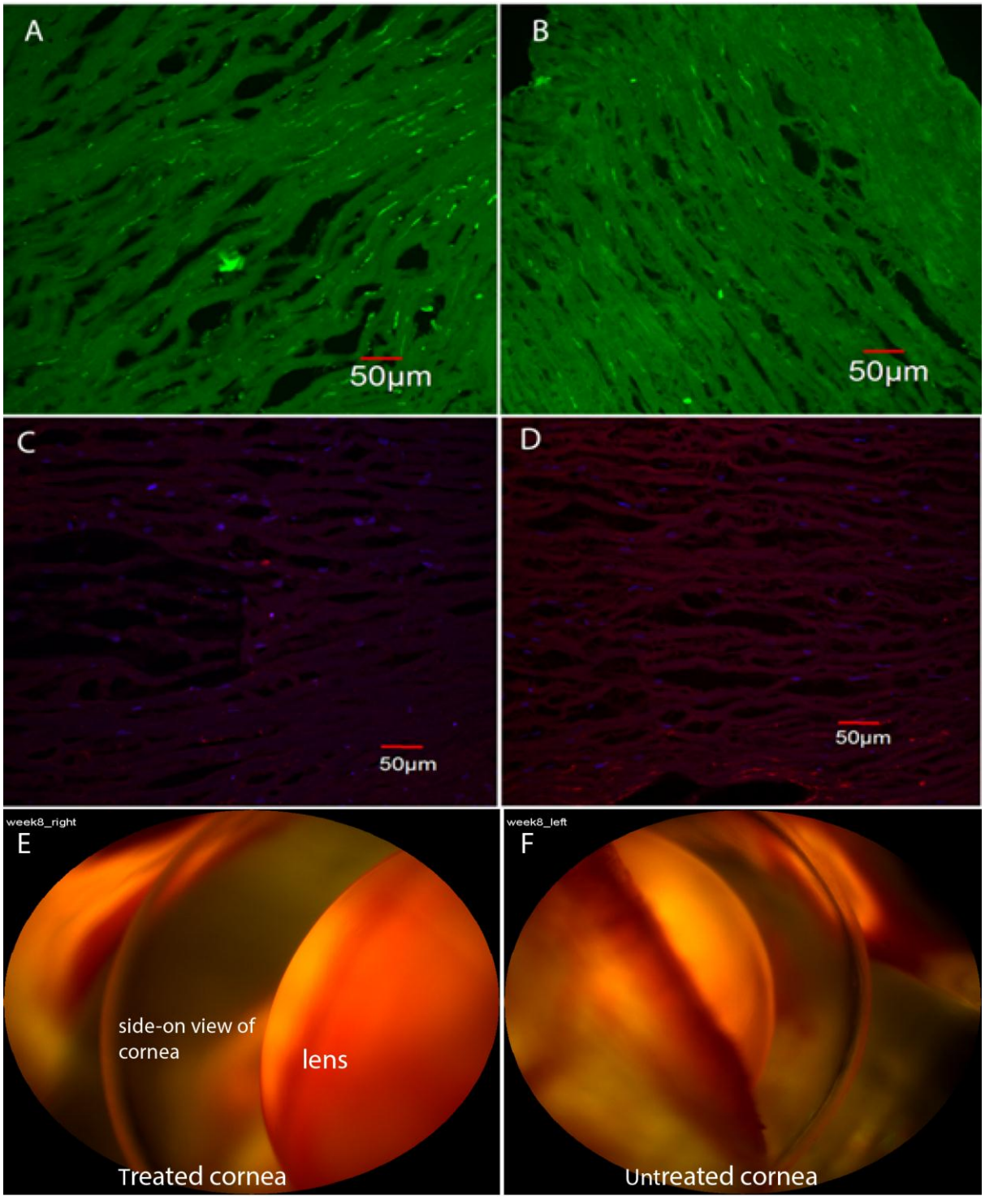
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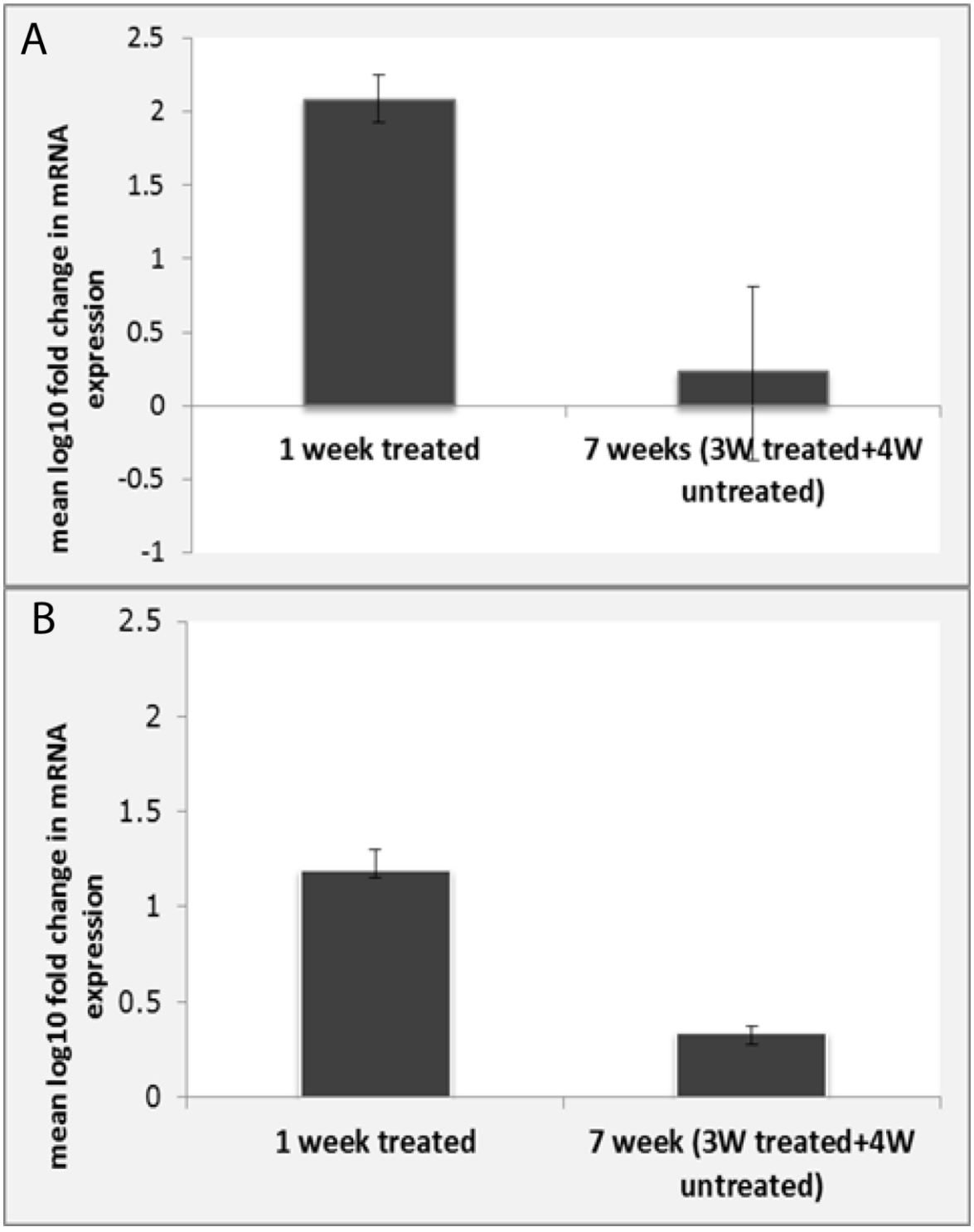
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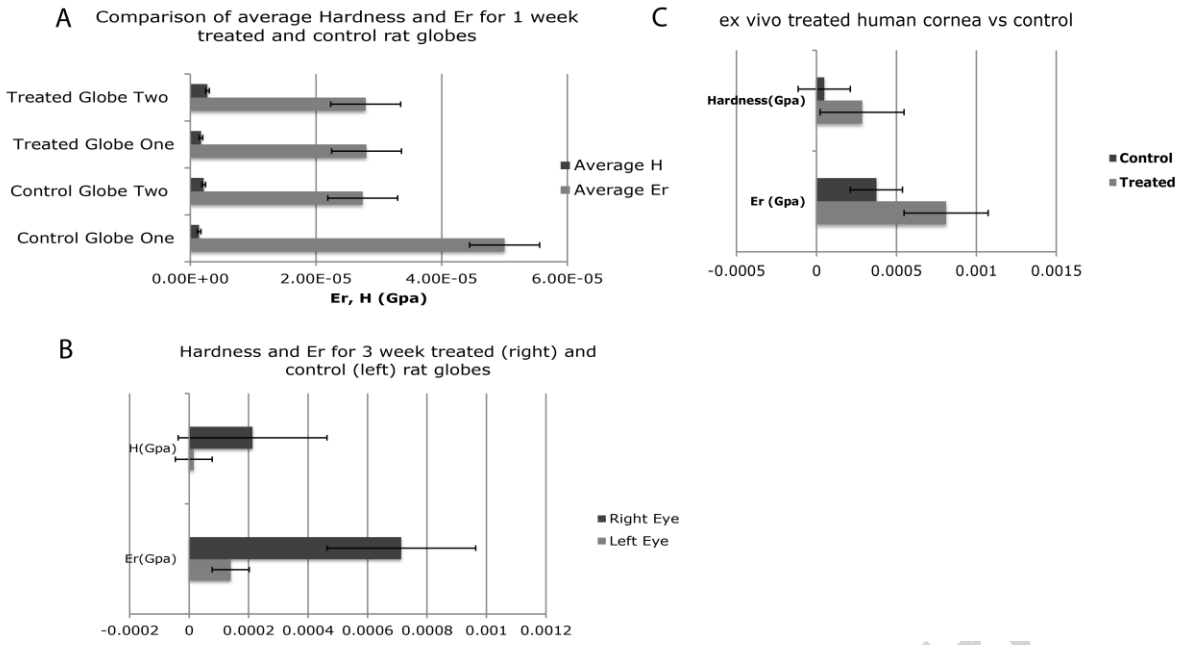

Highlights

- Keratocytes from the adult cornea are induced to produce a non-native collagen, collagen type II
- Collagen type II is induced by exogenous application of Transforming growth factor beta 3 and dexamethasone
- Collagen type II deposition in vitro and in vivo did not cause corneal opacity and resulted in increased corneal hardness and elasticity
- This method could be used to strengthen a weakened ectatic cornea