Cellular biomechanics of the sclera: Role of strain on cell-mediated extracellular matrix contraction and cellular differentiation

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Abstract

Myopia is a refractive error characterised by an abnormal increase in eye size. The sclera, a structure that maintains ocular integrity, is responsible for controlling eye size and hence is a potential target for myopia control. Active remodelling of the scleral extracellular matrix during myopia has been believed to alter the tissue’s biomechanical properties and result in ocular elongation. However, studies carried out in other connective tissues have demonstrated a possible role for the α-smooth muscle actin (α-SMA) expressing myofibroblasts in contributing to the tissue biomechanics. The effect of myofibroblasts at contributing to tissue biomechanics is further mediated by the stresses affecting the surrounding matrix. As the sclera contains a resident myofibroblast population and is under constant yet varying strain during eye growth and myopia, this study aimed to identify the effect of matrix strain in mediating matrix contraction and scleral myofibroblast differentiation.

The first experiment demonstrated the role of cellular strain (intrinsic and applied) in mediating contraction of the in vitro scleral cell-populated matrix. Increasing densities of scleral fibroblasts (2.5 x 10^2 – 3.75 x 10^3/mm^3) were seeded into three-dimensional collagen matrices, which were formulated to approximate the in vivo scleral matrix, demonstrated a cell density-dependent increase in matrix contraction when no external strain was applied. When these matrices were exposed to an external strain of 8.5% and 11.6%, which was calculated to reflect the in vivo strain under normal and myopic conditions, a rapid increase in contraction was observed within 6 hours, while contraction plateaued after 24 hours. The matrix contraction achieved at 24 hours after application of external strain was significantly higher than that achieved by the matrices maintained under intrinsic strain at that time point.
The role of myofibroblasts in mediating the contraction of the cell-populated matrices observed in experiment one was investigated in the second experiment using immunocytochemical studies. The results of the second experiment demonstrated a cell density-dependent increase in the population of myofibroblasts in matrices maintained under intrinsic strain for 5 days. However, the α-SMA stained cells in matrices exposed to external strains appeared pyknotic at the end of 5 days. Specifically, these cells were smaller in volume and circular with even smaller nuclei. In addition, these cells lacked α-SMA containing stress fibres apparent in the matrices maintained under intrinsic strain. Since a rapid increase in matrix contraction was observed within 6 to 24 hours after the application of strain, the early expression of myofibroblasts (until 24 hours) was investigated. In these matrices there was an increase in α-SMA staining at various early time points (≤ 6 hours), while at 24 hours these cells became pyknotic and lacked stress fibres. These changes are consistent with the plateauing of matrix contraction from 24 hours to 120 hours after the application of strain. Such a finding suggests that rapid initial increase matrix contraction when exposed to 8.5% strain is mediated by the rapid myofibroblast differentiation, while possible apoptosis underpins the lack of significant contraction after 24 hours. This apoptosis is likely to result from removal of strain on scleral cells due to remodelling of the surrounding extracellular matrix.

The sclera is under constant yet varying strain during eye growth due to fluctuations in IOP from events such as rapid eye movements. Thus, the sclera must alter its contractile properties within few seconds to maintain ocular integrity. This study demonstrated that scleral cells are capable of rapid (<1hr) response to imposed strain and undergo a change in phenotype producing highly contractile myofibroblasts in an in vitro environment. Such a finding suggests that in addition to changes in the matrix components, scleral
cells may also play an important role in controlling the scleral biomechanics and thus maintaining ocular integrity during eye growth and myopia development. Furthermore, the transient nature of these myofibroblasts in this study may have implications for myopia treatments that involve strengthening the sclera.
Declaration

The experiments in this thesis constitute work carried out by me unless otherwise stated. Due acknowledgement has been made in the text to all other material used. The thesis is less than 30,000 words in length, exclusive of tables, figures, bibliography and appendices, and complies with the stipulations set out for the degree of Master of Philosophy by the University of Melbourne.

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Preface

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ii) the in vivo and in vitro scleral cell density calculations were performed by Dr. Andrew Jobling, Dr. Alex Gentle and Luke Xiang-Yu Chong.

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Chapter 1: Literature Review

The human eye helps us perceive objects clearly through its refractive and neuro-sensory properties. It consists of complex structures such as the light sensory retina, containing numerous signalling pathways and cells and refractive components such as the cornea and lens. These ocular components and signalling pathways are instrumental in achieving a sharp focus of images on the retina. One condition wherein the image is not focussed on the retina is called myopia, which is characterised by an increase in axial length of the eyeball. A great deal of research pertaining to myopia tells us that changes in the sclera, the outer coat of the eye, are instrumental in determining the eye size during myopia development. This thesis will investigate the importance of mechanical strain in modulating the cellular properties of the sclera. Specifically, the effect of imposed strain on cell-mediated matrix contraction, differentiation of scleral myofibroblasts and the time course changes in their differentiation will be investigated.

1.1 Coordinated eye growth - Emmetropization

Parallel rays of light entering the eye must be focussed on the retina by its refractive components, the cornea and lens, in order to perceive a clear image. Hence, for the human eye to see images clearly, proper coordination between these refractive components and axial length of the eye is necessary. During normal ocular development, there is a change in axial length, which is compensated by a proportionate change in refractive power of the cornea and lens to render clear images on the retina; this coordinated process is called emmetropization (Sorsby, et al., 1961, Yackle &
FitzGerald, 1999). Failure to attain emmetropization results in refractive errors, such as myopia (short-sightedness) and hyperopia (long-sightedness). Myopia is a condition wherein the parallel rays of light entering the eye are focussed in front of the retina, whereas in hyperopia these parallel rays of light are focussed behind the retina (Curtin, 1985). Figure 1.1 shows the diagrammatic representation of myopia induced by an increase in axial length of the eye.

**Figure 1-1: Refractive states of the eye.** *An emmetropic eye is one wherein the image is focussed on the retina due to proper coordination between the ocular refractive components and the axial dimension of the eye. However, a change in the axial length of the eye can result in the image being focussed in front of (myopia or near-sightedness) or behind (hyperopia or long-sightedness) the retina. Alterations in the ocular refractive components can also induce myopia, however increase in axial length is the most common cause of myopia and hence depicted in the above figure.*
It has been estimated that refractive error is the major cause of visual impairment in the world, affecting close to 80% of people aged 12 and above in the US alone (Vitale, et al., 2006). While the optical effects of these refractive errors can be corrected with spectacles, contact lenses and surgeries, refractive errors can also pose a greater risk of blindness among certain Asian countries. The World Health Organisation (WHO) estimated that about 45 million Southeast Asians are visually impaired, due to refractive errors, with about 26% of them blind (Resnikoff, et al., 2004). In particular, WHO classified myopia as one of the leading causes of visual impairment in the world (Pararajasegaram, 1999) and studies have shown also that it is the fourth and fifth leading cause of blindness in Japan and Hong Kong, respectively (Lim & Jones, 1982). As far as socioeconomic factors are concerned, refractive errors pose a direct economic burden, with 90 million US dollars spent exclusively on spectacle corrections in Singapore (Seet, et al., 2001) and at least 3.8 billion dollars are spent on optical corrections for refractive error in US per year (Vitale, et al., 2006a). Australia is no exception, spending hundreds of millions of dollars exclusively for spectacles, contact lenses and refractive surgeries (Rose, et al., 2001).

### 1.2 Classification of Myopia

Myopia can be classified based on two main factors, age of onset and its severity. Juvenile-onset myopia progresses rapidly during childhood and stabilises on reaching adolescence (Goss & Cox, 1985, Morgan & Rose, 2005), whereas the adult onset myopia starts after adolescence with lesser progression than juvenile-onset myopia (Jiang & Woessner, 1996). Curtin further classified myopia, into physiologic or simple myopia (less than -3.00 diopters (D)), intermediate myopia (-3.00D to -5.00D) and
pathologic or high myopia (more than -6.00D), based on its degree and aetiology (Curtin, 1985).

Physiologic or simple myopia is a condition wherein the postnatal ocular development is normal but there is a mismatch between the refractive components, of the eye, and its axial length. Intermediate myopia is characterised by an increase in posterior segment length, which results from abnormal postnatal ocular growth. Clinically, fundus changes such as crescent formation and lattice degenerations are noted. Finally, pathologic myopia or high myopia is characterised by excessive ocular enlargement associated with degenerative changes such as formation of posterior staphyloma (bulging of sclera posteriorly), myopic crescent (Figure 1-2) and ultimately vision loss (Curtin, 1985, Tong, et al., 2002).

Figure 1-2: Clinical retinal features of pathologic myopia. A: Fundus photograph of a patient’s right eye with posterior staphyloma (marked by an arrow), whose visual acuity is 20/200. B: This is a fundus photograph of a patient’s right eye showing myopic retinal detachment (visual acuity of 16/200).
The distribution of myopia is variable worldwide, with levels of 70% to 80% in certain Asian populations (Saw, et al., 1996) while agricultural countries such as Vanuatu and Solomon islands exhibit low prevalences of 2.9% and 0.8%, respectively (Garner, et al., 1988, Verlee, 1968). In addition to geographic variations, various studies have shown myopia to vary with age, sex, ethnicity, education and lifestyle. Ethnic variances in prevalence of myopia were observed among school children in Sydney, with lower prevalence among European Caucasian (4.6%, 95% CI 3.1-6.1) and Middle Eastern children (6.1%, 95% CI 1.3-11.0) as compared to East Asian children (39.5%, 95% CI 25.6-53.5) (Ip, et al., 2008a). Another study demonstrated the racial differences in prevalence of myopia with higher prevalence amongst non-Hispanic whites (35.2%) than in non-Hispanic blacks (25.1%) in the US (Vitale, et al., 2008). In addition, Lin et al demonstrated an age-dependency, particularly in early childhood, with 20% of the 7-year-old Taiwanese children being myopic and this figure increased to 61% and 80% at the age of 12 and 15 years. This study also demonstrated that the overall prevalence rates were higher in girls as compared to boys (Lin, et al., 2001). Many such studies that looked into the relationship between age and myopia prevalence showed that myopia progresses rapidly in school children and stabilises by the age of early twenties (Fan, et al., 2004, Lin, et al., 2004). In fact, a decrease in prevalence of myopia was noted among older individuals (above 40yrs) as compared to schoolchildren (Attebo, et al., 1999, Katz, et al., 1997).

A study by Kempen et al demonstrated that 25.4% and 16.4% of individuals above 40 years of age were myopic (>1.00D) in the United States and Australia respectively. Within this group, 4.5% and 2.8% have myopia of over -5.00D respectively (Kempen, et
al., 2004). In India, 34.6% had myopic correction of over -0.50D as opposed to 4.5% who had high myopia (> -5.00D) (Krishnaiah, et al., 2009). In other Asian countries such as Singapore 73.9% were myopic (> -0.50) as opposed to 41.8% in Japan, however, the prevalence of high myopia (> -5.00D) was higher in Japan (8.2%) as opposed to Singapore (5.7%) (Quek, et al., 2004, Sawada, et al., 2008)

In addition to the high prevalence of high myopia in Asian countries, Lin et al demonstrated that the rate of myopia among schoolchildren aged 12 and 15 years increased by 24.3% and 16.8%, respectively, over a 17-year period. Furthermore, the rate of high myopia (over -6.00 D) increased by 10.1% among 18 year olds, during the same period (Lin, et al., 2004). These increasing trends in the prevalence of myopia suggest that the cost of myopia treatment and the pathological complications will increase in the near future and hence impose bigger financial and health burdens. From the above-mentioned studies, it can be clearly understood that myopia is a global health issue. Despite prevalence, classification and clinical characteristics of myopia being well documented, the aetiology of myopia is still debatable.

1.4 Aetiology of myopia

Even though the aetiology of myopia is not clearly understood, various studies have outlined two important factors that contribute to the development of myopia. Firstly, environmental factors encompassing the occupation and/or daily activities of an individual and secondly, the genetic factors.
1.4.1 Environmental influences

The development of myopia can be influenced by various environmental factors such as level of education, occupation, amount of near work and outdoor activity and lifestyle. A study by Wong et al demonstrated that Chinese adults with tertiary level of education (more than 11 years of education) had increased levels of myopia as compared to those with no education (-2.35 +/- 3.30D vs. 0.38 +/- 2.00D, p<0.001) (Wong, et al., 2000). In addition to the Asian population, a similar increase in myopia, with an increase in the level of education, was demonstrated in various countries such as the US (Katz, et al., 1997) and Australia (Attebo, et al., 1999). Ting et al demonstrated the occupational variances in the prevalence of myopia, with 87% myopes among microscopists (Ting, et al., 2004), who demand a considerable amount of near work, as compared to a study by Wong et al that reported a prevalence of 52.4% and 25.9% among professional staff and labourers respectively (Wong et al., 2000). On the other hand, various studies have shown that an increase in outdoor activity decreases the prevalence of myopia (Dirani, et al., 2009, Jones, et al., 2007, Rose, et al., 2008). In support of this, children living in the inner city of Sydney recorded a 17.8% prevalence of myopia as compared to 6.9% in outer suburbs (Ip, et al., 2008b). Consistent with the above study, Saw et al, observed a similar trend in distribution of myopia among Chinese schoolchildren (19.3%, in the city vs. 6.6%, in rural areas) (Saw, et al., 2001).

1.4.2 Genetic influences

While varying prevalence and progression of myopia across geographic locations, age and occupation may be attributed to environmental influences, the presence of familial links in myopia suggests a role for genetic aspects in myopia development. In
1966, Sorsby et al, demonstrated a strong correlation between the development of myopia and ocular components, among monozygotic (MZ) and dizygotic (DZ) twins (Sorsby, et al., 1966). However recently, a study by Dirani et al, among Caucasian population, demonstrated a high intra-pair correlation of adult-onset myopia among MZ twins ($r=0.61$) compared to their DZ counterparts ($r=0.16$) (Dirani, et al., 2008). This finding was consistent with previous heritability studies on twins by various researchers (Hammond, et al., 2001, Hu, 1981, Lin & Chen, 1987, Sorsby & Fraser, 1964, Teikari, et al., 1991). Further evidence in support of genetic influences in myopia development comes from studies that focussed on the type of inheritance in high myopia. While earlier, studies proposed an autosomal recessive (AR) pattern of inheritance in high myopia (Edwards & Lewis, 1991, Macklin, 1927), recent work has suggested that high myopia follows an autosomal dominant (AD) inheritance pattern. Such recent studies further identified specific loci for AD high myopia as 12q (Young, et al., 1998a), 18p (Young, et al., 1998), 17q (Paluru, et al., 2003) and 2q (Paluru, et al., 2005). Such a range of genetic loci may be reflective of the multifarious nature of high myopia. Further highlighting its genetic component, high myopia is associated with other ocular abnormalities like Retinitis Pigmentosa and systemic syndromes such as Marfan’s syndrome and Stickler’s syndrome (Jacobi, et al., 2005), which arise due to specific genetic abnormalities.

In addition to twin studies and inheritance studies, work has shown that children with myopic parents are more likely to develop myopia (Hui, et al., 1995, Krause, et al., 1993). This was consistent with the finding that the rate of axial length elongation, over a year, was higher among children with both myopic parents (0.37mm) as compared those with one (0.26mm) and no myopic parent (0.20mm) (Lam, et al., 2008). Another study by Hirsch et al showed that 55% of high myopes (more than -7.00D) have myopic
parents, this number dropped down to 15% for individuals, with less than -1.00D myopia (Hirsch & Ditmars, 1969), suggesting that parental myopia could be an important predictor for the development of high myopia. The many studies (Edwards, 1998, Jones et al., 2007, Mutti, et al., 2002, Zadnik, et al., 1994) highlighting a strong correlation between parental myopia and childhood myopia suggest that genetic factors play an important role in development and progression of high myopia.

The varying prevalence of myopia across different ethnicities, geographic locations and age groups raised many questions as to whether the aetiology of myopia is genetic or influenced by environmental factors. Even though studies suggest a familial link in myopia, it must also be understood that families share the same environment and hence, such high correlations cannot be purely attributed to genetic factors. Studies carried out on myopia development among twins and studies pertaining to distribution of myopia among different work groups tend to suggest that myopia has a multifactorial aetiology not simply from purely genetic or environmental factors. However, changes in the ocular structure during myopia development are consistent, irrespective of the aetiology being genetic or environmental. Specifically, the structure and integrity of sclera, a structure that maintains the eye size, is altered during myopia development. Further work in human myopes and animal models of myopia suggest that sclera plays a major role in ocular remodelling during myopia. These studies demonstrated that changes in scleral biochemical and biomechanical properties are instrumental in controlling the eye size during myopia development, thus indicating that sclera as a potential target for myopia control.
1.5 Sclera and its composition

The outer coat of the eye, the sclera, is a rigid structure that provides mechanical strength to the eye. It withstands ocular expansion in response to changes in intra-ocular pressure and maintains ocular integrity from external injury. The sclera is essentially a fibrous connective tissue, rich in extracellular matrix (McBrien & Gentle, 2003, Rada, et al., 2006). As like other connective tissues, the scleral extracellular matrix is composed of collagens, proteoglycans, protease enzymes and is maintained by a resident population of scleral cells. The turnover of these matrix components and activity of scleral cells maintain the scleral biochemical and biomechanical properties, thus regulating eye size.

Collagens

Collagens are proteins that are found in various connective tissues such as cartilage and skin, with a half-life of nearly 15 years in the skin (Verzijl, et al., 2000). So far, 28 different types of collagen have been identified throughout the human body, which perform a variety of functions (Veit, et al., 2006). The major protein content of the scleral extracellular matrix is collagen, which accounts for 90% of the scleral dry weight in mammals. During foetal development, the distribution of scleral collagen shows an anteroposterior trend, with more immature fibrils deposited at the posterior sclera (Foster & Sainz de la Maza, 1994). Scleral collagen fibrils exhibit a gradient in diameters ranging from 96, 148 and 161 nm in the inner, middle and outer layers of sclera (Curtin, et al., 1979). The larger diameter fibrils provide strength in a given tissue (Kadler, et al., 1996), whereas smaller diameter fibrils are responsible for tensile properties (Kadler, et al., 2007). Different types of collagen have been identified in the
sclera, with type I constituting the majority of scleral collagen, 99% (Zorn, et al., 1992). Other collagen subtypes reported in the sclera include types III and V (smaller collagen fibrils) (Norton & Miller, 1995), where type V is instrumental in determining the fibril diameter during fibrillogenesis (Birk, 2001). In addition, fibril associated collagen such as type VI (Marshall, et al., 1993), attach to the collagen fibrils and are believed to mediate inter-fibrillar interactions in the scleral matrix (McBrien & Gentle, 2003). Collagen types VIII (Sawada, et al., 1990) and XIII (Sandberg-Lall, et al., 2000) are also reported in the sclera but their function is not clearly understood. The concentration of these scleral collagens vary with age, with collagen types I and VI increasing, whereas types IV, V and VIII decrease with age (Fullwood, et al., 1995).

**Proteoglycans**

Proteoglycan is a scleral component that contributes 0.7-0.9% of the scleral dry weight, despite their relatively low quantity; proteoglycans perform an important role in maintaining scleral function (Di Girolamo, et al., 1997). Proteoglycans are distributed throughout the extracellular matrix and are believed to play a major role in collagen fibril assembly and tissue hydration (Rada et al., 2006). Proteoglycans exist as one or more core proteins attached to glycosaminoglycan (GAG) side chains, which are sulphated (Rada, et al., 1997). Subsequently, these GAGs are negatively charged and play a major role in biomechanical properties of a tissue (Buschmann & Grodzinsky, 1995). (Rada et al., 1997). Dermatan and chondroitin sulphate based proteoglycans, such as decorin and biglycan, are smaller proteoglycans (Rada, et al., 2000) that are thought to regulate collagen fibril assembly within the sclera. Decorin is an abundant proteoglycan in the human sclera (Rada et al., 1997), but its role is yet to be clearly defined. However, in other connective tissues, decorin has been suggested to maintain
the biomechanical properties, during stress, by attaching itself to collagen fibrils (Cribb & Scott, 1995). Aggrecan, a large sulphated proteoglycan, which is abundant in the posterior sclera, regulates tissue hydration (Kuc & Scott, 1997, Rada et al., 2000). These proteoglycans are suggested to be located between the collagen fibrils and regulate their assembly (Rada et al., 2006, Siegwart & Strang, 2007). In addition to the sulphated proteoglycans, hyaluronon, a non-sulphated proteoglycan, is also found in the sclera (Kawamura, et al., 1995) and is thought to play a role in tissue hydration (Bohlandt, et al., 2000, Meyer & Stern, 1994). Apart from the above-mentioned proteoglycans, the human sclera also contains a group of structurally related proteins called Small Leucine-Rich Proteoglycans (SLRPs) (Johnson, et al., 2002). Decorin and biglycan belong to the family of SLRPs (Hocking, et al., 1998).

Interestingly, the distribution of GAGs is uneven throughout the sclera, with higher concentration of dermatan sulphate found around the papilla and higher concentrations of chondroitin sulphate based proteoglycan in the posterior pole of sclera, while hyaluronic acid was high at the equator of the sclera (Trier, et al., 1990). However, the importance of regional variations in GAGs concentration is still unclear. Similar to collagens, the proteoglycans also undergo a change in their concentration with the smaller proteoglycans decreasing after the fourth decade, as opposed to larger proteoglycans, whose concentration remain constant with age (Rada, et al., 2000a).

**Protease enzymes and growth factors**

Protease enzymes such as matrix metalloproteinases (MMPs) are zinc dependent enzymes, which play a major role in collagen degradation and extracellular matrix (ECM) remodelling in various connective tissues. So far, over 20 different types of
enzymes of the MMP family have been identified in the human body (Woessner, 1991, Woessner, 1994). Types such as, MMP-1 (Gaton, et al., 1999), MMP-2, MMP-3 (Gaton, et al., 2001) and MMP-9 (Di Girolamo, et al., 1997) have been observed in the sclera. Changes in MMP activities are involved in extracellular matrix degradation and result in a number of pathological conditions such as rheumatoid arthritis and cancer (Galis, et al., 1994, Vu & Werb, 2000). The activity of MMPs are regulated by the presence of low molecular weight, tissue inhibitors of matrix metalloproteinases (TIMPs) (Woessner, 1991). Two different types of TIMPs, TIMP-1 and TIMP-2, were reported in sclera of a mammalian model, tree shrew (Siegwart & Norton, 2001). A proper balance between MMP-2 activity and TIMP-2 regulation is essential to maintain the integrity of the scleral matrix and thus regulate normal ocular growth (McBrien & Gentle, 2003).

Studies have also demonstrated the presence of various growth factors in the sclera, with fibroblast growth factor (FGF-2) (Gentle, et al., 2002) and transforming growth factor-β (TGF-β) (Jobling, et al., 2004), playing important roles in matrix remodelling during normal ocular development in mammalian sclera. A study carried out amongst the cultured human smooth muscle cells demonstrated that FGF-2 activity increased the expression of MMP-1 and decreased the production of collagen type I, suggesting their possible role in matrix turnover (Pickering, et al., 1997). In sclera, it has been demonstrated that the FGF-2 content decreases from anterior to posterior sclera, suggesting that FGF-2 may play a role in scleral development (Gentle et al., 2002).

TGF-β is a multifunctional cytokine that regulates cell growth proliferation and formation of matrix components in various connective tissues. A study carried out amongst cultured chick embryo fibroblasts demonstrated an increase in the expression of fibronectin and collagen when exposed to TGF-β (Ignotz & Massague, 1986).
Furthermore, addition of TGF-β to the granulation tissue of wounds accelerates the wound healing process, which is marked by increased collagen deposition and wound strength (Mustoe, et al., 1987). Later a study by Jobling et al demonstrated the presence of TGF-β isoforms (β1, β2, β3) in mammalian sclera, in the ratio 2:33:1 (Jobling et al., 2004). However, their role in sclera is yet to be clearly understood, although they are critical in mediating scleral matrix turnover, such as collagen synthesis, GAG remodeling and MMP-2/TIMP-2 regulation (Roberts, et al., 1990). A diagrammatic representation of the major constituents of the sclera extracellular matrix is shown in Figure 1-3.

Figure 1-3: Schematic representation of the scleral structure and biochemistry. Major matrix constituents such as the collagen fibril types I, III V, VI and XII, proteoglycans (hyaluronan, biglycan and aggrecan), growth factors (TGF-β1 and FGF-2), matrix degrading enzymes (MMPs) and their regulators (TIMPs) are depicted in the above figure, interspersed with scleral fibroblasts. Figure adapted from (Fransson, et al., 1993).
1.6 Scleral changes during the development of myopia

As the sclera constrains eye growth, changes in the cell and matrix components will result in alterations in eye size. Numerous studies have detailed changes in scleral properties during myopia development. For ease of description, the scleral changes during myopia have been classified into structural and biomechanical changes. The biomechanical changes have been further subdivided into extracellular matrix changes and cellular changes. Ultimately, it is these changes that result in a thinner and weaker tissue. This weakened sclera is likely to be less able to regulate eye growth, leading to an increase in eye size that results in myopia.

1.6.1 Structural changes

The human sclera undergoes various structural changes during the development of myopia, with one common feature being scleral thinning. Curtin et al demonstrated that a highly myopic sclera is 50% thinner than the emmetropic sclera at its posterior pole, which ultimately leads to the formation of posterior staphyloma, an area of regionalised thinning (Curtin, 1977). In 1983, Avetisov et al demonstrated a similar reduction in posterior scleral thickness of a highly myopic eyes (-7.00D to -9.00D) as compared to the age matched emmetropic ones (myopic: 0.89 +/-0.06 mm vs. emmetropic: 1.29 +/-0.11 mm). Such scleral thinning was observed in the equatorial sclera as well (myopic: 0.56 +/- 0.08 mm vs. 0.71 +/- 0.07 mm) (Avetisov, et al., 1983). McBrien et al, in 2001, observed a similar decrease in thickness at the posterior pole of sclera in a mammalian model of myopia, the tree shrew (myopic sclera 68 +/- 5µm vs normal sclera 89 +/- 4µm). This study also revealed that the sclera thins to approximately 20% during the
first 12 days of onset of experimental myopia, which accounts for 12.00 to 14.00D of myopia. Further thinning progresses slowly over the next 3-8 months, which accounts for up to 20.00D of myopia. Studies in tree shrews revealed that in addition to scleral thinning, a marked loss of scleral tissue was observed in the first 12 days of myopia development that accounts for nearly 17% loss of scleral dry weight. Incidentally, the loss of scleral tissue was observed more at the posterior pole, which is consistent with the posterior scleral thinning (McBrien, et al., 2001). Such results suggest that scleral changes in myopia are a result of an active remodelling rather than passive scleral stretching. Another major structural change observed in the human sclera, during myopia, is a decrease in collagen fibril diameter, particularly at the posterior pole (control: 107 nm vs. myopic sclerae: 82 nm) (Curtin et al., 1979). Moreover, a significant reduction in median collagen fibril diameter (control: 75 nm [50-127] vs. myopic sclerae: 61 nm [41-108]) was observed in tree shrews after 6 months of myopia development and these changes were localised to the posterior pole, as in humans (McBrien et al., 2001). Collectively, these structural changes result in a weaker sclera that is less resistant to intraocular forces or applied stress and thus contribute to altered biomechanical properties.

1.6.2 Biomechanical changes

Various studies have elucidated the significance of scleral biomechanical changes during myopia development and their contribution towards increased axial length. Avetisov and colleagues demonstrated that the myopic sclera of humans exhibited more extensibility when subjected to applied strain (the measure of change in tissue properties with force) as opposed to normal sclera (Avetisov et al., 1983). Furthermore, the above
study also demonstrated that the posterior pole of a myopic sclera had less tensile strength (resistance to applied stress), compared to the control (emmetropic) sclera. Despite these changes in the posterior sclera, no such differences were observed at the anterior sclera of myopic and emmetropic eyes (Avetisov et al., 1983). Phillips and McBrien have also demonstrated the importance of scleral biomechanical changes, in particular, scleral elasticity during myopia (Phillips & McBrien, 1995). Tree shrew scleral strips (myopic and control) were exposed to increasing loads and the amount of scleral extension was measured (Figure 1-4; A, B). It was observed that the amount of extension in myopic sclerae was 25% higher than the control sclerae, with no significant change in modulus of elasticity (the measure of stiffness of a material) and a significant reduction in posterior scleral thickness, (myopic: 149 +/- 4 µm vs. control sclerae: 164 +/- 3 µm). Furthermore, the posterior myopic scleral strips failed (tissue fracture) when the load was increased to 162 g, versus 198 g for control the sclera. On the other hand, the equatorial scleral strips of myopic tree shrews failed at a load of 62 g as opposed to 106 g in control sclera. These findings suggest that scleral thinning could exclusively contribute to an increased scleral elasticity in myopic eyes, owing to an unaltered modulus of elasticity. Interestingly, this increase in scleral elasticity accounted for only 20% of the total ocular enlargement, suggesting that altered scleral elastic properties are not solely responsible for an increase in eye size during myopia. The scleral creep rate is a more critical biomechanical property, which contributes to further scleral extension with load and thus axial length elongation. Creep rate is the measure of amount of scleral extension with time on application of a constant load (Phillips & McBrien, 1995). Figure 1-5 shows, while the initial increase in scleral extension could be attributed to the scleral elastic component, further extension in contributed by the creep rate.
Figure 1-4: Measurement of the elastic properties of posterior and equatorial sclera. The scleral strips were held at either ends and subjected to increasing loads using the miniature tensile testing machine (A). The graph (B) shows that posterior and equatorial myopic scleral strips (Post/Equat MYO) recorded an increase in the extension compared to the control ones (Post/Equat CON). Graph adopted from (Phillips & McBrien, 1995).

Figure 1-5: Pictorial representation of the scleral elastic and creep components contributing to scleral extension. Elasticity, the measure of scleral extension with increasing loads accounts for initial increase in extension and creep rate, the measure of scleral extension on application of a constant load accounts for further extension. The above figure was modified from (McBrien, et al., 2009).
Further research on scleral creep rate by Siegwart and Norton in 1999 indicated that four days of -5.00D lens wear (an optical method to induce myopia) increased the scleral creep rate by 200-300% in tree shrews, suggesting a rapid remodelling of the scleral biomechanical properties during myopia. Further, a reduction in creep rate to a value below that of the control sclerae was observed during recovery from myopia (Figure 1-6). Interestingly, this reduction was detected in less than two days of recovery from myopia, indicating that the rapid change in creep rate is bidirectional. Providing evidence that creep rate is a biologically important measure, an increase and decrease in scleral creep rate in tree shrews that had -5.00D lens for 21 days corresponded with an increase and decrease in the axial length (Siegwart & Norton, 1999).

**Figure 1-6: Changes in the scleral creep rate during -5.00D lens wear and during recovery (Adapted from Siegwart and Norton, 2005).** It can be observed from the graph that the scleral creep rate increases rapidly during myopia (treated eye) and decreases rapidly, on removing the -5.00D lens for 2 days demonstrating that the scleral creep rate is bidirectional.
Further work by Phillips and colleagues, in tree shrews, demonstrated that an increase in scleral creep rate in myopic eyes strongly correlates with an increase in vitreous chamber depth (Pearson, r=0.746, P<0.05), which further emphasizes the importance of creep rate in altering the eye size (Phillips, et al., 2000).

The above-mentioned studies strongly demonstrate the importance of scleral biomechanical properties such as creep rate in contributing to an increase in axial length during myopia. It is also evident from these studies that the sclera undergoes active remodelling during myopia and not just passive stretching; ultimately, these changes alter the biomechanical properties of the sclera, producing an increase in creep rate, leading to ocular elongation and myopia. This active biomechanical remodelling could be driven by changes in the scleral extracellular matrix components such as collagen, GAGs, etc, and/or by changes in the biomechanical properties of the scleral cells themselves.

1.6.2.1 Extracellular matrix changes

Collagen

As already mentioned collagen is the major component of the scleral extracellular matrix, in mammals (Zorn et al., 1992). One of the major biochemical changes during myopia development involves the production and turnover scleral collagen. Avetisov et al demonstrated a decrease in posterior scleral collagen content (-8.16%) in highly myopic humans (-7.00D to -9.00D) (Avetisov et al., 1983). Later in 1995, Norton and Rada demonstrated a similar reduction of collagen content in the posterior sclera of myopic tree shrews (-11.8%) (Norton & Rada, 1995). Reduced collagen content at the
posterior sclera suggests a decrease in collagen synthesis and/or an increase in collagen degradation. Gentle et al studied these two mechanisms in tree shrew sclera by administering $[^3\text{H}]$-Proline, a radiolabelled collagen precursor (Gentle, et al., 2003). To assess collagen synthesis, proline was administered 5 days after myopia development. It was observed that the proline content was significantly reduced at the posterior sclera of myopic tree shrews compared to control (-36 +/- 4%, P<0.001), implying a reduced collagen synthesis during myopia development. To assess the amount of collagen degradation, Proline was injected before myopia induction and its incorporation was recorded for 5, 12 and 24 days after myopia development. It was observed that the proline content at the posterior sclera was greatly reduced after 5 days (-8 +/- 2%, P<0.05) and 12 days (-15 +/- 4%, P<0.05) of myopia induction, suggesting increased collagen degradation. These findings demonstrate an increased collagen turnover at the posterior sclera during myopia is a result of reduced synthesis and increased degradation. The turnover of scleral collagen mirrors the loss of posterior scleral dry weight during myopia development (McBrien & Gentle, 2003) suggesting it may be the major determinant of scleral thinning. However, the avian sclera is characterised by increased matrix production in its cartilaginous layer hence thicker than the fibrous sclera. It was also suggested that changes in fibrous layer of chick sclera is similar to the degradative changes observed in myopic sclera of mammals (Kusakari, et al., 2001). In addition to collagen turnover, Gentle et al demonstrated that the mRNA expression of collagen type I was reduced by 20% in myopic sclerae while no significant change in the expression of collagen types III and V were observed (Gentle et al., 2003). Siegwart and Norton also recorded a similar decrease in the expression of collagen I mRNA (-34%) after 11 days of myopia development (Siegwart & Norton, 2002). Thus during myopia development an increase in the ratio of collagen types V/I and III/I was
observed, such altered collagen type ratios results in almost 40% reduction in the size of collagen fibrils (Birk, et al., 1990). These studies provide a possible explanation for an increase in the concentration of small diameter collagen fibrils, at the posterior sclera during myopia development.

**Proteoglycans**

In addition to collagen turnover, scleral GAG content was also reduced during myopia development in humans and mammalian models of myopia (Avetisov et al., 1983, Norton & Rada, 1995). Various studies in mammalian and avian models of myopia have employed sulphate incorporation as a standard measurement index to assess the scleral GAG synthesis (McBrien, et al., 1999, Rada, et al., 1991). One such study by McBrien et al demonstrated that 5 days of form deprivation (a method to induce myopia) resulted in a 36% reduction in posterior scleral GAG synthesis in myopic tree shrews compared to control animals. To further detail proteoglycans, studies have also looked at the expression of core proteins such as aggrecan, decorin, lumican and biglycan. Siegwart and Strang demonstrated a decrease in the mRNA expression of aggrecan (-60%, p<0.05), four days after myopia development, and a four fold increase in its expression, 2 days after recovery from myopia (Siegwart & Strang, 2007). Owing to the presence of aggrecan between the collagen bundles, these proteoglycans might regulate the organisation of collagen fibrils, during the development of, and recovery from, myopia. Such altered collagen fibril assembly might contribute to altered scleral creep rate during myopia development. In addition, the above study demonstrated a similar significant (p<0.05) regulation of lumican and biglycan during the development of and recovery from myopia. However, decorin, a major proteoglycan that was thought to regulate collagen fibril assembly, demonstrated
no change in its expression during the development of and recovery from myopia. (Siegwart & Strang, 2007). Further, a study by Moring et al demonstrated a rapid decrease in the hyaluronic acid (−27.9% +/- 6.6%) as early as one day after -5D lens wear and this level remained lower during the 11 days of -5D lens wear, compared to the control eyes. However, one day after recovery from myopia, levels of hyaluronic acid returned to baseline (Moring, et al., 2007). This study also suggested that such rapid regulation in the hyaluronic acid content, as early as 1 day of myopia development and/or recovery, may result in altered scleral creep rate previously detailed (Siegwart & Norton, 1999).

Matrix metalloproteinase and Growth factors

Various studies have been carried out in mammalian and avian models of myopia to elucidate the role of MMP-2 in matrix remodelling during myopia development. Guggenheim and McBrien demonstrated that form deprivation myopia resulted in a threefold increase in the levels of active MMP-2 (Guggenheim & McBrien, 1996), with a more significant increase in the posterior sclera than in equatorial sclera. Furthermore, this study also demonstrated a decrease in MMP-2 levels in eye recovering from myopia, suggesting that regulation of MMP-2 is dependent on the direction of eye growth. Siegwart and Norton demonstrated a similar regulation of MMP-2 mRNA expression in tree shrews, with an increase to 44% and 66% after 4 and 11 days of myopia induction respectively. After 4 days of recovery from myopia, the expression of MMP-2 mRNA decreased to 20% as compared to the age-matched normal tree shrews (Siegwart & Norton, 2002).
Siegwart and Norton also identified changes in the mRNA expression of factors that control MMP-2 activity such as TIMP-2 and MT1-MMP, a transmembrane proteinase that initiates MMP-2 activity. They found that during myopia, there was an increase in the mRNA expression of MT1-MMP, suggesting a possible mechanism for increased MMP-2 expression. However, the same study showed that the levels of TIMP-2 were not significantly lowered during myopia development. (Siegwart & Norton, 2005).

In addition to the above-mentioned changes, growth factors such as TGF-β have been suggested to play an important role in scleral remodelling during myopia development. An in vivo study by Jobling et al, in tree shrews, demonstrated a significant decrease in the expression of TGF-β isoforms (β2, -27%; β3, -42%), one day after myopia induction. A significant reduction in all TGF-β isoforms was also evident after 5 days of myopia induction (β1, -37%; β2, -50%; β3, -36%). Owing to the fact that TGF-β isoforms are involved in remodelling the extracellular matrix in other connective tissues, it has been suggested that such a differential expression of TGF-β isoforms could be instrumental in various stages of scleral remodelling during myopia development (Jobling et al., 2004).

Historically, it has been believed that changes in scleral extracellular matrix, such as the ones described above, result in a thinner and weaker sclera that could not withstand ocular expansive forces and result in increased scleral creep rate, finally leading to an increased axial length. However, recent studies carried out in other connective tissues suggest that cellular contraction also plays a major role in controlling the biomechanical properties of a tissue (Dallon & Ehrlich, 2008, Ehrlich & Rittenberg, 2000, Hinz, et al., 2007, Tomasek, et al., 2002)
1.6.2.2 Cellular factors

Various studies have demonstrated the importance of cellular factors in altering the tissue biomechanics. Most of the work done to date employed a fibroblast-populated collagen lattice (FPCL), which is an in vitro, three-dimensional (3D), collagen rich matrix, seeded with cells. The importance of such a 3D matrix in the study of cell mediated regulation of matrix biomechanics will be detailed in chapter two; however, the use of an in vivo-like matrix enables researchers to study various cell-mediated properties such as cell locomotion and matrix remodelling towards matrix contraction (Bell, et al., 1979, Ehrlich & Rittenberg, 2000, Harris, et al., 1980).

Harris et al showed that movement of cells within a FPCL causes reorganisation of the collagen fibrils which produces a tractional force, resulting in contraction of the matrix (Harris, et al., 1981). In addition, Ehrlich and Rittenberg showed when cells attach to the extracellular matrix they elongate as the matrix polymerizes. This elongation pulls the collagen fibrils towards the fibroblasts, which results in compaction of the collagen fibrils and ultimately results in contraction of the collagen lattice (Ehrlich & Rittenberg, 2000). Finally, studies have demonstrated that exposing the FPCLs to mechanical tension results in cellular contraction of the matrix, which is due to the phenotypic differentiation of cells to form highly contractile cells called myofibroblasts (Dallon & Ehrlich, 2008, Kessler, et al., 2001).

Myofibroblasts

While three mechanisms of matrix contraction have been discussed, only one mechanism, the myofibroblast-mediated matrix remodelling, has been extensively investigated. Myofibroblasts arise from the differentiation of chiefly fibroblasts and
contain stress fibres, expressing the smooth muscle protein, alpha-smooth muscle actin (α-SMA), a commonly used marker for myofibroblast differentiation (Hinz, et al., 2007, Kessler et al., 2001). The contractile property of stress fibres containing α-SMA is at least two times greater than that of normal stress fibres (Hinz, et al., 2001a); hence, α-SMA is believed to be responsible for the contractile property of myofibroblasts. Myofibroblasts are not commonly found in all connective tissues, with the first evidence of myofibroblasts coming from studies undertaken by Gabbiani et al, who demonstrated their presence at the site of wound healing (Gabbiani, et al., 1971). This study showed that, during wound contraction, the fibroblasts at granulation tissues exhibited various morphological changes, such as the presence of numerous fibrils and a modified nucleus with nuclear folds, such changes have been suggested to be characteristic of cellular contraction amongst smooth muscle cells (Lane, 1965) and myocardial fibres (Bloom & Cancilla, 1969). Furthermore, the presence of attachment sites, is suggestive of a mechanism to transfer tractional forces to the surrounding environment (Gabbiani et al., 1971). Previous studies have detailed similar cellular morphology in highly contractile, smooth muscle cells (Hogan & Feeney, 1963, Lane, 1965, Pease & Paule, 1960). Thus, these findings suggested that fibroblasts, at granulation tissues, assume smooth muscle like morphology and contract wound openings, by cellular contraction. However, in the event of corneal wounding, the corneal keratocytes differentiate to activated fibroblasts, which further differentiate to corneal myofibroblasts and promote wound healing (Jester, et al., 1999). Further work on wound healing by Kessler et al showed that, in the event of wounding, the fibroblasts differentiate into myofibroblasts, which lay down stress fibres, at the site of wound healing, to facilitate wound contraction and scar formation. Once the healing process is complete, these myofibroblasts disappear by a process called apoptosis (Kessler, et al., 2001). A similar differentiation of
myofibroblasts was observed in the injured cornea by Jester et al, who demonstrated that the wound contraction process was initiated 7 days after wounding and was accompanied by the expression of α-SMA (the marker for myofibroblast differentiation). During day 14 and day 28, this expression of α-SMA was evident throughout the site of wound contraction, suggesting that wound contraction is facilitated by myofibroblasts through an increased expression of the highly contractile α-SMA. On completion of the wound contraction process, the expression of α-SMA disappeared, suggesting apoptosis of myofibroblasts after wound healing (Jester, et al., 1995). Even though myofibroblasts are not commonly found in all connective tissues, studies have demonstrated their constant presence in few connective tissues such as the lung septa (Kapanci, et al., 1992), uterine submucosa (Glasser & Julian, 1986) and periodontal ligaments (Beertsen, et al., 1974). In 1998, Poukens et al demonstrated a constant population of myofibroblasts in the human sclera (Poukens, et al., 1998), appearing between 17 months to 4 yrs of age and increasing in number with age.

**Scleral myofibroblasts**

Even though the role of myofibroblasts in the human sclera is yet to be clearly understood, the above study suggested a possible role of these contractile cells in controlling the eye size during an increase in intraocular pressure (IOP). In addition, Phillips and McBrien observed the presence of myofibroblasts in tree shrew sclera and suggested their possible role in controlling eye size (Phillips & McBrien, 2004). Briefly, the IOP was increased to 100 mmHg in the eyes of chicks (whose sclera is predominantly cartilaginous) and tree shrews (whose sclera is fibrous) and the amount of axial length elongation was recorded over a 60-minute period before IOP was returned to baseline. It was observed that, after an initial increase in axial length in both
tree shrews (101.2%) chicks (103.9%), the former stayed constant and in fact decreased at the end of 60 minutes (-64µm +/- 33µm); however, the chick eye increased by 7.9% by the end of 60 minutes as compared to its initial value (Figure 1-7). On reducing the IOP to 15mmHg, the tree shrew’s axial length was 0.88% smaller than that recorded before the start of the experiment (Figure 1-7; A), while the chick’s axial length was 4.9% larger than its initial value. Such a rapid alteration in scleral biomechanical properties (in less than 60 minutes) discounts the role of biochemically derived matrix changes and suggests cellular based alteration. Immunohistochemical studies carried out on the chick and tree shrew sclera showed that myofibroblasts are present in the tree shrew sclera, while these highly contractile cells were not observed in the avian sclera.

![Figure 1-7: Percentage increase in axial length of chicks and tree shrews with an increase in IOP.](image)

In tree shrews, an increase in IOP to 100 mmHg resulted in a rapid initial increase in axial length, with no further increase for an hour. However, the chicks, in addition to an initial increase in axial length, continued to increase further for an hour. As encircled in the above graph, (A) a reduction in axial length is evident (in both chicks and tree shrews) on reducing the IOP to 15 mmHg, however chick’s eyes were longer at the end of the experiment (Adapted from Phillips and McBrien, 2004).
1.7 Factors responsible for differentiation of myofibroblasts

The above findings suggest that myofibroblasts, as in other connective tissues, might play an important role in rapid contractile properties of the scleral matrix and thus influence (control) eye size. As myofibroblasts have been shown to play an important role in altering the biomechanical properties of a connective tissue and owing to its presence in the sclera, the factors that promote the differentiation of scleral myofibroblasts are of critical importance.

1.7.1 Transforming growth factor –β (TGF-β)

One of the factors that promote myofibroblast differentiation is TGF-β, evidence of which comes from a study by Mustoe et al, who demonstrated that addition of TGF-β isoforms to incisional wounds increase the rate of wound healing in rats, additionally, the final wound strength was 220% higher as compared to control (Mustoe et al., 1987). The above findings suggest that TGF-β isoforms increases the myofibroblast differentiation at the site of wound healing, which may promote the rapid wound healing process. Another study by Desmouliere et al confirmed this action when they demonstrated that addition of TGF-β1 to subcutaneous fibroblasts increased the expression of α-SMA cells (45.3%) as compared to controls (7.5%) (Desmouliere, et al., 1993).

Studies investigating scleral myofibroblasts have also identified TGF-β as a potential modulator of myofibroblast differentiation. A study by Jobling et al demonstrated, that addition of a TGF-β isoform combination (2β1: 33β2: 1β3, 0.55ng/ml) to tree shrew scleral fibroblasts, seeded in an in vitro collagen I rich matrix,
produced an increase in matrix contraction (>650%, P<0.001) (Figure 1-8; A), as compared to that achieved by the matrix without TGF-β isoforms. This finding suggests that TGF-β increases matrix contraction by inducing the differentiation of highly contractile myofibroblasts. Further, immunocytochemical studies confirm this hypothesis with an increase in α-SMA expression observed, after the addition of TGF-β isoforms (Figure 1-8; C), further, stress fibres containing α-SMA were evident at higher magnifications (Figure 1-8; D).

![Graph showing matrix contraction over time](image)

**Figure 1-8: Effect of TGF-β on matrix contraction and α-SMA expression.**

Addition of TGF-β isoforms (2β1: 3β2: 1β3, 0.55ng/ml) resulted in an increase in matrix contraction (A), which is associated with an increased expression of α-SMA (C), as compared to the matrices with no TGF-β (B). Higher magnification reveals presence of stress fibres containing α-SMA (D). The above figure was adopted from (Jobling, et al., 2009)
Further immunocytochemical staining using a general cell marker (vimentin), a cell nuclei marker (4′,6-diamidino-2-phenylindole (DAPI)) and α-SMA revealed an increase in the expression of α-SMA but no change in the expression of vimentin and DAPI in matrices treated with TGF-β isoforms (Figure 1-9; A, B). Such a finding suggests that addition of TGF-β does not increase a small population of myofibroblasts but rather converts fibroblasts to myofibroblasts. This study strongly demonstrates that TGF-β isoforms are critical in regulating scleral myofibroblast differentiation (Jobling et al., 2009).

![Figure 1-9: Immunocytochemical staining of scleral fibroblast populated matrices before (A) and after being treated with TGF-β. The expression of vimentin (red), and DAPI (blue), was unaltered in matrices before and after adding TGF-β; however, the expression of the contractile protein α-SMA (green) increased after adding TGF-β. The above figure was adopted from (Jobling, et al., 2009)](image)

1.7.2 Stress/strain

Stress is the external load or force applied on a tissue, while strain is the measure of change in tissue properties with force. Similar to TGF-β, importance of mechanical
stress in wound healing has been demonstrated in various studies. One of the in vitro studies used to demonstrate the importance of stress in wound healing showed that an increase in mechanical tension at the site of wound healing, by splinting, resulted in an early expression of $\alpha$-SMA compared to unsplinted wounds. Moreover, the expression of $\alpha$-SMA decreased after the healing process in unsplinted wounds whereas, no change in $\alpha$-SMA expression was observed in splinted ones, suggesting that continued matrix strain is important in the continued presence of myofibroblasts (Hinz, et al., 2001b). In addition, Gilbert and colleagues observed that fibroblasts, when subjected to cyclic stretching, demonstrate an increase in the expression of $\alpha$-SMA and these cells align along the direction of stretching (Gilbert, et al., 2007). This strain-mediated myofibroblast differentiation is particularly important in the sclera because it is under constant stress during normal eye growth as well as due to rapid changes in the IOP.

1.7.3 Strain and its role in matrix remodelling

Application of strain is not only crucial in causing early matrix contraction, it is also important in causing altered expression of matrix components such as collagen, proteoglycans, MMPs, etc. Studies have demonstrated that application of mechanical tension on fibroblasts increases the expression of collagen type I in various connective tissue systems such as, anterior cruciate ligaments (Kim, et al., 2002), human patellar tendons (Yang, et al., 2004) and the pulmonary system (Breen, 2000). Having known that fibroblasts differentiate into myofibroblasts in response to stress, such altered expression of matrix components is possibly mediated by myofibroblast differentiation.
1.7.3.1 Strain and scleral cells

Studies highlighting the effect of strain on scleral cells are limited; however, one such study by Yamaoka et al demonstrated that scleral fibroblasts subjected to mechanical stretching resulted in a significant decrease in TIMP-1 synthesis after 72 hours of stretching as opposed to the control fibroblasts (p<0.05). However, the levels of MMP-1 and MMP-2 were not greatly altered between the stretched and control fibroblasts (Yamaoka, et al., 2001). Later in 2007, Shelton and Rada demonstrated an altered expression of scleral ECM genes on application of equibiaxial stretch on scleral fibroblasts. In this study, scleral fibroblasts were seeded on to a 2D in vitro matrix and subjected to equibiaxial stretch. It was observed that the levels of active MMP-2 were increased (+59.72%) accompanied by a decrease in TIMP-2 mRNA expression (-22%); however no significant change in proteoglycan synthesis was observed after 48 hours of application of strain. This study suggested that an increase in strain causes an increase in the expression of the matrix-degrading enzyme MMP-2, which might contribute to degradation of the scleral ECM such as posterior scleral thinning as seen during myopia (Shelton & Rada, 2007).

An in vitro study by Jobling et al, demonstrated that various densities of scleral fibroblasts seeded in a 3D collagen rich matrix exhibit a difference in percentage contraction, with most contraction achieved by the matrix with the highest population of scleral fibroblasts (Figure 1-10). Based on the role of myofibroblasts in other connective tissues and in granulation tissues of wound healing, the mechanism of matrix contraction was suggested to be an increase in intrinsic strain, the strain created by increasing the cell number within the collagen matrix. This causes an increase in
myofibroblast differentiation, which resulted in increased matrix contraction (Jobling et al., 2009).

**Figure 1-10:** Percentage contraction of the *in vitro* 3D collagen matrix with increasing densities of scleral fibroblasts. Various densities of scleral fibroblasts were seeded into an attached collagen I matrix and was released after 5 days, with matrix contraction monitored for every 24 hours. An increase in matrix contraction could be observed with an increase in cell density, the mechanism of which could be an increase in intrinsic stress within the densely populated matrices. Figure adopted from (Jobling, et al., 2009).

Even though the above study demonstrated that addition of TGF-β isoforms to the scleral fibroblast-populated matrices increases the expression of α-SMA, addition of lower concentration of TGF-β isoforms (as that observed during 1 and 5 days after myopia development) reduced the α-SMA expression (Figure 1-11). However, *in vivo* findings of this study demonstrate an insignificant increase in the expression of α-SMA after 1 and 5 days of myopia development, which is contrary to the above *in vitro* findings. Further *in vitro* experiments demonstrated that addition of lower
concentrations of TGF-β isoforms to these cell-populated matrices when maintained under a physiologically relevant strain, resulted in an increased expression of α-SMA (Figure 1-11) (Jobling et al., 2009). We already know that in addition to a decrease in the expression of scleral TGF-β isoforms during myopia (Jobling et al., 2004), the sclera undergoes significant thinning and altered synthesis of matrix components, which results in increased strain on the sclera (McBrien & Gentle, 2003, McBrien, et al., 2009). This increase in scleral strain has been suggested to increase the α-SMA expression during myopia, demonstrating that TGF-β and strain may have opposing effects during myopia (Jobling et al., 2009).

Figure 1-11: α-SMA expression in the scleral fibroblast populated matrices treated with lower concentrations of TGF-β when subjected to no external strain and an applied strain of 8.5%. Addition of lower concentrations of TGF-β isoforms (as that observed in vivo after 24 hours and 5 days of myopia development) to the in vitro cell-populated matrices maintained under no strain resulted in a decreased α-SMA expression compared to those matrices exposed to 8.5% strain, which recorded an increase in α-SMA expression.
To summarize, myofibroblasts are highly contractile cells that are not commonly found in connective tissues but a constant population has been identified in the human and tree shrew sclera. These myofibroblasts are critical in altering the biomechanical properties of a connective tissue and may contribute to the maintenance of eye size. The differentiation of myofibroblasts could be mediated by TGF-β and imposed strain; studies from our own laboratory have shown TGF-β to regulate the differentiation of scleral myofibroblasts and suggested a possible role of strain and TGF-β in contributing to an increased eye size during myopia development. However, the effect of physiologically relevant levels of strain in causing myofibroblast differentiation and thus matrix contraction are yet unknown. As the sclera is under continued, yet varying strain during ocular development and eye movements, this research project aims to identify the role of strain in mediating matrix contraction and scleral myofibroblast differentiation.

1.8 Hypothesis

Scleral fibroblasts differentiate into myofibroblasts, on application of uniaxial strain. These myofibroblasts cause an increase in contraction of the extracellular matrix and express the stress protein, α-SMA.

1.8.1 Aims

Aim I: To characterise the effect of strain on cell mediated contraction of the extracellular matrix in a 3D environment.

Aim II: To show that an increased matrix contraction during stress is due to the differentiation of highly contractile myofibroblasts.
Chapter 2: The effect of strain on the contraction of scleral fibroblast populated 3D collagen matrices

2.1 Introduction

Scleral biomechanical properties are of critical importance in maintaining eye size during normal ocular growth and myopia development. Changes in the scleral extracellular matrix constituents, such as an increase in collagen and proteoglycan turnover and an increased MMP-2 activity, during myopia, result in a thinner and weaker sclera. Historically, these matrix changes were considered to result in a more extensible sclera, resulting in an increased eye size. However, studies carried out in other connective tissues have demonstrated that cells play a role in contributing to the biomechanical properties of a tissue (Tomasek et al., 2002). One cell type of particular interest in mediating tissue biomechanics is the myofibroblast. Myofibroblasts are highly contractile cells that express the smooth muscle protein α-SMA and arise from fibroblast differentiation, with stress and TGF-β being their potential mediators. These cells are not commonly found in all connective tissues, however, a permanent population of myofibroblasts were reported in the human (Poukens, et al., 1998) and tree shrew sclera (Phillips & McBrien, 2004), but their role in contributing to the scleral biomechanics is not yet fully understood. This chapter aims to identify the effect of strain on contraction of a scleral fibroblast-populated 3D collagen matrix. Recent in vitro studies aimed at characterising the properties of various cell types, highlighted the importance of using a physiologically relevant 3D matrix. The use of such a system is particularly important when investigating the effect of mechanical tension on cell properties since the matrix is critical for the cell to detect changes in strain, while it may also shield the cells from imposed strain.
2.1.1 2D vs. 3D in vitro matrices

Researchers have studied the morphology and functions of cells in two different environments, two-dimensional (2D) and three-dimensional (3D) matrices. However, in 1982, Brown showed that cell migration of certain cell types such as neutrophil granulocytes are not well described in a 2D matrix, as motility of the cell is restricted by factors such as substrate adhesion, while the cells undergo migration in a 3D matrix (Brown, 1982). In addition to cell migration, Elsdale and Bard compared the morphology of fibroblasts in 2D and 3D in vitro environments. They showed that fibroblasts are characterised by the formation of pseudopodia in a 2D matrix whereas they assume a bipolar spindle shape (as they are in vivo), within a 3D matrix (Elsdale & Bard, 1972). Such studies highlight the importance of employing a 3D in vitro matrix in detailing various cellular properties. The use of a 3D matrix is of even more importance if connective tissue resident cells are being investigated, since the 3D matrix buffers the applied stress/strain and thus alters the cellular response (Wang & Ingber, 1994). Gruber et al, demonstrated that human meniscal cells seeded in a 3D matrix, play an active role in remodelling the matrix by altering the synthesis of ECM proteins (Gruber, et al., 2008). However, such changes in matrix constituents could not be clearly elucidated in a 2D matrix as the cellular response is affected by factors such as cell migration, substrate adhesion, etc. In addition, cells in a 3D matrix exert a similar force on their matrix as that experienced by them in vivo and hence changes in cellular morphology, contraction and matrix remodelling can be investigated in such an environment (Grinnell, et al., 2006, Tomasek et al., 2002). Recent in vitro studies that looked at the effect of cellular stress/strain on the extracellular matrix have largely employed a 3D matrix (Garvin, et al., 2003, Stegemann & Nerem, 2003).
Owing to various advantages of a 3D matrix, this study aims to identify the effect of intrinsic and applied physiological strain in causing contraction of a scleral fibroblast-populated collagen matrix. The \textit{in vitro} culture system employed in this study is composed of a 3D collagen matrix. The Flexcell® Tissue Train® culture system (Flexcell International, Hillsborough, NC) will be used to accurately control imposed strain so as to investigate its effect on cell phenotype. This culture system more closely resembles the \textit{in vivo} extracellular matrix and most importantly, physiologically relevant levels of strain can be manipulated in this system.

2.2 Materials and methods

2.2.1 Animal model and tissue isolation

Tree shrew (\textit{Tupaia belangeri}) is the animal model employed in this study. It is a well-established mammalian model to study the development of myopia as their ocular anatomy and ocular growth patterns are similar to those found in humans (Norton & McBrien, 1992).

Fifteen days after eye opening, a period during which tree shrews demonstrate the most post-natal rapid development of their ocular components, (Norton & McBrien, 1992), the animals were terminally anaesthetised (ketamine 90mg/kg, xylazine 10mg/kg, followed by sodium pentobarbital 120mg/kg) and the eyeballs were removed by dissection. The whole eyeball was rinsed in PBS and trimmed of orbital fat. The eyeball was cut, posterior to the limbus, into anterior and posterior segments, with the posterior eye cup flat mounted and the vitreous, retina and the choroid removed with the aid of a cotton tip. The optic nerve head was punched out using a 1.5 mm surgical trephine.
Finally, the isolated scleral tissue was transferred to a 25 ml vial containing Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen, VIC, Australia) supplemented with 20% Fetal bovine serum (FBS) (JRH Biosciences, VIC, Australia), 25mM hydroxyethyl piperazine ethane sulfonic acid (HEPES) and Penicillin/Streptomycin, 100 units/ml, (Invitrogen, VIC, Australia). The scleral tissues were subsequently transferred to a 6-well culture plate (Nunc, Roskilde, Denmark) and 2 drops of growth media added gently on top of the tissue. The explant was placed in a humidified incubator (5% CO₂ and 95% air) at 37°C. Once the tissue was attached to the culture plate, 2 ml growth media was added to the scleral tissue and changed every 2-3 days to facilitate cell growth. The culture plates were monitored daily for cell growth around the tissue.

2.2.2 Cell passaging

Upon achieving ~70% confluency, cells were rinsed with PBS (137mM sodium chloride, 2.7mM potassium chloride, 2mM potassium phosphate monobasic and 10mM sodium phosphate dibasic; pH 7.4), to remove FBS present in the growth media and 1ml trypsin (Invitrogen, VIC, Australia) was added per well for 2 minutes at 37°C. Once the cells were completely detached from the surface, as monitored under a microscope at 5x magnification, cells were centrifuged for 2 minutes (at 2000 rpm) and the precipitated cells were resuspended in growth media. Cell density was estimated using a 0.1mm haemocytometer (Boeeco, Germany), on to which 10μl each of the cells and trypan blue dye was added and the number of viable cells were counted under a microscope (Leica) at 10x magnification. The trypan blue dye uses the dye exclusion method of staining the cells, wherein the dye stains the dead cells (blue) and not the viable cells, hence when the mixture of the dye and cells is visualised in a
haemocytometer, the number of viable cells (per ml) in the growth media could be estimated.

After counting the cells and estimating the number of cells obtained from passaging, cells were transferred to new culture flasks, with surface area 75 cm² (T75) or 25 cm² (T25) (Nalge Nunc International, United States). Growth media was added into these flasks (10ml, and 6ml respectively), to promote further cell growth. Cell densities of 0.8 x 10⁶ to 1 x 10⁶ cells/ml were transferred in a T75 flask and cell densities 0.4 x 10⁶ to 0.6 x 10⁶ cells/ml were added to a T25 flask. These flasks were monitored for confluency and passaged as above. This procedure was repeated until the required number of cells was achieved to setup an experiment. Cells from passage numbers from 4 to 7 were only used in the experiments, as previous work from our lab have shown fibroblasts to loose their spindle-shaped morphology above these passage numbers and assume a more rounded morphology.

2.2.3 Cell density and preparation of the collagen matrix

The aim of this study was to detail the effect of cell density and imposed matrix strain on the contractile capacity of scleral cells. Thus selecting the appropriate range of cell densities for this experiment was critical. The cell densities chosen for this experiment were 2.5 x 10², 3.75 x 10², 5 x 10², 1.25 x 10³, 2 x 10³, 3.25 x 10³ cells/mm³. The highest density of scleral fibroblasts used in this study is the maximum practically achievable while the lowest density was chosen to provide a baseline value for contraction, as previous studies from our lab have shown that the lowest density of scleral cells causes only little/no contraction of the surrounding matrix (Jobling et al.,
In addition, previous work done in our lab has indicated that exposing a collagen matrix without any cells to applied strain resulted in no reduction in surface area (data not shown). Hence, the matrix with less number of cells (which recorded a ~10% contraction in previous studies) was employed as a control in this study.

Later, in a study carried out in our lab the posterior scleral strip from tree shrews was isolated and stained for fibroblasts to count the number of cells in the given area (see Appendix I for calculations). Based on the above calculations, it was estimated that the posterior sclera contains $7.16 \times 10^4$ cells/mm$^3$. However, the in vivo scleral cell density was not available during the start of the experiment hence we could not closely approximate the in vivo situation by using the estimated scleral cell density. The main aim of this experiment was not to exactly mimic the in vivo cell numbers but to determine the effect of increasing cell number in response to intrinsic and applied strain.

Having identified the scleral cell densities to be used in the experiments, the scleral cells grown in culture flasks were passaged to isolate the total number of cells required for an experiment ($7.63 \times 10^3$ cells/mm$^3$). After passaging, the cells were pelleted and subsequently resuspended in a variable volume of FBS to achieve $7.63 \times 10^3$ cells/mm$^3$. A collagen rich matrix was prepared using bovine type I collagen (MP biomedicals, Cellagen, Aurora, OH) and DMEM (5 times concentrated), with cells and FBS added to the above matrix to make a total volume of 200µl (refer to table 2-1 for concentration of above matrix constituents). This fibroblast populated collagen matrix was seeded into the three-dimensional, in vitro, Flexcell® Tissue Train® culture system.
The three-dimensional Flexcell® Tissue Train® culture system

The three-dimensional Flexcell® Tissue Train® culture system is a novel culture system wherein the amount of mechanical strain to be applied on the scleral cell-populated matrix can be accurately delivered to the 3D matrix via a vacuum pump. The accurate definition of strain is particularly important since previous work has shown that strain is a potent mediator of myofibroblast differentiation.

Table 2-1: Components of the collagen rich matrix and their concentration in each cell-populated matrix

<table>
<thead>
<tr>
<th>Matrix components</th>
<th>2.5 x 10^2</th>
<th>3.75 x 10^2</th>
<th>5 x 10^2</th>
<th>1.25 x 10^3</th>
<th>2 x 10^3</th>
<th>3.25 x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS (µl)</td>
<td>1.54</td>
<td>2.31</td>
<td>3.08</td>
<td>7.7</td>
<td>12.3</td>
<td>0</td>
</tr>
<tr>
<td>5x DMEM (µl)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Collagen (µl)</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Cells (µl)</td>
<td>18.46</td>
<td>17.69</td>
<td>16.92</td>
<td>12.3</td>
<td>7.7</td>
<td>20</td>
</tr>
<tr>
<td>Total volume (µl)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

The Flexcell® Tissue Train® culture system allows the effect of mechanical strain on matrix contraction to be determined. This novel system consists of a base plate (Figure 2-1, A), which incorporates vacuum outlet and inlet ports (two tubes seen to the left of base plate). This base plate allows culture plates with deformable bases to be incorporated (three rectangular white colored culture plates with red colored rubber caskets around them can be seen in the Figure 2-1, A). For setting up a three-dimensional fibroblast populated collagen matrix with various cell densities, the trough loader (Figure 2-1; B) was fitted on to the base plate with the culture plate placed on top of it and fastened with the rubber gasket. The vacuum inlet and outlet tubes (two tubes seen to the left of base plate) were connected to the Flexcell strain unit (Flexcell tension plus system and the cylinder that applies vacuum) via vacuum tubing. The amount of
vacuum to be applied to the culture plates was accurately defined using the software, Flexsoft V5.0. Once the Tissue Train setup was ready, the three-dimensional fibroblast populated collagen matrix was prepared. Firstly, FBS and 5 times concentrated DMEM were mixed well in a microfuge then collagen was added and mixed, after which the fibroblasts were added. Collagen is highly acidic (pH 3) hence it was added after FBS and 5 times concentrated DMEM, which reduces its pH so that the final pH of the mixture is 7.3-7.6, so that the fibroblasts are not killed by the high acidity of the collagen.

Once the program started, the Flexcell strain unit applied vacuum through the vacuum tubes onto the base plate. The holes in the trough loader deformed the flexible base of the culture plate to form as trough where the cells along with its matrix components (FBS, 5 times concentrated DMEM and bovine collagen type I) were seeded (Figure 2-1, C, D). After seeding the cells and matrix components, the culture plates along with the base plate were placed in the CO$_2$ incubator at 37°C for 3 hours to form a gel, which was held in place by the anchor points on either side. After 3 hours, 3ml culture media (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 25mM HEPES, Penicillin/Streptomycin (100 units/ml), and 10% FBS) was added onto each gel. The culture plate was detached from the base plate, making sure that the ends of the matrices were well attached to the anchor points, and scanned using a high-resolution scanner to record the baseline dimensions of the matrix. The culture plates were then kept in the incubator at 37°C, with matrix contraction monitored until 6 hours to record early matrix contractions after which they were scanned every 24 hours for 5 days. These groups of matrices were monitored to determine baseline contraction rates in response to intrinsic strain (strain created by increasing the cell density within the matrix).
Figure 2-1: The Flexcell tissue train culture system. The above figure represents the features of the Flexcell culture system. A: Base plate, on to which culture plates were fitted along with the loading post and fastened tightly with the gaskets. B: Loading posts, the trough loader, for seeding the matrices, and the arctangle loading post, for applying uniaxial strain (arctangle is a rectangle with curved ends). C: Top view of the culture well with trough loader placed beneath, which contains vacuum holes that deform the flexible substrate to form a trough for seeding the cells with the matrix components. D: Side view of the culture well demonstrating that on application of vacuum through the vacuum holes, the flexible substrate along with the anchor points are pulled down to form a trough. Once the matrix is seeded, it is held in its place by the anchor points. Image adapted from Tissue train tech report, Rev 2.2
In a separate set of experiments, matrices were subjected to mechanical tension using the Flexcell tension plus system, which employs a vacuum to apply uniaxial tensile strain to the matrix. Even though the scleral cells may experience biaxial or multi-axial strain \textit{in vivo}, an \textit{in vitro} stress-strain culture system mimicking the \textit{in vivo} strain conditions is not available yet, hence an uniaxial strain was applied on these cell-populated matrices to characterise the effect of strain on scleral cells. For applying mechanical tension, after adding media, the culture plate was placed in the base plate (Figure 2-2; A) fitted with an arctangle loading post (Figure 2-2; B).

The vacuum tubes were connected to the Flexcell tension plus system as per the manufacturer’s protocol and the Flexsoft software was programmed to apply a defined level of strain. Once the program starts, the matrices were pulled down at either end of the anchor points across the arctangle loading post, resulting in an uniaxial strain across the matrices (Figure 2-2, B). Over time, the cells contract the matrix resulting in a reduction in matrix surface area (Figure 2-2, C).

In order to estimate the \textit{in vivo} strain levels, previous studies were used and certain assumptions were made. Work from McBrien et al demonstrated that a normal 12 days old tree shrew sclera is 120 +/- 13µm thick compared to 95 +/- 12µm in a myopic sclera, demonstrating a 21% scleral thinning during myopia development (McBrien et al., 2001). Based on the scleral thickness profile from the above study, the strain on the sclera during myopia and normal ocular development was calculated assuming that the scleral cells experience a constant strain at all regions. It was calculated that a normal sclera experiences a pressure of 52.3 KPa, as opposed to 66.1 KPa in a thinner myopic sclera (see Appendix II for calculations).
Figure 2-2: Mechanism for applying uniaxial strain on matrices. A: Side view of the cell populated matrix with arctangle loading post beneath the culture plate. B: Application of vacuum pulls the matrices down at either end of the anchor points, which elongates the matrix (as indicated by the direction of uniaxial strain) resulting in an uniaxial strain across the matrix. C: Top view of the fibroblast-populated matrix shows an increase in contraction, over time, on application of uniaxial strain. Image modified from (Garvin, et al., 2003).
The manufacturer’s pressure-strain curve for the tissue train system was used to convert the above values to 8.5% and 11.6% strain in the Flexcell tension plus system, respectively. In other words, application of 8.5% and 11.6% strain on the matrices, seeded in the flexcell culture plates, approximates the *in vivo* strain on scleral matrix experienced due to the normal IOP (assumed to be 15 mmHg in tree shrews) and that experienced by the cells after scleral thinning due to myopia development respectively. Further assumptions made for this calculation will be discussed in detail later.

### 2.2.3.2 Data analysis

The fibroblast populated matrices were maintained at intrinsic (n=5), 8.5% (n=5) and 11.6% (n=5) strains, with contraction monitored for 5 days using a high-resolution scanner. The scanned images of the cell-populated matrices were viewed using Adobe Photoshop and the reduction in overall pixel (surface area) of the matrix was estimated using the polygonal lasso tool. The percentage reduction in pixels over time was calculated to determine the reduction in surface area with strain. A figure showing the contraction of the matrix with one particular density of cells (1.25 x 10³ cells/mm³) maintained under intrinsic strain is shown in the appendix III. The percentage increase in matrix contraction was plotted over time using the software, Graphpad Prism 5. The significance of matrix contraction with time and/or with applied strain was analysed using two-way ANOVA with Bonferroni post-hoc test and the matrix contraction curves were fitted with Michaelis-Menten equation.
2.3 Results

2.3.1 Effect of intrinsic strain on scleral fibroblast mediated contraction of a 3D collagen matrix

All fibroblast-populated matrices demonstrated an increase in contraction at the end of 5 days as compared to the 0-day value (Figure 2-3). The maximum contraction was achieved by the matrix with the largest number of scleral fibroblasts (3.25 x 10^3 cells/mm^3: 67.3% +/- 2.5%) while the least cell-populated matrix demonstrated the minimum (2.5 x 10^2 cells/mm^3: 24.6% +/- 1.3%) at 5 days. The difference in contraction between these two densities was significant after 24 hours (3.25 x 10^3 cells/mm^3 [27.6 +/- 5.7%] vs. 2.5 x 10^2 cells/mm^3 [4.6% +/- 1.4%], p<0.001, two-way ANOVA).

Figure 2-3: Percentage contraction achieved by the scleral fibroblast populated matrices when under intrinsic strain. Increasing densities of scleral fibroblasts seeded in a 3D collagen matrix demonstrate an increase in contraction with an increase in cell density. The percentage contraction was estimated by calculating the percentage reduction in surface area of the matrix over 5 days (n=5, except 3.25 x 10^3, which is n=3). The data are presented as the mean percentage matrix contraction +/- SEM and the data points are fitted with Michaelis-Menten equation.
In addition to the highest and the lowest cell densities, a cell density-dependent increase in matrix contraction was observed across most cell densities as observed in table 2-2. Most of the significant matrix contractions outlined in table 2-2 were evident as early as 24 hours after starting the experiment, except between the densities 3.75 x 10^2 vs. 5 x 10^2 cells/mm^3 and 1.25 x 10^3 vs. 3.25 x 10^3 cells/mm^3, where the matrix contraction was significant only at 72 and 120 hours respectively. The variation (Mean +/- SEM) in amount of matrix contraction across each sample was minimal hence, the data shown in the following table 2-2. 2-3 and 2-4 are repeatable.

Table 2-2: Comparison of matrix contraction across various cell densities under intrinsic strain. A Two-way ANOVA, with Bonferroni post-hoc test was used to compare the significance of matrix contraction achieved across various cell densities and at various time points when maintained under intrinsic strain, (the strain created within the matrix on increasing the cell density). Repeated comparison of cell densities was omitted on increasing the cell density from 2.5 x 10^2 to 3.25 x 10^3.

<table>
<thead>
<tr>
<th>Comparison between cell densities (cells/mm^3)</th>
<th>P value (two-way ANOVA)</th>
<th>% difference in matrix contraction</th>
<th>Earliest time at which significant matrix contraction was observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10^2 vs. 3.25 x 10^1 2 x 10^3 1.25 x 10^3 5 x 10^2 3.75 x 10^2</td>
<td>p&lt;0.001 p&lt;0.001 p&lt;0.001 p&lt;0.001 p&gt;0.05</td>
<td>23.34% 26.71% 23.84% 10.72% N/A</td>
<td>24 hours 24 hours 24 hours N/A</td>
</tr>
<tr>
<td>3.75 x 10^2 vs. 3.25 x 10^1 2 x 10^3 1.25 x 10^3 5 x 10^2 3.75 x 10^2</td>
<td>p&lt;0.001 p&lt;0.001 p&lt;0.001 p&lt;0.05</td>
<td>16.48% 19.86% 17% 9.62%</td>
<td>24 hours 24 hours 24 hours 72 hours</td>
</tr>
<tr>
<td>5 x 10^2 vs. 3.25 x 10^1 2 x 10^3 1.25 x 10^3 1.25 x 10^3</td>
<td>p&lt;0.05 p&lt;0.01 p&lt;0.01</td>
<td>12.62% 16% 13.12</td>
<td>24 hours 24 hours 24 hours</td>
</tr>
<tr>
<td>1.25 x 10^2 vs. 3.25 x 10^1 2 x 10^3 1.25 x 10^3</td>
<td>p&lt;0.05 p&gt;0.05</td>
<td>12.13% N/A</td>
<td>120 hours N/A</td>
</tr>
<tr>
<td>2 x 10^3 vs. 3.25 x 10^4</td>
<td>p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
2.3.2 Effect of 8.5% strain on scleral fibroblast mediated contraction of a 3D collagen matrix

Cell populated matrices, were also exposed to mechanical strain of 8.5%, which was calculated to approximate the strain on scleral cells during normal IOP (see Appendix II for calculations). Application of 8.5% strain on these fibroblast-populated matrices resulted in an increase in matrix contraction across all cell densities, with a significantly higher contraction achieved by the matrix with highest density of fibroblasts (48.4% +/- 1.7%, at 120 hours) and as compared to that achieved by the least cell-populated matrix (11.6% +/- 1.5%, at 120 hours, p<0.001) (Figure 2-4). The highest cell density showed a significant increase in contraction as early as 2 hours after the application of strain (3.25 x 10³ cell/mm³: 14.3 +/- 0.8%, 2.5 x 10² cells/mm³: 1.2 +/- 0.3%, p<0.001). This is more rapid than that observed in matrices under intrinsic strain, which required at least 24 hours.

Application of strain resulted in a more rapid increase in matrix contraction up until 24 hours after which the contraction curve plateau towards 120 hours. All densities showed little increase in contraction after 24 hours (Figure 2-4). The increase in matrix contraction at 24 hours was statistically significant compared to the contraction achieved by matrices under intrinsic strain across all cell densities (p<0.001). For example, the highest cell density achieved a significantly higher contraction of 48 +/- 1.7% as compared to 27.6 +/- 6% in the matrices exposed to intrinsic strain (p<0.001).
Figure 2-4: Percentage contraction achieved by the scleral fibroblast populated matrices when exposed to 8.5% strain. The matrices exposed to an uniaxial strain of 8.5% for 5 days using the Flexcell tension plus system demonstrated an increase in matrix contraction with cell density. It can be noted from the graph that application of strain results in a rapid increase in matrix contraction up until 24 hours, followed by little increase in contraction out to 120 hours. The data are presented as the mean percentage matrix contraction +/- SEM, (n=5 and the data points are fitted with Michaelis-Menten equation.

Table 2-3 shows the statistical significance of matrix contraction achieved by the cell-populated matrices at the end of 120 hours and the earliest time at which the matrix contraction achieved by a cell-populated matrix is significantly higher than other cell densities. It can be observed from the table that most of the significant increases in matrix contraction were achieved within 6 hours of application of strain except between the densities, 5 x 10^2 vs. 2 x 10^3 cells/mm^3, 5 x 10^2 vs. 1.25 x 10^3 cells/mm^3 and 2.5 x 10^2 vs. 3.75 x 10^2 cells/mm^3, wherein the significance was noted at 24 hours. It can also be observed from the table 2-3 that a density dependent increase in matrix contraction was not observed amongst the three most densely populated matrices (1.25 x 10^3 to 3.25 x 10^3 cells/mm^3). Such a finding is more easily visualised in figure 2-4, which
demonstrates the grouping of contraction curves amongst the densely populated matrices as opposed to the sparsely populated matrices (2.5 \times 10^2 to 5 \times 10^2 cells/mm^3).

Table 2-3: Comparison of matrix contraction across various cell-populated matrices exposed to 8.5% strain. A Two-way ANOVA, with Bonferroni post-hoc test was used to compare the significance of matrix contraction achieved across various cell densities and at various time points. Repeated comparison of cell densities was omitted on increasing the cell density from 2.5 \times 10^2 to 3.25 \times 10^3.

<table>
<thead>
<tr>
<th>Comparison between cell densities (cells/mm^3)</th>
<th>P value (two-way ANOVA)</th>
<th>% difference in matrix contraction</th>
<th>Earliest time at which significant matrix contraction was observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 \times 10^2 vs. 3.75 \times 10^2</td>
<td>p&lt;0.001</td>
<td>13.15%</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>15.11%</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>15.41%</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>13.74%</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>11.9%</td>
<td>24 hours</td>
</tr>
<tr>
<td>3.75 \times 10^2 vs. 5 \times 10^2</td>
<td>p&lt;0.05</td>
<td>9.25%</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>10.67%</td>
<td>6 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>11.29%</td>
<td>6 hours</td>
</tr>
<tr>
<td></td>
<td>p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5 \times 10^2 vs. 1.25 \times 10^3</td>
<td>p&lt;0.05</td>
<td>10.22%</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>10.9%</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>10.37%</td>
<td>24 hours</td>
</tr>
<tr>
<td>1.25 \times 10^3 vs. 2 \times 10^3</td>
<td>p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2 \times 10^4 vs. 3.25 \times 10^4</td>
<td>p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2.3.3 Effect of 11.6% strain on scleral fibroblast mediated contraction of a 3D collagen matrix

The final set of matrices were exposed to 11.6% strain, which was calculated to reflect the strain on the thinner sclera after 12 days of myopia development (see Appendix II for calculations). It was assumed that the scleral cells numbers in normal and myopic sclera to be same at the start of the experiment. However, a recent study by Backhouse and Phillips demonstrated that there was no difference in scleral cell
numbers in myopic and control guinea pigs (Backhouse & Phillips, 2010). These cell-populated matrices also demonstrate an increase in contraction with an increase in cell density (Figure 2-5), with a significantly higher contraction achieved by the most densely populated matrix (52% +/- 3%), at 120 hours, and the minimum by the least cell-populated matrix (10.7% +/- 1%, p<0.001). As with 8.5% strain data, a significant increase in matrix contraction was observed at 2 hours after application of strain. In addition, the majority of the matrix contraction occurred at/before 24 hours with little/no contraction from 24 to 120 hours. This increase in matrix contraction at 24 hours was statistically significant from those matrices exposed to intrinsic strain, except for the densities $5 \times 10^2$ and $3.75 \times 10^2$ cells/mm$^3$, wherein significance was achieved at 4 hours and 72 hours respectively (p<0.001).

Figure 2-5: Percentage contraction achieved by the scleral-fibroblast populated matrices when exposed to 11.6% strain. The matrices exposed to 11.6% strain for 5 days demonstrate a rapid initial increase in contraction until 24 hours. A cell density dependent increase in matrix contraction was not observed amongst the top three densely populated matrices and amongst the three sparsely populated matrices, as these matrices group into two bands of contraction. The data are presented as the mean percentage matrix contraction +/- SEM, (n=5) and the data points are fitted with Michaelis-Menten equation.
In addition, the maximal contraction achieved by these matrices was less than that achieved in matrices exposed to intrinsic strain. It could be noted from the figure 2-5 that the contraction curves achieved by the densely populated matrices are tightly grouped together as are the contraction curves for the sparsely populated matrices, which resembles two bands of low and high cell density curves. Hence, from table 2-4 it can be seen that the matrix contraction achieved by the highest density is not significantly greater than that achieved by \(2 \times 10^3\) and/or \(1.25 \times 10^3\) cells/mm\(^3\), which is similar to that seen in matrices exposed to 8.5% strain. However, this was observed amongst the sparsely populated matrices as well.

Table 2-4: Comparison of matrix contraction across various cell-populated matrices exposed to 11.6% strain. A Two-way ANOVA, with Bonferroni post-hoc test was used to compare the significance of matrix contraction achieved across various cell densities and at various time points. Repeated comparison of cell densities was omitted on increasing the cell density from \(2.5 \times 10^2\) to \(3.25 \times 10^3\).

<table>
<thead>
<tr>
<th>Comparison between cell densities (cells/mm(^3))</th>
<th>P value (two-way ANOVA)</th>
<th>% difference in matrix contraction</th>
<th>Earliest time at which significant matrix contraction was observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.5 \times 10^2) vs. (3.25 \times 10^3)</td>
<td>p&lt;0.05 p&lt;0.01</td>
<td>9.7% 11.83%</td>
<td>2 hours</td>
</tr>
<tr>
<td>(2 \times 10^3)</td>
<td>p&lt;0.05 p&lt;0.05</td>
<td>9.6% N/A</td>
<td>4 hours</td>
</tr>
<tr>
<td>(1.25 \times 10^3)</td>
<td>p&lt;0.05 p&gt;0.05</td>
<td>N/A</td>
<td>2 hours</td>
</tr>
<tr>
<td>(5 \times 10^2)</td>
<td>p&lt;0.05 p&lt;0.05</td>
<td>8.9% 9.3%</td>
<td>2 hours</td>
</tr>
<tr>
<td>(3.75 \times 10^2) vs. (3.25 \times 10^3)</td>
<td>p&lt;0.05 p&lt;0.05</td>
<td>8.7% N/A</td>
<td>4 hours</td>
</tr>
<tr>
<td>(2 \times 10^3)</td>
<td>p&lt;0.05 p&gt;0.05</td>
<td>N/A</td>
<td>2 hours</td>
</tr>
<tr>
<td>(1.25 \times 10^3)</td>
<td>p&gt;0.05 p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(2 \times 10^3) vs. (3.25 \times 10^4)</td>
<td>p&gt;0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
2.3.4 Modelling of matrix contraction curve

The results of this study demonstrate that application of strain enhances the initial contraction of scleral fibroblast-populated collagen matrices. It can be observed from figure 2-3 and/or table 2-2 that the matrices exposed to intrinsic strain follow a cell density dependent increase in contraction. On the other hand, such a trend in contraction is not evident in matrices exposed to 8.5% (Figure 2-4, table 2-3) and/or 11.6% strain (Figure 2-5, Table 2-4). However, the exact mechanism behind contraction of these cell-populated matrices is not known. Hence, in order to understand the kinetics of matrix contraction at various levels of strain, the contraction curves were fitted using Allosteric sigmoidal and Michaelis-Menten equations, since these equations fit the curves that follow a rapid initial increasing trend followed by plateauing of the curve. An Allosteric sigmoidal curve fit assumes that the matrix contraction thus achieved is due to the cooperativity of various mechanisms and Michaelis-Menten curve fit assumes that only one particular mechanism mediates the matrix contraction. Statistical analyses were used to determine which curve fit best described the data. As there was no significant difference between the goodness of the fit ($R^2$) between the two curve fits and strain is the only factor that could be controlled for in this study, the simpler Michaelis-Menten equation was used to obtain the contraction kinetics such as the maximum projected contraction of a cell-populated matrix ($V_{max}$) and the time taken for half-maximal contraction to occur ($K_m$).

Figure 2-6 shows the contraction kinetics of matrices exposed to intrinsic, 8.5% and 11.6% strain. It can be observed that matrices exposed to intrinsic strain have a higher $V_{max}$ (A) and $K_m$ (B) value with large variations, especially in the matrices with lower densities of scleral fibroblasts. This is unsurprising, since the least cell-populated matrix
do not exhibit a typical Michaelis-Menten kinetics (i.e., the contraction curve do not exhibit a well defined initial increase followed by less/no contraction phase), suggesting that Michaelis-Menten is not the best fit for a contraction curve that demonstrates a gradual increase in contraction over time. However, the matrices exposed to 8.5% and 11.6% strain demonstrate a typical Michaelis-Menten kinetics, hence, in order to maintain consistency and ease of comparison among different levels of strain; all the contraction curves were fitted with Michaelis-Menten equation. It could be observed from figure 2-6 that the Vmax and Km values for matrices exposed to 8.5% and/or 11.6% strain are lower when compared to those recorded in matrices exposed to intrinsic strain; however, this decrease is not statistically significant due to the variation in the intrinsic strain data. The trend in contraction kinetics followed by the Vmax and Km values in these matrices are such that intrinsic strain > 8.5% strain > 11.6% strain.
Figure 2-6: Contraction kinetics of the fibroblast-populated matrix contraction when exposed to intrinsic, 8.5% and 11.6% strains. The maximum projected matrix contraction, $V_{\text{max}}$, (A) and $K_m$ (B) is the time taken for half-maximal contraction to occur. The $V_{\text{max}}$ values were higher in matrices exposed to intrinsic strain as compared to those exposed to 8.5% and/or 11.6% strains. In addition, the time taken for half-maximal matrix contraction to occur reduced on increasing the strain from intrinsic to 8.5% and 11.6%.
2.3.5 Importance of strain in early matrix contraction

It can be observed from the results that the application of strain dramatically increases early (<24 hours) matrix contraction. In order to investigate the effect of strain on early matrix contraction, the matrix contractions achieved at 1, 2, 4, 6 and 24 hours were analysed separately (Figure 2-7). It can be observed that application of 8.5% and/or 11.6% strain results in an earlier increase in matrix contraction compared to those matrices experiencing intrinsic strain.

On application of 8.5% strain, the matrix with highest density of scleral fibroblasts (3.25 x 10^3 cells/mm^3) demonstrated a significant increase in strain-mediated matrix contraction as early as 2 hours, whereas in the least cell-populated matrix (2.5 x 10^2 cells/mm^3) such significance was observed only at 24 hours. However, the remaining matrices (3.75 x 10^2 to 2 x 10^3 cells/mm^3) demonstrated an increase in matrix contraction at 4 hours when exposed to 8.5% strain.

On application of 11.6% strain, a strain-dependent increase in early matrix contraction was evident as early as 1 hour in the three most densely populated matrices (1.25 x 10^3 to 3.25 x 10^3 cells/mm^3). On the other hand, the sparsely populated matrices (2.5 x 10^2 and 3.75 x 10^2 cells/mm^3) demonstrated a significant strain-dependent contraction at 2 and 4 hours respectively, while the 5 x 10^2 cells/mm^3 density did not show such significance even after 24 hours.

It can also be observed from the graph 2-7 that the matrix contraction achieved by the matrices exposed to 11.6% strain is lower than that achieved those exposed to 8.5% strain at the end of 24 hours. Such a decrease in contraction in matrices exposed to 11.6% strain is significant in matrices with 3.75 x 10^2 cells/mm^3 (p<0.001), 5 x 10^2 cells/mm^3 (p<0.001), 1.25 x 10^3 cells/mm^3 (p<0.01) and 3.25 x 10^3 cells/mm^3 (p<0.05).
Figure 2-7: Significance of applied strain in causing an increase in early contraction of the scleral fibroblast populated collagen matrix. The figures 2-3, 2-4 and 2-5 were re-plotted to compare the percentage matrix contraction achieved across various levels of strain. It can be observed from the above graph that application of strain increases the contraction the cell-populated matrices, particularly the densely populated matrices achieve early and significant increase in matrix contraction. The data are presented as the mean percentage matrix contraction +/-SEM and were analysed using two-way ANOVA (n=5 for 8.5% and 11.6% strain, n=3 for intrinsic strain).
All cell-populated matrices maintained under intrinsic strain demonstrated a gradual increase in contraction after 24 hours up until 120 hours. On the other hand, the application of 8.5% and/or 11.6% strain on these matrices resulted in little/no contraction after 24 hours (Figure 2-8). However, the densely populated matrix, $2 \times 10^3$ cells/mm$^3$ exposed to 8.5% strain achieved a significantly higher matrix contraction, as opposed to those under intrinsic strain, at 48 hours ($p<0.001$) and 72 hours ($p<0.05$), while the highest cell density showed significance at 48 hours ($p<0.001$). The two most densely populated matrices ($2 \times 10^3$ and $3.25 \times 10^3$ cells/mm$^3$) exposed to 11.6% strain achieved a significantly higher matrix contraction, as opposed to those under intrinsic strain only at 48 hours ($p<0.05$).

Having shown that application of strain results in a significant increase in early matrix contraction, the amount of total matrix contraction that is contributed by the application of strain was estimated by subtracting the matrix contraction achieved at intrinsic strain from that observed when 8.5% and 11.6% strain was applied (Figures 2-9 and 2-10 respectively).

It can be observed from figure 2-9 that the difference between the contraction achieved at 8.5% and intrinsic strain is positive until 24 hours of application of strain (Figure 2-9), demonstrating that the initial increase in matrix contraction is mediated by the application of strain. However, further changes in the matrix, after 24 hours, were not explained by the applied strain. The fact that the application of 8.5% strain results in a reduction in maximal contraction compared to intrinsic strain is reflected in the negative trend post 72 hours.
Figure 2-8: Comparison of late contraction achieved by the fibroblast-populated matrices when exposed to different levels of strain. The figures 2-3, 2-4 and 2-5 were re-plotted to compare the percentage matrix contraction achieved after 24 hours across various levels of strain. The matrices exposed to 8.5% and/or 11.6% strain undergo little/no contraction after 24 hours, as opposed to a gradual increase in those under intrinsic strain. The data are presented as the mean percentage matrix contraction +/- SEM and were analysed using two-way ANOVA (n=5 for 8.5% and 11.6% strain data, n=3 for intrinsic strain data).
Similar to matrices exposed to 8.5% strain, the amount of strain-mediated contraction was calculated in matrices exposed to 11.6% strain over 120 hours. The contraction difference between 11.6% and intrinsic strain increases and clusters on the positive side of the graph as early as 6 hours after application of strain (Figure 2-10), demonstrating that the rapid initial increase in matrix contraction is mediated by the application of 11.6% strain. In addition, matrices exposed to 11.6% strain also results in reduced maximal contraction compared to those under intrinsic strain, which is reflected in the negative trend starting from 48 hours.

Figure 2-9: Amount of matrix contraction that is contributed by the application of 8.5% strain. The data from figures 2-3 and 2-4 were used to plot the difference in contraction achieved by matrices exposed to intrinsic and 8.5% strain at various time points. Clustering of the contraction difference values across all cell densities towards the positive direction until 24 hours demonstrates that the initial increase in matrix contraction is mediated by the application of 8.5% strain. However, later shift towards the negative values after 24 hours could not be explained by the application of strain, also the final matrix contraction is underestimated by the applied strain at 120 hours.
Figure 2-10: Amount of matrix contraction that is contributed by the application of 11.6% strain. The data from figure 2-3 and 2-5 were used to plot the difference in contraction achieved by matrices exposed to intrinsic and 11.6% strain at various time points. As opposed to figure 2-9, the contraction difference plateaus at 24 hours and shifts towards negative side as henceforth. In addition, the final matrix contraction was further underestimated on application of 11.6% strain from 72 to 120 hours.

2.3.6 Underlying assumptions in the current study

The assumptions made to calculate the in vitro cell numbers, the in vitro strain to be applied on the cells and the curve fit used to fit the contraction curves achieved by these cell-populated matrices at various levels of strain are discussed below.

2.3.6.1 In vitro cell numbers

Even though the cell densities chosen in this study are an order of magnitude less than those calculated to be present in vivo (Appendix I), the contraction achieved by the densely populated matrices (1.25 x 10^3 vs. 2 x 10^3 vs. 3.25 x 10^3 cells/mm^3) exposed to external strain are not significantly different from each other at the end of 5 days. Such a
finding suggests that an increase in cell number towards the calculated *in vivo* density could result in a similar contraction as that observed in the present study using this 3D culture system.

### 2.3.6.2 *In vitro* strain calculation

Certain assumptions were made to calculate the *in vitro* strain to be applied that approximates the *in vivo* strain. Firstly, calculations of applied strain were based on the assumption that the normal IOP of tree shrew sclera is 15mmHg. Secondly, it was assumed that the scleral cells experience a constant strain throughout the posterior pole, resulting from a 21% loss of scleral thickness after 12 days of myopia development (McBrien et al., 2001). We already know that the sclera, which maintains the ocular integrity, experiences a constant yet varying strain in all directions; hence, application of uniaxial strain on the *in vitro* matrices in the present study is not an ideal estimate of the strain experienced by the scleral cells *in vivo*. However, a culture system to apply biaxial or multiaxial strain on 3D cell-populated collagen matrices was not available at the time of commencing this study. Even though, a 2D culture system that enables the application of biaxial strain on the cells was available, we decided that cells residing in a 3D environment, as that observed *in vivo*, is more important to study the cellular biomechanics than a 2D matrix, owing to various advantages of a 3D matrix mentioned in section 2.1.1.
2.3.6.3 Curve fitting

The contraction curves in this study were fitted with two fits, a simple Michaelis-Menten equation and Allosteric sigmoidal curve fit. The Michaelis-Menten equation assumes that the matrix contraction thus achieved is exclusively strain dependent; on the other hand, Allosteric sigmoidal curve fit assumes cooperativity of various mechanisms in the matrix contraction. However, the mechanisms behind cell-mediated contraction are unknown. That being the case no equation would accurately reflect the underlying process involved in the contraction achieved in this study. We therefore used equations (Michaelis-Menton and allosteric models) that would allow us to derive some basic parameters such as contraction rate and maximum contraction. We included the Michaelis-Menten fit because this provided the best fit of the data ($R^2$ value).
2.4 Discussion

2.4.1 Scleral cell-mediated *in vitro* matrix contraction

It can be observed from the results of this study that the scleral fibroblast-populated matrices maintained under either intrinsic or applied strain demonstrate an increase in contraction over 120 hours. The scleral cell-populated matrices follow a Michaelis-Menten-like curve fit only when exposed to external strain (8.5% and 11.6%) and not when maintained under intrinsic strain (justification of Michaelis-Menten curve fit will be discussed later in this section). A similar Michaelis-Menten like response defined by an initial rapid contraction followed by a slower contraction phase was demonstrated amongst scleral fibroblast populated matrices that were attached to the walls of culture plates for 5 days and released for 4 days (Jobling et al., 2009). In addition to the scleral cells, avian tendon fibroblasts seeded in a 3D collagen matrix follow a similar increase in contraction until 24 hours followed by a slower contraction (Garvin et al., 2003), demonstrating a Michaelis-Menten like curve fit.

The mechanism behind the contraction of the scleral cell-populated matrix could be, firstly, collagen fibril reorganisation mediated by the spreading of fibroblasts within the collagen matrix, which creates a tractional force on the surrounding matrix thus mediating matrix contraction. A study by Harris et al demonstrated that chick heart fibroblasts seeded in a silicone substrate undergo cell spreading and create a tractional force, which is characterised by wrinkling of the substrate. Furthermore, reorganisation of collagen fibrils was observed when these fibroblasts were seeded onto collagen gels, as observed by formation of thick collagen bundles and alignment of these fibrils in one plane. This study suggested that cellular tractional force created by cell spreading mediates collagen fibril reorganisation (Harris et al., 1981). Another study by Grinnell
and Lamke demonstrated that human dermal fibroblasts seeded on the top and bottom of the collagen matrix spread onto the collagen fibrils within 30 minutes after seeding the cells. These fibroblasts spread all over the collagen fibrils after 6 days, which is marked by densely packed collagen fibrils and marked reduction in matrix thickness to 10-20% of its original thickness (Grinnell & Lamke, 1984). The findings of the above studies suggest that a similar collagen fibril reorganisation by scleral fibroblasts is likely to result in contraction of the cell-populated matrix.

In addition to collagen fibril reorganisation, strain-mediated differentiation of myofibroblasts may also mediate the contraction of the scleral cell-populated matrix, as various studies on wound healing have shown that the contractile properties of myofibroblasts mediate the rapid contraction of wound openings (Desmouliere, et al., 2005, Gabbiani et al., 1971, Jester et al., 1999). Even though various mechanisms have been suggested to mediate the in vitro matrix contraction, the exact mechanism is not clearly understood.

2.4.2 Effect of strain on scleral fibroblast populated matrices

Even though the matrices maintained under intrinsic and applied strain demonstrate an increase in contraction over 5 days, the contraction curves achieved by these matrices demonstrate subtle differences in the cell density-dependent contraction, and the contraction kinetics until and after 24 hours.
2.4.2.1  Cell density-dependent contraction

Matrices maintained under intrinsic strain follow a cell density-dependent increase in contraction as opposed to those exposed to applied strain (8.5% and 11.6%). Jobling et al demonstrated a similar cell-density dependent increase in matrix contraction on seeding the scleral fibroblasts onto collagen gels (Jobling et al., 2009). However, this is the first study to demonstrate a cell density-dependent increase in matrix contraction amongst scleral fibroblasts in a more physiologically relevant 3D environment. As already mentioned the mechanism of contraction in matrices maintained under intrinsic strain could be mediated by cell spreading and tractional force generation resulting in collagen fibril reorganisation. Hence, the cell number is a critical factor underpinning the amount of contraction achieved in matrices maintained under intrinsic strain, i.e., the densely populated matrices undergo increased cell spreading and collagen fibril reorganisation, as compared to the sparsely populated ones, resulting in a cell density-dependent contraction. Additionally, it is already known that myofibroblast differentiation is critical in mediating contraction of a matrix, hence, an increase in the number of fibroblasts within the matrix results in increased strain, which could result in increased strain-mediated myofibroblast differentiation and thus matrix contraction. On the other hand, the sparsely populated matrices are characterised by reduced intrinsic strain and thus reduced strain-mediated myofibroblast differentiation, which could also underpin the reduced matrix contraction and thus result in a cell density-dependent increase in matrix contraction. This possibility will be assessed in the next experimental chapter.
2.4.2.2 Contraction kinetics- until 24 hours

Matrices maintained under intrinsic strain demonstrate a gradual increase in contraction over 5 days, whereas those exposed to 8.5% and 11.6% strain recorded a rapid increase in contraction until 24 hours. The above finding demonstrates altered contraction kinetics between the matrices maintained under intrinsic and applied strain. In order to understand the contraction kinetics of these matrices, the contraction curves were fitted with Michaelis-Menten curve fits and it was observed that the time taken by these cell-populated matrices to achieve half-maximal contraction (Km) is faster in matrices exposed to applied strain (8.5% and 11.6%) as opposed to those maintained under intrinsic strain. Such a finding suggests that application of strain alters the biomechanical response of the scleral cells, resulting in even faster contraction of the surrounding matrix. A similar finding was observed by Costa et al amongst human aortic endothelial cells, wherein application of cellular stress resulted in contraction of the cell-populated substrate. On further investigation, it was observed that, 15 seconds after the stress-induced substrate contraction, the highly contractile cellular actin stress fibres exhibit a thin and disorganised morphology, however, within 60 seconds these fibres assume a thick and straight morphology, as found in vivo (Costa, et al., 2002). The Costa study demonstrates the rapidity of cellular stress mediated contraction of the substrate and suggests rapid remodelling of the actin stress fibres play an important role in substrate contraction. Additionally, owing to its contractile properties, the rapid initial increase in contraction could also be mediated by the differentiation of myofibroblasts containing stress fibres.
**2.4.2.3  Contraction kinetics- from 24 hours to 120 hours**

Matrices exposed to applied strain recorded no significant increase in contraction after 24 hours of application of strain, unlike those maintained under intrinsic strain. Such a response could be due to possible dehydration of the matrix resulting from rapid expulsion of the water content out of the matrix due to the rapid initial matrix contraction, which ultimately results in increased collagen content within the matrix. Such increased collagen content within the matrix inhibits the contraction of a cell-populated matrix. A study by Bell et al demonstrated a similar increase in contraction on seeding human dermal fibroblasts onto collagen matrices until 24 hours, followed by slower contraction henceforth. It was also observed in this study that these dermal fibroblasts seeded onto collagen matrices squeeze the water content out of the matrix as the matrix contracts. Furthermore, this study also demonstrated that an increase in collagen content within the matrix limits the contraction of the matrix (Bell, et al., 1979), the mechanism of which will be discussed later in this section. Another possible explanation for lack of contraction after 24 hours is an alteration of the matrix property on application of strain. Using scleral fibroblasts Shelton and Rada reported an increase in the synthesis of active MMP-2 (+59.72%, p<0.05), a matrix-degrading enzyme and reduced TIMP-2 synthesis (-22%, p<0.05), which controls the activity of MMP-2 (Shelton & Rada, 2007). Furthermore, in another study, human scleral fibroblasts subjected to mechanical stretching demonstrated a decrease in TIMP-1 synthesis and no change in the expression of MMP-1 and MMP-2 as compared to those secreted by the control fibroblasts (Yamaoka et al., 2001). The above findings suggest that the fibroblast-populated matrices subjected to mechanical stretching are characterised by altered MMP/TIMP balance. In addition, an increased collagen synthesis was observed in fibroblast-populated matrices exposed to various levels of strain (Breen, 2000, Kim et
Collagen content within a fibroblast-populated matrix is significant because an increase in collagen content around the fibroblasts tends to shield the fibroblasts from the strain and thus result in reduced strain-mediated matrix contraction (Brown, et al., 1998, Muellner, et al., 2001). In support of the above argument, a study by Bell et al demonstrated that the contraction achieved by the human dermal fibroblast-populated matrices is inversely proportional to the concentration of collagen within the matrix. The diameter of the fibroblast-populated matrix with the least concentration of collagen (220µg) reduced by 80%, within 24 hours of seeding, as opposed to 20% reduction in matrices with 570µg of collagen (Bell et al., 1979). Hence, increase in matrix degradation (MMPs/TIMPs) and altered synthesis (collagen) may result in production of a matrix that is stiffer and less able to be contracted by the intrinsic cell population.

Another possible reason for lack of significant contraction after 24 hours of application of strain could be cell death via apoptosis. Studies carried out in other connective tissues have demonstrated that strain-mediated differentiation of myofibroblasts results in extracellular matrix contraction, however these cells are transient disappearing by apoptosis on relieving the strain (Hinz et al., 2007, Kessler et al., 2001). Having previously discussed the relationship between matrix contraction and collagen concentration, a possible alleviation of applied strain due to increased collagen content within the scleral fibroblast-populated matrix, may lead to apoptosis of scleral cells. In support of the above argument, a study by Grinnell et al demonstrated that release of mechanical tension on the fibroblasts-populated matrices results in cell death via apoptosis (Grinnell, et al., 1999). Furthermore, Fluck et al demonstrated that human dermal fibroblasts seeded in 3D contractile collagen matrices, contract the matrix by 20% within 24 hours of seeding the cells and a subsequent reduction in cell number was
observed. When these matrices were stained for DAPI, cell nuclei fragmentation was observed (Fluck, et al., 1998), which is a characteristic feature of apoptotic cell death (Kerr, et al., 1972).

In the present study, in addition to the plateauing of the contraction curves, the strained matrices exhibited a lower maximal contraction compared to matrices with the same density of cells maintained under intrinsic strain. For example, at the end of 120 hours the least cell-populated matrix maintained under intrinsic strain contracted by 24.6% as opposed to 11.6% and 12.4% when exposed to 8.5% and 11.6% strain respectively. This reduction in maximal contraction could be explained by possible matrix remodelling or apoptosis following myofibroblast differentiation, which would ultimately result in the asymptotic nature of the contraction curve after 24 hours.

2.4.3 Conclusion

This study demonstrated the effect of strain (intrinsic and applied) in mediating the contraction of a scleral cell-populated matrix. Specifically, the results of this study demonstrate that the scleral cells seeded in a collagen matrix contracts the matrix in a cell density-dependent manner over 5 days. Further, when these matrices were exposed to external strain, a rapid initial increase in contraction was observed, demonstrating the rapid contractile properties of scleral cells. The above findings suggest that rapid contraction of matrix when exposed to myopic scleral strain (additional strain due to scleral thinning in myopia) could play a critical role in controlling the rapid scleral biomechanical changes such as an increased creep rate during myopia development. Even though, maintaining the cell-populated matrices at 8.5% strain and increasing the strain to 11.6% may more closely approximate the strain on scleral cells during myopia.
development, this experiment was designed to explore the effect of different levels of strain on the response of scleral cells and its surrounding matrix. The *in vitro* system was not designed to mimic *in vivo* scleral extracellular matrix and/or scleral strain. Having characterised the response of sclera cells to strain future experiments may be carried out by applying cyclic strain on the scleral cells.

Studies carried out in other connective tissues and in the sclera have suggested myofibroblasts to mediate such rapid matrix contraction with strain; however, the precise mechanism is unclear. However, if the mechanism of matrix contraction observed in this study is due to a similar myofibroblast differentiation it would strongly implicate that the contractile capacity of myofibroblasts could be critical in controlling the rapid changes in the scleral biomechanical properties during myopia development.

In order to properly determine the phenotypic changes occurring due to strain, further immunocytochemical staining of these fibroblast-populated matrices will be assessed in the next chapter.
Chapter 3: The role of strain in differentiation of scleral myofibroblasts during contraction of in vitro extracellular matrices

Myofibroblasts are highly contractile cells that arise from differentiation of primarily fibroblasts in response to factors such as TGF-β and mechanical tension. These cells express the smooth muscle protein, α-SMA, which is responsible for the cell’s rapid contractile properties. The first evidence of these contractile cells comes from the studies carried out on wound healing, which demonstrated the importance of these cells in contraction of wound openings and scar formation. Furthermore, it was demonstrated that an increase in mechanical tension around the granulation site of wounds enhances myofibroblast differentiation and hastens the wound healing process (Hinz et al., 2001b). Stress-mediated differentiation of myofibroblasts and contraction of the matrix is potentially important to the sclera. We know that the sclera is under constant yet varying tension from alterations in IOP and must remodel its matrix constituents rapidly in order to maintain the ocular integrity during ocular growth and pathological conditions such as myopia (McBrien & Gentle, 2003, Rada et al., 2006). A constant population of scleral myofibroblasts may play a critical role in maintaining the ocular integrity during periods of fluctuations in IOP (Phillips & McBrien, 2004). In particular, the scleral remodelling during myopia development and thus ocular enlargement is an active process; hence, rapid contractile properties of scleral myofibroblast cells might be instrumental in controlling the scleral biomechanics. Even though a constant population of myofibroblasts has been identified in the human and tree shrew sclera, their role is not fully understood (Phillips & McBrien, 2004, Poukens et al., 1998)
From the previous chapter, it was observed that scleral cells seeded in a 3D collagen rich matrix contract the surrounding matrix, with the kinetics being dependent on surrounding matrix strain. Application of external strain on these matrices is critical in causing early and rapid contraction of the scleral cell-populated matrix. As the time course is shorter than that would be expected if this rapid contraction was due to altered expression of various matrix components, it is likely that it is due to the cell itself. Furthermore, the extent of the contraction is more than that would be expected from the cells of fibroblast phenotype, owing to the lack of stress fibres and the contractile α-SMA. The literature highlights that mechanical tension is one of the factors that causes an increase in fibroblast to myofibroblast differentiation (Dallon & Ehrlich, 2008, Tomasek et al., 2002). Having identified the effect of strain on matrix contraction; this chapter aims to demonstrate that the cell density-dependent increase in matrix contraction is mediated by the differentiation of highly contraction α-SMA. In addition, this chapter will also investigate the lack of significant matrix contraction after 24 hours of application of external strain. In order to confirm the differentiation of scleral myofibroblasts in these scleral fibroblast-populated matrices seeded in the 3D in vitro system, the expression of α-SMA will be assessed by immunocytochemical staining, since α-SMA is a commonly used myofibroblast-specific maker (Darby, et al., 1990, Gabbiani et al., 1971).

3.1 Materials and Methods

The scleral cell-populated collagen matrices employed in the first experiment were used in this study to assess the extent of fibroblast to myofibroblast differentiation. After completion of the 5-day experiment described in the previous chapter the matrices
exposed to intrinsic strain, were fixed in 4% paraformaldehyde for 30 minutes and washed with PBS (0.14M NaCl, 2.7nM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.4). The matrices exposed to 8.5% and 11.6% strains were fixed while the strain was still maintained with the Flexcell Tension Plus system. This was done because previous studies have shown that removal of strain initiates a rapid loss of α-SMA containing stress fibres (Grinnell, et al., 1999, Varedi, et al., 2000). Once fixed and washed with PBS all matrices were detached from the anchor points on the culture plates, transferred to 1.6ml microfuge tubes with 1ml PBS, to prevent dehydration of the matrix and stored at 4°C until used for immunocytochemical staining.

### 3.1.1 Immunocytochemistry

The Flexcell tissue train culture system is a novel cell culture system; hence staining of the flexcell matrices required modification of the protocol that was employed in previous studies in our lab (Jobling et al., 2009, McBrien, et al., 2006). The above studies employed collagen-coated cover slips onto which the scleral fibroblast were grown and later stained for α-SMA.

The fixed and stored gels were cut into two halves using a double honed razor blade, in a petridish so that one-half was used for α-SMA and DAPI staining, while the other half served as a within gel negative control. Both halves of the matrices were blocked with 1% goat serum (GS) (Sigma, St. Louis USA) in PBS and incubated overnight at room temperature, after which the primary antibody, α-Actin (1A4) mouse monoclonal (Santa Cruz, CA, USA), was added to one half of matrix (diluted to 1:50 in 1% GS in PBS). The other half served as a negative control incubation and had the α-SMA antibody replaced with PBS alone. After an overnight incubation at room
temperature, the gels were washed three times with PBS, with an incubation time of 30 minutes, 1 hour and 24 hours after the 1st, 2nd and 3rd washes, respectively. These longer incubation times were required to washout the antibody from the gel, as previous pilot experiments with shorter time periods resulted in extensive background staining (data not shown). The secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Eugene, USA), was added the following day (diluted to 1:500 in 1% GS) to both halves of the matrix, and the samples were protected from light from this point forward. After one-hour incubation, the matrices were washed with PBS as above and incubated overnight at room temperature. For staining the cell nuclei, DAPI (1µg/ml) was diluted in distilled water and added to both halves of the matrix. After a 7-minute incubation at room temperature, the matrices were washed three times with PBS (with a 30-minute interval between washes) and mounted onto a slide using Aqua Polymount (Polysciences, Pennsylvania, USA). After overnight incubation at room temperature in the dark, slides were stored at 4°C until use. Slides were observed under a deconvolution microscope (Zeiss Axioplan 2) and photos were taking using Axiocam and Axiovison 4.7 software.

The fibroblast populated collagen matrices, at the end of 5 days, were of varying thickness due to different levels of contraction. The stained and mounted matrices were visualised under a deconvolution microscope and multiple photographs were taken through the z-axis of the 3D matrix (z-stack). Previous studies have shown that application of strain on fibroblast-populated matrices causes the fibroblasts to align at the centre of the matrix and along the direction of strain (Eastwood et al., 1998, Garvin et al., 2003). Hence, in this study the images were taken at the centre of the 3D matrix and additionally, from the ends of the matrix (close to the anchor points) to identify any gradient in distribution of fibroblasts and myofibroblasts across the matrix.
Once the pictures were obtained, they were grouped in order from the uppermost layer to the lowest using the software Adobe Acrobat Professional. As neighbouring image planes, which make up the z-stack may contain the same cell body, processing was performed in a way to minimise the probability of double counting cells. The DAPI and α-SMA staining was marked in each picture using the “pencil tool in Adobe Acrobat”. Each clear image of the cell was marked and if the blurred image of the same cell appears in the same spot in the next z-stack picture, it was not included in the cell count.

### 3.1.2 Assessment of early myofibroblast differentiation on application of external strain

Having demonstrated previously that the cell-populated matrices undergo a rapid increase in contraction until 24 hours of application of strain, the matrices exposed to applied strain (8.5%) were stained for early differentiation of myofibroblasts. In this study, collagen matrices (FBS, 5 times concentrated DMEM and bovine type I collagen) with $5 \times 10^2$ cells/mm$^3$ were seeded in four separate culture plates and maintained under 8.5% strain (as detailed in chapter 2). In order to assess early differentiation of myofibroblasts, matrices were setup as described in section 3.1. These matrices were fixed 2, 4, 6 and 24 hours after application of strain and the gels stained for α-SMA and DAPI using the protocol described in section 3.1.1.

The cell density chosen for this study ($5 \times 10^2$ cells/mm$^3$) exhibited a well-defined strain-dependent increase in matrix contraction when compared to the intrinsic strain contraction curves. The time points were chosen until 24 hours since these matrices demonstrated an increase in contraction only for 24 hours of application of strain after
which they plateaued. The 8.5% strain condition was chosen over 11.6% since the matrices exposed to the higher levels of strain did not exhibit a statistically significant increase in matrix contraction from that achieved by the matrices exposed to 8.5% strain.

### 3.1.3 Data analysis

Myofibroblast differentiation was quantified as the number of $\alpha$-SMA expressing cells compared to the number of DAPI stained nuclei and expressed as a percentage. As in the previous chapter a Michaelis-Menten curve fit was used to fit the matrix contraction achieved by the cell populated matrices for the early time points ($5 \times 10^2$ cells/mm$^3$ at 2, 4, 6 and 24 hours). One-way ANOVA was used to analyse the significance of the percentage $\alpha$-SMA expression in matrices exposed to various levels of strain and two-way ANOVA (with Bonferroni post-hoc test) was used to compare their significance across various levels of strain and cell densities.
3.2 Results

3.2.1 Myofibroblast differentiation at varying levels of strain

3.2.1.1 Intrinsic strain

Z-stack photos of the matrices stained with α-SMA (green) and DAPI (blue) were obtained from the centre (n=3, Figure 3-1) and edge (n=3, Figure 3-2) of the cell-populated matrices to identify the extent of myofibroblast differentiation and the presence of a gradient in the distribution of myofibroblasts across the matrix. Since the scleral cells are residing in a 3D environment and the z-stack photos were taken from the uppermost to the lowest layer of gel, only a few α-SMA stained cells were in focus at one particular time. The images shown in figure 3-1 and 3-2 are the best photos chosen from a series of z-stack images.

Continual presence of the stellate-shaped myofibroblasts, marked by the expression of α-SMA and presence of stress fibres were observed across all the scleral cell-populated matrices maintained under intrinsic strain (Figure 3-1 and 3-2). Furthermore, figure 3-1 shows that the matrix with the least density of scleral cells ($2.5 \times 10^2$ cells/mm$^3$), which recorded minimal contraction in the first experiment is characterised by less α-SMA expression as compared to the matrix with $3.25 \times 10^3$ cells/mm$^3$, which recorded the maximum contraction. Since the matrices are attached to the anchor points on their either ends, an increase in the intrinsic strain (the strain created within the matrix on increasing the cell density) within the matrix, results in increased contraction at the centre of the matrix. On the other hand, the attachment sites on either ends of the matrix tend to pull the matrix in opposite directions. Hence, the direction of intrinsic strain acts across the matrices’ attachment site.
Figure 3-1: Immunocytochemical staining showing myofibroblast differentiation at the centre of the scleral cell-populated matrices maintained under intrinsic strain for 5 days. The images shown above are selected from a series of z-stack images obtained throughout the full thickness of the matrix. It can be observed that the myofibroblast marker, α-SMA (green), is continually expressed across all cell densities and in fact, it increases with an increase in cell density. The cell nuclei are stained with DAPI (blue). Magnification- 40x; scaling- 50µm.
Figure 3-2: Immunocytochemical staining showing myofibroblast differentiation at the end (anchor points) of the scleral cell-populated matrices maintained under intrinsic strain for 5 days. The images shown above are selected from a series of z-stack images obtained throughout the full thickness of the matrix. Similar to that observed at the centre of the matrix, a continual presence of α-SMA (green) can be observed across all cell densities, which increases with cell density. The cell nuclei are stained with DAPI (blue). Magnification- 40x; scaling- 50µm.
Further, the percentage of myofibroblast differentiation was quantified in these cell-populated matrices maintained under intrinsic strain. The dimension of each z-stack photo in the matrix along the X and Y-axis was 220µm x 174µm, however, owing to a cell density-dependent increase in matrix contraction, the thickness of the matrix across the z-axis (depth of the matrix) varied between 73 +/- 21µm (3.25 x 10^3 cells/mm^3) and 154 +/- 33µm (2.5 x 10^2 cells/mm^3). Such a finding demonstrates a cell density-dependent decrease in matrix thickness at the end of 120 hours. Multiple images were obtained throughout the thickness of the matrix with each image separated by 7 to 8µm, to ensure no cells were missed. Additionally, cells were marked in image so that there was no chance of double counting while quantifying the number of myofibroblasts.

Table 3-1 shows the absolute number of myofibroblasts, percentage of myofibroblast differentiation and the total number of cells as identified by the number of DAPI stained nuclei at the centre of the matrix. It can be observed from table 3-1 that the percentage of myofibroblasts at the centre of the matrix increases with cell density. For example, nearly 53% of the cells in the sparsely populated matrix (2.5 x 10^2 cells/mm^3) were identified as myofibroblasts, as opposed to ~89% in the densely populated gels (3.25 x 10^3 cells/mm^3). This finding was statistically significant (p<0.001). Furthermore, a significant increase in the percentage of myofibroblasts was observed between the least cell-populated matrix (2.5 x 10^2 cells/mm^3) and all other matrices (p<0.05 or higher). Similarly significant was the percentage of myofibroblasts between the sparsely populated matrix (3.75 x 10^2 cells/mm^3) and the densely populated matrices (2 x 10^3 and 3.25 x 10^3 cells/mm^3). However, the cell density-dependent increase in myofibroblast differentiation was not significant amongst the top four cell densities (5 x 10^2 to 3.25 x 10^3 cells/mm^3). Table 3-2 shows the statistical significance of myofibroblast differentiation across various cell densities.
Table 3-1: Population of myofibroblasts at the centre of the scleral cell-populated matrices maintained under intrinsic strain for 5 days. The number of myofibroblasts was estimated by quantifying the α-SMA expression from the series of z-stack photos obtained from the centre of the matrix. Similarly, the total number of cells was estimated by quantifying the expression of DAPI stained nuclei throughout the thickness of the gels. The data are represented as mean +/- SEM (n=3).

<table>
<thead>
<tr>
<th>Initial cell density (per mm$^3$)</th>
<th>Absolute no. of Myofibroblasts</th>
<th>Percentage of myofibroblasts (%)</th>
<th>Total number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10$^2$</td>
<td>16 +/- 3</td>
<td>53.2 +/- 2.7</td>
<td>29 +/- 5</td>
</tr>
<tr>
<td>3.75 x 10$^2$</td>
<td>41 +/- 4</td>
<td>68.9 +/- 3</td>
<td>59 +/- 4</td>
</tr>
<tr>
<td>5 x 10$^2$</td>
<td>54 +/- 3</td>
<td>75.6 +/- 3.7</td>
<td>71 +/- 3</td>
</tr>
<tr>
<td>1.25 x 10$^3$</td>
<td>68 +/- 5</td>
<td>81 +/- 2.8</td>
<td>84 +/- 4</td>
</tr>
<tr>
<td>2 x 10$^3$</td>
<td>83 +/- 4</td>
<td>84.2 +/- 1.8</td>
<td>99 +/- 5</td>
</tr>
<tr>
<td>3.25 x 10$^3$</td>
<td>104 +/- 6</td>
<td>89 +/- 1.5</td>
<td>117 +/- 8</td>
</tr>
</tbody>
</table>

Table 3-2: Significance of density-dependent myofibroblast differentiation at the centre of the matrices maintained under intrinsic strain for 5 days. Significant increase in the percentage of myofibroblast differentiation was observed when comparing the matrix with lowest density of scleral cells with all other matrices. However, the percentage of myofibroblasts in the matrix with highest cell density (3.25 x 10$^3$ cells/mm$^3$) was not significantly higher than that observed in matrices with 2 x 10$^3$, 1.25 x 10$^3$ and 5 x 10$^2$ cells/mm$^3$.

<table>
<thead>
<tr>
<th>Comparison between cell densities (cells/mm$^3$)</th>
<th>P value (One-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10$^2$ vs. 3.25 x 10$^3$</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>3.75 x 10$^2$ vs. 2 x 10$^3$</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>5 x 10$^2$ vs. 1.25 x 10$^3$</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>1.25 x 10$^3$ vs. 2 x 10$^3$</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>2 x 10$^3$ vs. 3.25 x 10$^3$</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
The percentage of myofibroblast differentiation at the anchor points (the edge of the matrix) also demonstrated a limited cell-density dependent increase ($2.5 \times 10^2$ cells/mm$^3$: ~69% vs. $3.25 \times 10^3$ cells/mm$^3$: ~92%, p<0.001) (Table 3-3). The significant increase in myofibroblast differentiation was observed only between a few matrices ($2.5 \times 10^2$ vs. $3.25 \times 10^3$ and $2 \times 10^3$ cells/mm$^3$, p<0.01; $3.75 \times 10^2$ vs. $3.25 \times 10^3$ cells/mm$^3$, p<0.01) (Table 3-4), demonstrating very limited cell density dependent myofibroblast differentiation at the edges. Furthermore, from tables 3-1 and 3-3 it can be observed that the number of scleral cells at the centre of the matrix is higher than that observed at the anchor points (at least p<0.05). With respect to myofibroblast differentiation at the centre and at anchor points (Figure 3-3), it was observed that the percentage of myofibroblasts at the centre is significantly higher than that observed at the anchor points only in the least cell-populated matrix ($2.5 \times 10^2$ cells/mm$^3$, p<0.01).

Table 3-3: Population of myofibroblasts at the end (anchor points) of the scleral cell-populated matrices maintained under intrinsic strain for 5 days. Similar to that at the centre of the matrix, the number of myofibroblasts and scleral cells was estimated by quantifying the α-SMA and DAPI expression from the series of z-stack photos obtained from the edges. The data are represented as mean +/- SEM (n=3).

<table>
<thead>
<tr>
<th>Initial cell density (per mm$^3$)</th>
<th>Absolute no. of Myofibroblasts</th>
<th>Percentage of myofibroblasts (%)</th>
<th>Total number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2.5 \times 10^2$</td>
<td>7 +/- 1</td>
<td>69 +/- 1</td>
<td>11 +/- 2</td>
</tr>
<tr>
<td>$3.75 \times 10^2$</td>
<td>19 +/- 3</td>
<td>76 +/- 3</td>
<td>25 +/- 4</td>
</tr>
<tr>
<td>$5 \times 10^2$</td>
<td>27 +/- 3</td>
<td>80 +/- 4</td>
<td>33 +/- 3</td>
</tr>
<tr>
<td>$1.25 \times 10^3$</td>
<td>36 +/- 3</td>
<td>83 +/- 3</td>
<td>44 +/- 4</td>
</tr>
<tr>
<td>$2 \times 10^3$</td>
<td>62 +/- 4</td>
<td>90 +/- 2</td>
<td>70 +/- 3</td>
</tr>
<tr>
<td>$3.25 \times 10^3$</td>
<td>76 +/- 3</td>
<td>92 +/- 2</td>
<td>83 +/- 5</td>
</tr>
</tbody>
</table>
Table 3-4: Significance of myofibroblast differentiation at the end (anchor points) of the matrices maintained under intrinsic strain for 5 days. Even though a cell density-dependent increase in myofibroblast differentiation was observed at the anchor points, such a finding was not statistically significant across various cell densities (3.25 x 10^3 vs. 2 x 10^3 vs. 1.25 x 10^3 vs. 5 x 10^2 cells/mm^3).

<table>
<thead>
<tr>
<th>Comparison between cell densities (cells/mm^3)</th>
<th>P value (One-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10^2 vs.</td>
<td>p&lt;0.01</td>
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<td></td>
<td>p&lt;0.01</td>
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<td></td>
<td>p&gt;0.05</td>
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<tr>
<td></td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>3.75 x 10^2 vs.</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>5 x 10^2 vs.</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>1.25 x 10^2 vs.</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>2 x 10^3 vs. 3.25 x 10^3</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

Having already known the number of scleral cells at the end of 5 days in each cell-populated matrix maintained under intrinsic strain, the total number of scleral cells (per mm3) was estimated at the anchor points and centre of the matrix. It was observed that the cell density at the centre and end of the matrix is significantly higher than that seeded at the start of the experiment (at least p<0.05, two-way ANOVA) (Figure 3-4). Even though the number of fold increase in scleral cells seem to be similar for the densely populated matrices (1.25 x 10^3 to 3.25 x 10^3 cells/mm3), two-way ANOVA demonstrated a significant increase in the cell number at the centre of these matrices as compared to that at the anchor points (at least p<0.05).
Figure 3-3: Comparison of percentage of myofibroblast differentiation at the centre and anchor points of the cell-populated matrix maintained under intrinsic strain for 5 days. The percentage of myofibroblasts observed at the anchor points is higher than observed at the centre of the matrix, however, a statistically significant increase in percentage of myofibroblast at the anchor points was observed only for the least cell-populated matrix ($p<0.01$). The above graph was fitted with Michaelis-Menten equation as this proved to be the best fit with an $R^2$ value of 0.9 and 0.75 for the myofibroblast differentiation curves at the anchor points and centre of the matrix respectively. Data are represented as mean +/- SEM ($n=3$).
Figure 3-4: The fold increase in scleral cell density (per mm$^3$) at the centre and anchor points after 5 days of intrinsic strain. The above data was obtained by dividing the estimated scleral cell density at the end of 5 days by the actual number of cells seeded at the start of the experiment. It can be observed from the above graph that the estimated number of scleral cells at the end of 5 days is many folds higher at the centre and anchor points than the initial number of cells seeded. The above curve was fitted with log of Gaussian curve fit, for which the $R^2$ values were 0.9 and 0.7 for the curves followed by number of fold increase in cell number at the anchor points and centre respectively. The data are represented as mean +/- SEM (n=3)

3.2.1.2 Applied strain- 8.5% and 11.6%

The matrices exposed to applied strain (8.5% and 11.6%, n=3) were also stained for $\alpha$-SMA and DAPI and series of z-stack images were obtained throughout the thickness of the matrix with each image separated by 7 to 8µm. As opposed to the matrices exposed to intrinsic strain, the series of images were taken only at the centre of the matrix. The morphology of cells, their nuclei and number at the anchor points were similar to that observed at the centre of the matrix (data not shown). Staining of these
scleral cell-populated matrices demonstrated two phenotypic changes. Firstly, even though α-SMA staining could be observed in these matrices (green), these stained cells appeared rounded at the end of 120 hours (Figure 3-5 and 3-6) as compared to the stellate-shaped α-SMA stained myofibroblasts observed when these matrices were maintained under intrinsic strain. Secondly, the nuclei and α-SMA stained cells appear smaller than those observed in matrices maintained under intrinsic strain. For example, the DAPI-stained nucleus at the centre of the densely populated matrix (3.25 x 10³ cells/mm³) measured ~5 +/- 3µm when exposed to applied strain (8.5% and 11.6%) as opposed to ~16 +/- 5µm in the intrinsic strain matrices. In addition to the cell nuclei, the circular α-SMA stained cell at the centre of a densely populated matrix (3.25 x 10³ cells/mm³) measured ~16 +/- 3µm as opposed to the stellate-shaped myofibroblast with prominent stress fibres in intrinsic strain matrices measuring ~50 +/- 6µm. As well as being smaller, the scleral cells were not aligned in the direction of applied strain.
Figure 3-5: Immunocytochemical staining showing the expression of α-SMA and DAPI at the centre of the scleral cell-populated matrices exposed to 8.5% strain for 5 days. The images shown above are selected from a series of z-stack images obtained throughout the thickness of the matrix. It can be observed that, the cells exhibit a more rounded α-SMA staining and smaller nuclear size. Magnification- 40x; scaling- 50µm.
Figure 3-6: Immunocytochemical staining showing the expression of α-SMA and DAPI at the centre of the scleral cell-populated matrices exposed to 11.6% strain for 5 days. The images shown above are selected from a series of z-stack images obtained throughout the thickness of the matrix. Similar to matrices exposed to 8.5% strain, the cells exhibit a more rounded α-SMA staining and smaller nuclear size. Magnification- 40x; scaling- 50µm.
The expression of round shaped α-SMA stained cells and the smaller DAPI stained nuclei was quantified for the matrices exposed to applied strains. A significant decrease in the percentage of myofibroblast differentiation was observed in all matrices exposed to applied strain (8.5% and 11.6%) as opposed to the intrinsic strain matrices (two-way ANOVA, at least p<0.01) (Figure 3-7).

Figure 3-7: Percentage change in the myofibroblast differentiation in matrices exposed to applied strain from that observed when maintained under intrinsic strain for 5 days. The number of α-SMA expressing myofibroblasts was quantified throughout the thickness of the cell-populated matrices exposed to external strains and the percentage change in their expression, relative to that observed in the intrinsic strain matrices, is shown as mean +/- SEM in the above figure (n=3). A significant decrease in the α-SMA expression was observed in matrices exposed to 8.5% and 11.6% strain as opposed to the intrinsic strain matrices.
In addition, a significant reduction in the expression of DAPI stained scleral cells was observed in all matrices exposed to applied strain as opposed to those maintained under intrinsic strain (two-way ANOVA, at least p<0.05) (Figure 3-8).

**Figure 3-8**: Percentage change in the number of DAPI stained scleral cells in matrices exposed to applied strain from that observed when maintained under intrinsic strain for 5 days. The number of DAPI stained scleral cells was quantified throughout the thickness of the cell-populated matrices maintained under various levels of strain. A significant reduction in the population of scleral cells was observed in matrices exposed to 8.5% and 11.6% strain as opposed to the intrinsic strain matrices. The above data are represented as mean +/- SEM (n=3).
3.2.2 Early differentiation of myofibroblasts in scleral fibroblast-populated matrices exposed to 8.5% strain

Since the matrices exposed to external strain for 5 days and stained for α-SMA did not show any prominent stellate-shaped myofibroblasts with stress fibres expressing α-SMA and the cell nuclei appeared aberrant, separate set of experiments was carried out to identify the early differentiation of myofibroblasts in matrices exposed to applied strain. One particular density of scleral fibroblasts (5 x 10^2 cells/mm^3) was exposed to 8.5% strain and fixed at various early time points (2, 4, 6 and 24 hours) for immunocytochemical studies. Firstly, in order to ensure that the percentage of matrix contraction achieved in the current experiment is similar to that observed in the first experimental chapter, the matrix contraction was monitored at the above mentioned time points. It can be observed from the figure 3-9 that the contraction achieved by the cell-populated matrices is similar to that achieved in the first experiment across various early time points, except at 24 hours, wherein the matrices in current study demonstrated a slight reduced contraction (28.1% +/- 1.6% vs. 37.3% +/- 3.7%, p<0.05).

Having demonstrated a similar contraction as that observed in the first experiment, the expression of α-SMA and the DAPI stained nuclei was quantified in these matrices at 2, 4, 6 and 24 hours. Similar to the matrices stained at the end of 120 hours, z-stack photos were taken throughout the thickness of the matrix at the centre and anchor points of the matrix. As highlighted earlier, measures were taken to avoid over-counting or missing any cells, while quantifying the myofibroblast differentiation.
Figure 3-9: Comparison of matrix contraction achieved by the scleral fibroblast populated matrices in the first and current experiment when exposed to 8.5% strain. The cell-populated matrices were scanned at various early time points and the percentage reduction in surface area over time is plotted in the above graph. Two-way ANOVA demonstrated a significant decrease in contraction achieved by the matrices in the current study only at 24 hours (p<0.05). Michaelis-Menten equation was used to fit the data points in this graph ($R^2 = 0.9$ for the first and current experiment curve). The above data are expressed as mean +/- SEM (n=3 in the current experiment and n=5 in the first experiment).

Images selected from the series of z-stack photos are shown in the figure 3-10. Figure 3-10 demonstrates a continual presence of the stellate and non-stellate-shaped myofibroblasts until 6 hours after the application of 8.5% strain. Prominent projections from the cell bodies depicting stress fibres could also be noted at the centre and anchor points of the matrices after 4 and 6 hours from applying external strain. Furthermore, the scleral cells align with the direction of applied strain in the matrices fixed at 6 hours after the application of strain. The size of the DAPI stained nuclei and the stellate-shaped myofibroblasts is higher than that observed when these matrices were exposed to 8.5% strain for 5 days. For example, the DAPI stained nuclei measured 11 +/- 3µm at
the centre of the matrix at 6 hours and the stellate shaped myofibroblasts measures 43+/-5µm, as opposed to 4 +/− 2µm (DAPI) and 9 +/− 1µm (circular α-SMA stained cells) as that observed after 120 hours of exposure to 8.5% strain. After 24, these stellate-shaped myofibroblasts lose their morphology and assume a circular shape, which is similar to that observed at the end of 120 hours in the previous experiment.

The thickness of these matrices varied between 161 +/- 19 µm at 2 hours and 128 +/- 12 µm at the end of 24 hours, demonstrating a decrease in thickness with time. Table 3-5 shows the absolute number of myofibroblasts and the percentage of myofibroblast differentiation as opposed to that of the DAPI stained nuclei at the centre and anchor points of matrices exposed to 8.5% strain. It can be observed from the table 3-5, the number of α-SMA stained cells (stellate and non-stellate-shaped) increase until 6 hours and reduces after 24 hours of application of strain. This is evident at the anchor points and centre of the matrix. As with the intrinsic strain matrices stained after 5 days, the number of myofibroblasts and DAPI stained nuclei at the centre of the matrix is higher than that observed at the anchor points. Furthermore, a rapid increase in the percentage of myofibroblasts could be observed as early as 2 hours after the application of strain in these matrices.
Figure 3-10: Immunocytochemical staining showing the expression of α-SMA and DAPI at the anchor points and centre of the scleral fibroblast-populated matrices exposed to 8.5% strain for 24 hours. The images shown above are selected from a series of z-stack images obtained throughout the thickness of the matrix. In contrast with that observed after 120 hours, a continual presence of stellate-shaped myofibroblasts with stress fibres could be observed until 6 hours. However, such morphology is lost at 24 hours and the cells appear more rounded with smaller nuclei. The double headed arrow at the top right of each image denotes the direction of applied strain. Magnification- 40x; scaling- 50µm.
Table 3-5: Population of myofibroblasts and scleral cells at the end (anchor points) and centre of the scleral cell-populated matrices exposed to 8.5% for 24 hours. The percentage of myofibroblasts was calculated by estimating the number of α-SMA stained cells as opposed to the number of DAPI stained nuclei throughout the thickness of the matrix at the centre and anchor points. The data are expressed as the mean +/- SEM (n=3).

<table>
<thead>
<tr>
<th>Time</th>
<th>Absolute no. of myofibroblasts</th>
<th>% of myofibroblasts</th>
<th>No. of DAPI stained nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centre</td>
<td>Anchor points</td>
<td>Centre</td>
</tr>
<tr>
<td>2 hours</td>
<td>18 +/- 2</td>
<td>7 +/- 1</td>
<td>71 +/- 4</td>
</tr>
<tr>
<td>4 hours</td>
<td>42 +/- 2</td>
<td>22 +/- 2</td>
<td>77 +/- 1</td>
</tr>
<tr>
<td>6 hours</td>
<td>57 +/- 3</td>
<td>32 +/- 1</td>
<td>84 +/- 2</td>
</tr>
<tr>
<td>24 hours</td>
<td>22 +/- 2</td>
<td>11 +/- 1</td>
<td>71 +/- 1</td>
</tr>
</tbody>
</table>

An incremental increase in the number of α-SMA stained cells was observed at the centre and anchor points at 2, 4 and 6 hours after which a significant decrease in their population was observed at 24 hours as compared to that observed at 4 and 6 hours (p<0.01 and p<0.001, respectively) (Figure 3-11; A). Similarly significant was the reduction in the total population of scleral cells at 24 hours at the anchor points and centre of the matrix as compared to that at 4 and 6 hours (p<0.01 and p<0.001, respectively) (Figure 3-11; B).
Figure 3-11: Early changes in the population of myofibroblasts and total number of scleral cells across the scleral cell-populated matrices exposed to 8.5% strain for 24 hours. The number of myofibroblasts was estimated by quantifying the α-SMA expression and the number of scleral cells was estimated by quantifying the number of DAPI stained nuclei at the centre and anchor points of the matrix. Log of Gaussian curve fit, which was used to fit the above data points, $R^2$ value for which is 0.9 for all the above curves. Two-way ANOVA using Bonferroni-post hoc test demonstrated a significant decrease in scleral cell number (α-SMA and DAPI stained cells) at the end of 24 hours from that observed at 4 and 6 hours ($p<0.01$ and $p<0.001$, respectively) Each data point in the graph represents the mean +/- SEM (n=3).
3.3 Discussion

The results of the initial experiment demonstrated a cell density-dependent increase in contraction of the fibroblast-populated matrices when maintained under intrinsic strain and a rapid increase in contraction until 24 hours when exposed to external strains of 8.5% and 11.6%. Such a finding suggested a possible role of the highly contractile scleral myofibroblasts in mediating the contraction. Studies carried out in other connective tissues, have demonstrated that fibroblasts differentiate into myofibroblasts in response to mechanical tension, which contracts the surrounding matrix (Gabbiani et al., 1971, Kessler et al., 2001). Hence, this chapter aimed to identify myofibroblast differentiation in the matrices described in chapter 2 by labelling the matrices for α-SMA, a myofibroblast marker.

3.3.1 Myofibroblasts differentiation in matrices maintained under intrinsic strain for 5 days

Immunocytochemical staining of the scleral cell-populated matrices maintained under intrinsic strain showed myofibroblast differentiation across all cell densities, as observed by the expression of the contractile protein, α-SMA. Such a finding suggests that matrix contraction observed in the first experiment is mediated by the differentiation of fibroblasts into highly contractile myofibroblasts. Furthermore, increasing densities of scleral fibroblasts seeded in the collagen matrix creates an increasing intrinsic strain within the matrix, which could result in the increased strain-mediated differentiation of myofibroblasts. While a study by Jobling et al suggested a possible intrinsic strain-mediated scleral myofibroblast differentiation in densely populated matrices (Jobling et al., 2009), this is the first direct evidence to demonstrate
such a finding using scleral cells in a 3D environment. In addition to the sclera, studies carried out in other connective tissues have also demonstrated that an increase in intrinsic strain within a fibroblast populated matrix results in myofibroblast differentiation. One such study by Ehrlich and Rajaratnam demonstrated that a collagen matrix with increased populations of human dermal fibroblasts is characterised by the differentiation of myofibroblasts containing stress fibres, which also underpins the contraction of the above matrix (Ehrlich & Rajaratnam, 1990).

In addition to an increase in the myofibroblast differentiation, these scleral cells were also aligned along the major vector of intrinsic strain, which is across the anchor points on either side of the matrix. A study by Delvoye et al demonstrated a similar finding, showing that a dermal fibroblast populated collagen matrix, attached at either end, experiences a mechanical tension across the attachment points, which is directly proportional to the cell number. In addition, the cells in these matrices were aligned in the direction of the principal strain (Delvoye, et al., 1991). In addition to the dermal fibroblasts, a similar finding was observed amongst avian tendon fibroblasts in a more physiologically relevant 3D in vitro matrix and suggested that tractional forces exerted by the cells on its surrounding matrix mediates the cellular alignment (Garvin et al., 2003). Since the cell-populated matrices in the present study are attached to anchor points and maintained under intrinsic strain, the scleral cells experience essentially an uniaxial strain across either ends that are attached to anchor points, which is likely to mediate the alignment of scleral cells in the direction of strain.

In addition to the alignment of cells, it could be observed from figure 3-4 that the estimated scleral cell density at the centre and anchor points was higher than the seeded cell density. However, the cell population at the centre of the matrix, a region wherein
studies have shown the cells to experience less strain, was significantly higher than that at the anchor points across all matrices, suggesting cell proliferation. In support of the above finding, studies carried out in other connective tissues have shown that the cells at the centre of a cell-populated matrix are shielded from the imposed strain by a thicker collagen matrix and hence experience less strain than those at the attachment sites (Bellows, et al., 1982, Kolodney & Wysolmerski, 1992). Another study by Hannafin et al demonstrated amongst the anterior cruciate and medial collateral ligament fibroblasts that these cell types seeded onto collagen type I matrices and subjected to strain demonstrate a decrease in the proliferation of fibroblasts from 3-5 days after seeding as opposed to the non-strained control cells, which continued to proliferate (Hannafin, et al., 2006). Such a finding suggests that the scleral cells in this study are likely to experience less strain at the centre of the matrix compared to those at the anchor points, which ultimately results in increased cell proliferation at that region. This enhanced cell proliferation explains the increase in cell density at the end of 5 days as opposed to the cell density seeded initially.

Furthermore, tables 3-1 and 3-3 highlight two interesting findings of this experiment, firstly, the volume of the cell-populated matrix at the anchor points is less than that observed at the centre of the matrix and secondly, even though the number of scleral cells at the edges of matrix is low, the percentage of fibroblasts that differentiated to myofibroblasts is higher at the edges as compared to those at the centre of the matrix. Reduction in the volume of the matrix at the edges could possibly be explained by the finding that the cell-populated collagen matrices experience an increased strain at the attachment sties (anchor points in this study) as opposed to that at the centre of the matrix (Bellows et al., 1982, Kolodney & Wysolmerski, 1992). Furthermore, studies in other connective tissues have shown that fibroblasts when exposed to such increased
mechanical tension results in myofibroblast differentiation, which contracts the matrix owing to its rapid contractile properties (Hinz et al., 2001b, Kessler et al., 2001). In the current study, a similar increase in the strain-mediated myofibroblast differentiation, as that observed by increased percentage of myofibroblasts at the anchor points, could underpin the enhanced contraction of the matrix at that point thus resulting in reduced volume of the matrix.

3.3.2 Time course changes in myofibroblast differentiation in matrices exposed to external strain

Immunocytochemical staining of the scleral fibroblast-populated matrix (5 x 10^2 cells/mm^3) exposed to 8.5% strain demonstrated an increase in the expression of stellate-shaped myofibroblasts containing stress fibres and expressing α-SMA at various early time points (2, 4 and 6 hours). In addition, figure 3-9 shows an increase in contraction of the matrix at these time points. Similar to the findings of the current study, a study by Hinz et al demonstrated that rat lung fibroblasts seeded onto a stressed silicone substrate for 5 days, contracts the surrounding matrix to 63% of its original size and further immunocytochemical staining revealed a marked expression of the stress fibres containing the contractile protein, α-SMA. This study suggested that the contractile activity of stress fibres containing α-SMA is greater than that of normal stress fibres (Hinz et al., 2001a). Furthermore, an in vivo study on corneal wound healing by Jester et al demonstrated that a week after wounding, the rate of contraction of corneal wounds increases, marked by a corresponding increase in the expression of α-SMA and stress fibres. This study suggested that the presence of myofibroblasts with stress fibres expressing α-SMA is critical in the contraction of corneal wounds (Jester et
The above studies carried out in other connective tissues suggest that the rapid initial increase in contraction of the scleral cell-populated matrix observed in the first experiment is a result of the rapid strain-mediated differentiation of the highly contractile scleral myofibroblasts observed in the current experiment.

In addition, the number of α-SMA stained cells and DAPI stained nuclei was reduced after 24 hours of exposure to strain and was significantly lower at 120 hours as compared to that observed when these matrices were maintained under intrinsic strain. This finding suggests that the myofibroblasts containing stress fibres and expressing α-SMA, which is critical in mediating the rapid initial contraction, begins to disappear as early as 24 hours, which coincides with no significant increase in matrix contraction after 24 hours. Furthermore, the α-SMA expressing cells demonstrated a reduction in size and were more rounded with smaller nuclei from 24 hours of application of strain; such cellular changes are consistent with pyknosis, a series of the cellular and nuclear changes during the process of cell death. Thus, the disappearance of the DAPI stained nuclei and α-SMA stained myofibroblasts and presence of misshaped cells suggests possible cell death via apoptosis or necrosis.

In the current study, the scleral cell-populated matrices seeded in the Flexcell culture system were maintained under an uniaxial strain for 5 days. In order for cell death to occur, these fibroblasts must be relieved of the applied strain. There is no evidence of relief of strain in this culture system. However, studies carried out in other connective tissues have suggested a possible mechanism by which the strain on the fibroblasts might be relieved. Application of external strain on the fibroblast-populated matrices demonstrated a significant increase in collagen synthesis amongst the human patellar tendon fibroblasts (Yang et al., 2004), pulmonary fibroblasts (Breen, 2000) and
anterior cruciate ligament fibroblasts (Kim et al., 2002). This increase in collagen content around the fibroblasts has been shown to shield the amount of strain experienced by these cells (Muellner et al., 2001). Such an increase in the strain-mediated collagen synthesis could shield the scleral cells from imposed strain and thus initiate the process of cell death in the current study.

A similar disappearance of α-SMA stained myofibroblasts and stress fibres after mediating initial matrix contraction have been demonstrated in studies carried out in other connective tissues, especially in the granulation tissue of wounds. A study by Darby et al demonstrated that during the process of wound healing in rats, an increase in the expression of α-SMA was observed from 6 to 15 days after wounding, which corresponded with an increase in the contraction of wound openings. In addition, a decrease in the expression of α-SMA was observed 15 days after wounding and its expression disappeared once the wound closure was completed (after 30 days) (Darby, et al., 1990). Another study (Desmouliere, et al., 1995) pertaining to wound healing in rats demonstrated that from 16 to 35 days after wounding, a time during which the wound closes completely, an increase in apoptotic cells were visible with fragmented DNA, condensed chromatin and degeneration of mitochondria, which are characteristic features of apoptotic cell death (Searle, et al., 1982, Wyllie, et al., 1980). Owing to the finding that the mechanical tension within the granulation tissue of wounds alleviates once the healing process is complete (Hinz et al., 2001b) and such alleviation of strain on fibroblasts leads to cell death via apoptosis (Grinnell et al., 1999), it is likely that the disappearance of myofibroblasts observed in the current study is due to apoptotic cell death mediated by stress relaxation.
3.3.3 Conclusion

Scleral fibroblast populated collagen matrices that were maintained under intrinsic strain for 5 days and stained for α-SMA and DAPI, demonstrated a cell density-dependent increase in expression of stellate-shaped myofibroblast differentiation containing stress fibres. Such an increase in myofibroblast differentiation is likely to mediate the cell density-dependent increase in matrix contraction achieved by these cell-populated matrices. Quantification of scleral cells demonstrated an increasing gradient in their distribution from the anchor points to the centre of the matrix. Further estimation of the total number of scleral cells within the matrix at the end of 5 days demonstrated a significant increase in the cell density from that seeded initially. This increase was more significant at the centre of the matrix as opposed to that at the anchor points. This indicates that the cells at the centre of the matrix experience a lower strain at opposed to that the anchor points, which results in an increase in cell proliferation at the centre.

Unlike the intrinsic strain condition, the matrices exposed to applied strain (8.5% and 11.6%) were characterised by the presence of rounded α-SMA stained cells with smaller nuclei at the end of 120 hours. In addition, a significant decrease in the number of α-SMA stained cells and DAPI stained nuclei was observed in these matrices as opposed to that observed in those maintained under just intrinsic strain, suggesting possible cell death after 5 days. Interestingly, the presence of stellate-shaped myofibroblasts with stress fibres expressing α-SMA could be observed in the cell-populated matrix (5 x 10² cells/mm³) exposed to 8.5% strain until 6 hours. However, these myofibroblasts disappear and assume a rounded morphology at the end of 24 hours, as that observed after 5 days. Such a finding is consistent with the results of the
first experiment, which demonstrated that these matrices do not undergo any significant increase in contraction after 24 hours of application of strain. Furthermore, the presence of more rounded cells with smaller nucleus as early as 24 hours after application of strain suggests cell apoptosis due to alleviation of the applied strain. Such rapid differentiation of myofibroblasts and their disappearance after mediating the contraction of the collagen matrix demonstrates the rapid contractile properties of scleral myofibroblasts and how the mechanical environment of the surrounding matrix dramatically alters scleral cell phenotype and survival. This study has shown that scleral fibroblasts are able to contract their matrix and undergo differentiation to myofibroblasts when placed under strain. The effect of such factors in relation to eye growth and myopia will be discussed in the proceeding chapter.
Chapter 4: Summary and possibilities for future research

Myopia is a refractive error, which is characterised by an abnormal increase in eye size. This increase in eye size leads to various sight-threatening complications such as retinal degenerations and detachment. The outer coat of the eye, the sclera, is a structure that maintains ocular integrity and size; hence, studies have identified the sclera as a potential target to control eye size and thus myopia development. It was believed that the scleral matrix changes, such as reduced collagen synthesis and increased matrix degradation resulted in altered scleral biomechanical properties and lead to a thinner and weaker sclera that could not withstand ocular expansive forces finally leading to an increased axial length. However recently, studies carried out in other connective tissues suggested that cellular contraction, especially that provided by myofibroblasts may also play a role in controlling the biomechanical properties of a tissue. As the sclera contains a constant population of myofibroblasts (Poukens., et al) and the fact that the sclera is under constant, yet varying mechanical tension principally due to intraocular pressure, this study aimed to identify the role of cellular strain in mediating matrix contraction and differentiation of scleral myofibroblasts.

The first experiment aimed to identify the role of strain (intrinsic and applied) in mediating the contraction of a scleral cell-populated matrix. A cell density-dependent increase in contraction was observed in matrices maintained under intrinsic strain for 5 days, while a rapid increase in contraction was observed in matrices exposed to external strain until 24 hours, after which the contraction curve plateaued. The results of this study highlighted the contractile properties of scleral cells, demonstrating that these scleral cells are capable of rapid contractile properties in response to external strain. The second experiment aimed to identify whether this increase in matrix contraction was due
to myofibroblast differentiation. A cell density-dependent increase in the population of myofibroblasts, expressing α-SMA and containing stress fibres, was observed in matrices maintained under intrinsic strain for 5 days. On the other hand, such a differentiation of myofibroblasts was observed only until 6 hours in matrices exposed to external strain, while these α-SMA stained cells became smaller and assumed a circular morphology with smaller nuclei from 24 hours onwards. The results of this study demonstrate that the rapid matrix contractions achieved in the first experiment are mediated by the differentiation of scleral myofibroblasts, and their aberrant morphology after 24 hours underpins the lack of any further significant contraction.

4.1 Role of myofibroblasts in tissue biomechanics

The results of this study, which demonstrated a role of scleral myofibroblasts in mediating rapid contractions of an in vitro matrix is consistent with studies carried out in other connective tissues, which demonstrated the importance of these cells in contracting the in vivo granulation tissues of wounds in vivo (Darby et al., 1990, Hinz et al., 2001b). A study by Hinz et al demonstrated that application of mechanical tension to granulation tissues enhances the differentiation of myofibroblasts, which is directly proportional to the rate of wound contraction. Furthermore, disappearance of myofibroblasts and decrease in tissue contractility was observed on removal of mechanical tension (Hinz et al., 2001b), such a finding is consistent with the disappearance of myofibroblasts containing stress fibres after mediating initial matrix contraction suggesting a possible removal of strain could mediate the appearance of pyknotic cells. In addition to mediating matrix contractions, a strain-mediated myofibroblast differentiation also contributes to the biomechanical properties of a tissue.
such as increasing its mechanical strength (Garvin et al., 2003). The importance of matrix strength will be discussed later in this section.

The importance of myofibroblasts in contributing to the biomechanical properties of the sclera is yet to be fully elucidated. In order to understand the role of scleral myofibroblasts, future experiments will aim to measure the scleral biomechanical properties (creep rate and elasticity) after exposing scleral strips to specific agonist (such as endothelin-1 and TGF-β) and antagonist (such as Y27632, Rho kinase inhibitor and H-7, broad spectrum kinase inhibitor) of myofibroblast-mediated contraction. Once the biomechanical properties have been determined, these scleral strips would be stained for α-SMA expression. If myofibroblasts do contribute to sclera biomechanical properties, it is expected that the addition of agonists will increase the contraction of the scleral tissue and limit scleral elasticity and creep rate, whereas addition of antagonist will increase scleral creep rate and elasticity.

4.2 Role of myofibroblasts in eye growth

Studies have shown that the human eye, which is predominantly hyperopic at birth, undergoes an active regulation of its axial length with a corresponding change in the refractive components of the eye to reach emmetropization, which occurs between 6 to 8 years of age (Cook & Glassock, 1951, Kempf, et al., 1928). Even though Sorsby et al demonstrated that the human eyes are slightly hyperopic and does not necessarily reach emmetropization, the amount of hyperopia was minimal (Sorsby, et al., 1961). The results of this study, which demonstrated a rapid increase in the differentiation of myofibroblasts on application of strain and a rapid initial increase in matrix contraction tend to suggest that myofibroblasts in the sclera may possible play a role in contributing
to the biomechanical properties of the sclera and as such may be involved in the determination of eye size. However, a study by Poukens et al contradicts the above argument, since they demonstrated that scleral myofibroblasts first appear in humans between 17 months and 4 years of age and increase with age (Poukens et al., 1998). In addition, the above study demonstrated an increase in the population of myofibroblasts in the adult sclera as opposed to those observed during childhood. Such a finding suggests a possible role of these contractile cells in decelerating the eye growth in adults and preventing any abnormal increases in eye size in the adolescent and adult stages of life.

In addition to the increased population of myofibroblasts in the adult sclera, there is an increase in the biomechanical strength of the sclera, which is also likely to decelerate the rate of eye growth in adults. We already know that scleral collagens, which are smaller and scarcer during early stages of scleral development (6th week) increase in size and population towards adulthood and ultimately results in an adult sclera that is densely packed with collagen (Foster & Sainz de la Maza, 1994). It is possible that the increase in sclera myofibroblasts with age also results in an altered matrix, which is significantly stiffer than that found in younger eyes. Evidence from studies carried out in other connective tissues has shown that the presence of myofibroblasts does result in a matrix which is significantly stronger (Hinz et al., 2007)

Hence, based on the above discussion future experiments would be directed at identifying a developmental increase in the population of scleral myofibroblasts and demonstrating their importance in controlling ocular biometrics in an animal model of eye growth. In the tree shrew, developmental stages such as birth, eye opening, 7 and 20 days of visual experience, 3 months and 1 year of age would be useful to identify the
role of myofibroblasts in eye growth since these developmental times correspond to the rapid (0 to 20 days), slower (20 days to 3 months) and limited (1 year) eye growth phases in tree shrews (Norton & McBrien, 1992). Ocular biometrics and refractive error would be measured at the above-mentioned developmental stages and posterior eyecup of one eye could be fixed at each time point for assessing the differentiation of myofibroblasts using immunocytochemical studies. Furthermore, the posterior sclera of other eye could be used to identify α-SMA expression using gene expression analysis. If myofibroblasts are important in the latter stages of eye growth it is expected that the population of myofibroblasts will increase with age, characterised by increased α-SMA expression and a corresponding decrease in the rate of eye growth.

4.3 Ability of myofibroblasts to respond to rapid changes in the intraocular pressure

In addition to the normal scleral remodelling during eye growth, the sclera must alter its biomechanical properties quite rapidly during periods of rapid fluctuations in IOP in order to maintain the ocular integrity. Extensive research has been carried out to identify the variations in IOP within a day and these studies recorded higher IOP values after sleep, which decreases towards midnight. Back in 1964, a study by Katavisto demonstrated the peak IOP in non-glaucomatous individuals at 8am and lowest at midnight and demonstrated a diurnal variation of 3.17mmHg (Katavisto, 1964). In addition to diurnal variations, a study by Cooper et al demonstrated that rapid eye movements also causes a rapid fluctuations in IOP up to 10mmHg (Cooper, et al., 1979). The ability of the sclera to withstand such rapid ocular expansive forces and thus maintain ocular integrity suggests a possible role of scleral cells, especially
myofibroblasts, in contributing to the scleral contractile properties. In support of the above argument, the results of the current study demonstrate that application of external strain on the scleral cells results in rapid ($\leq$ 1 hour) differentiation of myofibroblasts, which facilitates the rapid contraction of the surrounding matrix. Phillips and McBrien also suggested a role for sclera myofibroblasts in regulating eye size after rapid changes in IOP, when they observed that tree shrew eyes resisted ocular elongation in response to increased IOP (100mmHg for one hour) (Phillips & McBrien, 2004). This rapid response argues against scleral matrix changes. While the current study only looked at time points greater than 1 hour after the application of strain, other studies have detailed more rapid alterations in myofibroblasts in response to changes in the matrix environment. A study by Costa et al demonstrated that application of external stress on the human aortic endothelial cells resulted in rapid contraction of the surrounding substrate and further investigation revealed the presence of the highly contractile actin stress fibres within 15 seconds of substrate contraction and these fibres exhibited a thin and disorganised morphology. Within one minute of stress-mediated substrate contraction, these stress fibres assumed a thick and straight morphology as that observed in vivo, suggesting that rapid remodelling of the cell’s actin stress fibres may play an important role in contracting the surrounding substrate (Costa et al., 2002).

In order to demonstrate the role of these scleral myofibroblasts to rapidly respond to alterations in IOP, future experiments could investigate earlier time points using the Flexcell system used in the current study. The applied strain could be raised to approximate the changes observed in IOP (due to eye movements for example) and the cell-populated gels could be fixed in situ after 1, 5, 10 minutes. After fixation, the expression of scleral myofibroblasts and changes in $\alpha$-SMA containing stress fibres
could be identified in these matrices using immunocytochemical and gene analysis techniques.

4.4 Role of myofibroblasts in myopia development

Myopia is characterised by an abnormal increase in eye size, resulting from a thinner and weaker sclera that could not withstand ocular expansive forces. As already mentioned the rate of eye growth is higher during childhood and reduces in adults, which is consistent with an increased population of myofibroblasts in adults that may decelerate the rate of growth using their contractile properties. The above argument is consistent with the results of this study, which demonstrated that an increase in the population of myofibroblasts within a matrix results in a highly contractile scleral matrix. However, the current study investigated only one modulator of myofibroblast differentiation, that being strain. Differentiation of myofibroblasts is also mediated by cytokines such as TGF-β (Jobling et al., 2009, Mustoe et al., 1987). Highlighting the complexity of the factors involved in modulating myofibroblasts, a study by Jobling et al. demonstrated that while a reduction in TGF-β isoforms resulted in decreased expression of α-SMA, the application of external strain reverses this effect leading to an upregulation in α-SMA, suggesting that strain and TGF-β may act in opposing manners during the development of myopia (Jobling et al., 2009).

In order to identify the role of scleral myofibroblasts in the development of myopia, future experiments could induce myopia in tree shrews and in a subset of eyes developing myopia, a retrobulbar injection of a myofibroblast contraction antagonist could be delivered. Over the duration of myopia development, ocular biometrics and refractive errors could be monitored and the expression of α-SMA and other matrix components could be subsequently investigated. If sclera myofibroblasts were involved
in maintaining normal eye growth, it would be expected that the addition of the antagonist would increase myopia development and an altered synthesis of matrix components will be observed in these eyes.

4.5 Importance of the current study towards treatment of myopia

The strain-mediated myofibroblast differentiation and contraction of the surrounding matrix observed in the current study has been suggested to increase the mechanical strength of an extracellular matrix in various connective tissues by possible matrix remodelling (Hinz et al., 2007, Huang, et al., 1993) and/or collagen cross-linking (Huang et al., 1993, Wakatsuki, et al., 2000). Even though an initial increase in myofibroblast differentiation was observed in the scleral cell-populated matrices exposed to external strain, these matrices were characterised by the presence of pyknotic cells from 24 hours. Such a finding suggests that owing to matrix remodelling, these scleral myofibroblasts no longer experience the imposed strain and underwent apoptosis. Previous studies have shown that myofibroblasts undergo apoptosis after release of mechanical strain (Grinnell et al., 1999, Varedi, et al., 2000).

Recent treatment methods of controlling myopia development and progression have been aimed at strengthening the sclera predominantly by mechanisms such as collagen cross-linking. A study by Wollensak et al demonstrated that even though cross-linking the scleral collagen significantly increased the stiffness of the posterior scleral strips, this study reported loss of scleral cells via apoptosis as identified by the presence of pyknotic cells. The authors suggest that increase in scleral stiffness is accompanied by the compensation of scleral cells (Wollensak, et al., 2005). However, as there is very little new cell division within the sclera, and the sclera matrix is secreted via scleral
fibroblasts (and presumably myofibroblasts), the loss of the resident population of sclera myofibroblasts may have serious and long lasting effects on the continued ability of the sclera to perform its role in the eye.

4.6 Conclusion

The scleral extracellular matrix remodelling during myopia has been believed to alter its biomechanical properties and result in increased eye size. This study investigated the role of cellular strain in modulating scleral cell biomechanics and observed that fibroblasts differentiate into myofibroblasts in a stressed environment, which contracts the surrounding matrix. Application of external strain resulted in rapid contraction of the surrounding matrix ($\leq 6$ hours) accompanied by early differentiation of myofibroblast, which later disappears ($\geq 24$ hours). These highly contractile myofibroblasts could be critical in controlling the scleral biomechanics and thus eye size during eye growth and possibly during myopia development.
References


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Appendices

Appendix I	Calculation of *in vitro* scleral cell density

The estimation of in vivo scleral cell density was based on a pilot study in our lab carried out by Mr Luke Xiang-Yu Chong. Posterior sclera was isolated from tree shrews 15 days after eye opening. The isolated tissue was dehydrated in ethanol and embedded in Araldite. The scleral tissue was stained with toluidine blue and visualised under light microscopy (see figure 6.1) and the cell number was counted. Table 6-1 shows the estimated *in vivo* scleral cell density and the calculated cell density to be seeded in the *in vitro* culture system. As highlighted in section 2.2.3 due to practical limitations with the *in vitro* system, this density was not achieved.

Table I-1: Estimation of *in vivo* scleral cell density and the calculation of *in vitro* cell density that approximates the *in vivo* situation.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density per mm$^3$ in the dehydrated posterior scleral samples</td>
<td>$7.16 \times 10^4$ cells/mm$^3$</td>
</tr>
<tr>
<td>Volume of the <em>in vitro</em> collagen matrix</td>
<td>$400 \text{mm}^3$ (25mm x 4mm x 4mm)</td>
</tr>
<tr>
<td>Total number of cells required to be seeded in the <em>in vitro</em> collagen matrix to approximate the <em>in vivo</em> situation</td>
<td>$2.86 \times 10^7$ cells/mm$^3$ ($7.16 \times 10^4 \times 400$)</td>
</tr>
</tbody>
</table>
Figure I-1 Posterior sclera of tree shrew stained with DAPI. *The posterior sclera of tree shrew was isolated after 15 days of eye opening and the nuclei of the scleral cells were stained with DAPI to quantify the cell number in the posterior sclera. The above image shows a section of posterior sclera obtained at 40x magnification.*

Appendix II Calculation of the *in vitro* strain levels that approximate the *in vivo* strain

The scleral strain levels were calculated using the scleral thickness profile from the control and myopic sclera after 12 days. A study by McBrien et al demonstrated that the dehydrated scleral thickness in myopic tree shrew is ~95µm after 12 days of myopia development, as opposed to 120µm in control sclera (McBrien et al., 2001). As the dimension of the collagen matrix in the Flexcell culture system is 400mm$^3$ (25 x 4 x 4), the *in vitro* dimension of the collagen matrix was estimated to be 9.5mm$^3$ (25mm x 0.095mm x 4mm) for the myopic condition as opposed to 12mm$^3$ (25mm x 0.12mm x 4mm) for the control (normal). Assuming that the control and myopic tree shrews
experience normal IOP (15mmHg) the *in vitro* strain to be applied on the collagen matrix was calculated (Table 6-2).

**Table II-1: Estimation of amount of *in vitro* strain to be applied on the cell-populated matrices that approximates the *in vivo* scleral strain.**

<table>
<thead>
<tr>
<th></th>
<th>Control sclera</th>
<th>Myopic sclera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of the scleral tissue after 12 days of myopia</td>
<td>120µm</td>
<td>95µm</td>
</tr>
<tr>
<td>Dimension of the <em>in vitro</em> matrix based on the above thickness profile</td>
<td>12mm³ (25mm x 0.12mm x 4mm)</td>
<td>9.5mm³ (25mm x 0.095mm x 4mm)</td>
</tr>
<tr>
<td>Approximation of mechanical tension experienced by the <em>in vitro</em> matrix</td>
<td>12mm³ of sclera experiences a tension of 15mmHg, so 400mm³ of the gel will experience 500mmHg</td>
<td>9.5mm³ of sclera experiences a tension of 15mmHg, so 400mm³ of the gel will experience 631.6mmHg</td>
</tr>
<tr>
<td>Pressure experienced by the <em>in vitro</em> matrix (in kPa)</td>
<td>66.7 kPa</td>
<td>84.2 kPa</td>
</tr>
<tr>
<td>Strain experienced by the <em>in vitro</em> matrix (calculated using the manufacturer’s protocol)</td>
<td>8.5%</td>
<td>11.6%</td>
</tr>
</tbody>
</table>
Appendix III  Figure showing the scanned image of the cell-populated matrix with $1.25 \times 10^3$ cells/mm$^3$.

Figure III-1: Scanned image of the matrix with $1.25 \times 10^3$ cells/mm$^3$. The above matrices were maintained under intrinsic strain for 5 day. A decrease in thickness of the matrix could be observed over 5 days. The reduction is surface area was calculated to demonstrate a percentage increase in matrix contraction over time.
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