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IDENTIFICATION OF ACTIN BINDING PROTEINS
ASSOCIATED WITH CROSS-LINKED ACTIN NETWORKS

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Abstract.

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Glucocorticoid therapy can lead to ocular hypertension and glaucoma. The purpose of this study is to examine mechanisms contributing to increased intraocular pressure using tissue culture models of steroid-induced ocular hypertension through identification of specific actin-binding proteins associated with cross-linked actin networks (CLANs). Human trabecular meshwork (TM) cells were cultured to confluence and treated with dexamethasone or vehicle for 14 days. Total RNA was extracted for gene expression analysis to confirm steroid-induced expression of actin binding proteins in human TM cells. Western blots confirmed expression of actin binding proteins and demonstrated the specificity of selected antibodies. Fluorescence microscopy of treated TM cells showed cytoskeleton rearrangements from linear actin stress fibers to cross-linked actin networks and the position of candidate proteins in relation to CLANs. Dexamethasone treatment of TM cells altered the expression of actin-associated proteins that may be important in the formation of CLANs and increased outflow resistance.

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CHAPTER I

INTRODUCTION

Classification of Glaucoma

Glaucoma is a complex group of diseases that have in common an increase in intraocular pressure sufficient to cause degeneration of the optic disk and visual field defects or even blindness. The second leading cause of blindness worldwide, glaucoma is projected to cause bilateral blindness in 4.5 million people by the year 2010. (Quigley and Broman, 2006) Currently, an estimated 2.5 million Americans are affected by glaucoma and approximately half of those are undiagnosed cases. (American Academy of Ophthalmology, 2000) Glaucoma is classified into three broad categories according to the age of onset, the anatomy of the angle as well as the cause of glaucoma. Each of these contains sub-classifications to further characterize this complex group of diseases.

To begin, glaucomas classified by age of onset include congenital, juvenile, and adult glaucoma. Congenital glaucoma usually manifests in the first year of life. Around the seventh month of fetal development, a halt in the maturation of the angle structures causes the iris to insert into the trabecular meshwork instead of the ciliary body. Buphthalmia, elevated intraocular pressure, and optic disk cupping are the most common symptoms early in the disease process. The treatment of choice is a surgical procedure known as goniotomy (Daniel Vaughan, 1989) which involves making an incision in the anterior trabecular meshwork to increase outflow. Decades of patient histories

demonstrate this procedure is able to permanently control intraocular pressure in 70-80% of cases. (Daniel Vaughan, 1989) Juvenile glaucoma is generally used to describe open angle glaucoma diagnosed in patients 35 years or younger. This type of glaucoma is typically inherited as an autosomal dominant trait that is phenotypically homogeneous whereas adult-onset is inherited as a complex trait with various phenotypes.(Wiggs et al., 1996) Families affected by juvenile glaucoma have been useful in genetic studies to sort out which loci may be involved in the progression of this disease. (Wiggs et al., 1995)

Another classification of glaucoma described by the anatomy of the angle consists of two major classifications, closed angle glaucoma and open angle glaucoma. Closed angle glaucoma occurs when there is a sudden increase in the intraocular pressure due to a block of the anterior chamber angle. This block essentially stops aqueous outflow leading to severe pain and sudden visual loss. An acute attack of closed angle glaucoma is likely to occur in an eye with an anatomically narrow angle and is considered a medical emergency in Caucasian or African anterior segments. (Daniel Vaughan, 1989) However, in the East Asian population closed angle glaucoma is more often a chronic asymptomatic disorder due to anatomical differences in ocular structures of the anterior segment. (Rotchford, 2005) The other, most common form of primary glaucoma is open angle. Open angle glaucoma has been described as a progressive optic neuropathy characterized by decreased retinal ganglion cells and atrophy of the optic nerve head. (Distelhorst and Hughes, 2003) Slowly progressing, open-angle glaucoma often shows no symptoms until visual impairment occurs, even though the angle and drainage routes are clinically observed to be unimpeded. Although the disease is nearly always bilateral,

one eye is frequently involved earlier and more severely than the other. (Kamal and Hitchings, 1998) Approximately one third of primary open-angle glaucoma cases are considered normal tension, or having intraocular pressures less than 22 mmHg. Therefore, optic disk changes are often the most important early findings. The disk margin thins and the optic nerve head gradually becomes wider and deeper. The lamina cribrosa cells supporting the optic nerve head become more exposed which leads to the displacement of large vessels and the optic disk becomes atrophic or "cupped". (Daniel Vaughan, 1989)

Normal tension glaucoma, a controversial sub-classification of open-angle glaucoma, describes a number of conditions in which there is evidence of glaucomatous damage (cupping of the optic disk, visual field defects, etc.) with normal or low intraocular pressure. Most cases can be classified into four groups. First, cases in which some type of glaucoma, often iatrogenic, has caused lingering changes and then regressed at the conclusion of treatment. Second is diurnal variation, in which patients usually have normal pressure measurements clinically but have elevations in pressure at other times. Next are normal tension cases in which glaucomatous damage is evident despite no diurnal elevation. This may be due to a structural weakness of the lamina cribrosa or vascular insuffienciency of the optic nerve head. The final category includes any variety of miscellaneous cases of damage to the optic nerve and retina, including vascular, congenital, degenerative and other diseases, or tumors involving the optic chiasm. (Daniel Vaughan, 1989) In 2005, Quigley argued that normal tension glaucoma is an outdated and misleading term. He goes on to say "damage to retinal ganglion cells means

it is no longer a disease of elevated intraocular pressure, although higher intraocular pressure increases the risk of damage and progression." The Collaborative Normal Tension Glaucoma Study gave statistical evidence that the lowering of intraocular pressure is beneficial for those with open-angle glaucoma in both the normal and higher pressure ranges. (Quigley, 2005)

Secondary glaucoma is the final classification of glaucoma which includes pseudoexfoliation (exfoliative syndrome), pigmentary glaucoma, developmental glaucoma, and glaucoma-like symptoms due to eye trauma, hemorrhage, surgical procedures, uveitis, neovascularization of the trabecular meshwork, etc. In pseudoexfoliation glaucoma, epithelial cells produce flake-like deposits that are seen on the lens surface, ciliary processes, zonule, posterior iris surface, loose in the anterior chamber, and in the trabecular meshwork. The disease is usually found in patients over the age of 60 and cataract may eventually develop. Lens replacement has no effect on the glaucoma but miotics, timolol, and epinephrine are moderately effective. Often laser trabeculoplasty or a filtering operation is necessary to increase aqueous outflow. For this reason, some surgeons prefer to perform lens replacement surgery along with filtering operations. (Daniel Vaughan, 1989) Another form of secondary glaucoma, pigmentary glaucoma, seems to be primarily a degeneration of the pigmented epithelium of the iris and ciliary body. The pigment granules slough off the iris as a result of abrasion against the underlying zonular fibers. The pigment is carried in aqueous fluid along the convection flow pathways and becomes lodged in the trabecular meshwork thus impeding the normal outflow. A number of pedigrees of autosomal dominant inheritance

of pigmentary glaucoma have been reported, however it occurs most often in myopic males between the ages of 25-40. This type of glaucoma responds to timolol and epinephrine but the prognosis is not favorable for individuals which require filtering surgery since the source cannot be removed. (Daniel Vaughan, 1989) Finally, a form of developmental glaucoma is associated with malformation of the anterior segment. (Shields et al., 1985) Phenotypes often exhibit dominant inheritance, variable expression, and incomplete penetrance all of which point to a multifactorial etiology. Approximately half of patients with anterior segment mutations will develop glaucoma and most human genes of anterior segment dysfunction are associated with transcription factors (PAX6, FOXC1, FOXC2, PITX2, LIMX1B). (Gould et al., 2004) Although pathogenic mechanisms are not established, ocular tissues appear exceedingly sensitive to changes in activity levels of these transcription factors. (Gould et al., 2004)

Prevalence

Estimates of the annual newly diagnosed cases of open angle glaucoma in population-based studies vary widely, however most can agree that the prevalence rises steeply with age. (Wolfs et al., 2000) The frequency of glaucoma in the general population over age 40 is about 1.5%. (Daniel Vaughan, 1989) This number approximately doubles at ages 50-59 and then doubles again after age 80 years. (Wolfs et al., 2000) Also, it has been shown that there is a high prevalence of POAG in African and African American populations while Hispanic populations report fewer cases, and still fewer cases of Caucasian POAG are reported. Asian populations are more prone to develop closed

angle glaucoma and therefore are even less likely to develop POAG. (Tielsch et al., 1991a) In addition, Asians and women are disproportionately affected as compared to other patient sub-populations suffering from all forms of glaucoma (Quigley and Broman, 2006) In a European study of patients suffering from pseudoexfoliation syndrome, open angle glaucoma in one eye at baseline was correlated with a five times higher risk of bilateral progression than if there is no glaucoma in either eye at the time of baseline measurement. (Vesti and Kivel a, 2000) Together, these studies suggest there is an underlying genetic element responsible for the susceptibility of a given population to this group of diseases. (Tielsch et al., 1991b)

Risk Factors

Some of the potential risk factors for ocular hypertensive subjects converting to primary open angle glaucoma include age, cup-disc ratio, level of intraocular pressure, myopia, steroid responsiveness, systemic vascular disease, family history of glaucoma and race.(Gordon and Kass, 1999) Many studies have tried to identify the most important risk factors leading to the progression of glaucomatous optic neuropathy in an attempt to better methods of diagnosis. Although these studies have reported variable findings, elevated intraocular pressure, age, family history and cupping of the optic nerve head lead to common visual field changes. (Leske et al., 2003) Since the 1600's, elevated intraocular pressure or "firmness of the eyeball" was generally accepted as a distinct sign of ocular disease. In 1859, von Graefe, Weber, and Muller independently described optic nerve head excavation as being linked to increased intraocular pressure. Von Graefe later

went on to say that "high intraocular pressure is the essence of glaucoma". (Flammer et al., 2002) Although it is not a diagnostic factor, elevated intraocular pressure remains the most important risk factor. (Distelhorst and Hughes, 2003) The clinically accepted mean pressure is 15.5mmHg with the normal range falling between 10-21 mmHg. According to the Baltimore Eye Survey, glaucomatous eyes are 8.6 times more likely to have pressure above 22 mmHg. (Tielsch et al., 1991b) Several studies, (Anderson, 2003; Feiner and Piltz-Seymour, 2003; Lee and Wilson, 2003) including the Ocular Hypertension Treatment Study (Gordon and Kass, 1999), collected data to suggest lowering intraocular pressure by 20 percent reduces the probability of developing glaucoma by approximately half. Weak correlation between visual field loss and level of pretreatment intraocular pressure at presentation, reflects multiple interactions of risk factors that modify the response of a particular nerve at a given intraocular pressure. (Hitchings, 1997) The severity of visual field loss is not merely a function of a "snapshot of intraocular pressure alone, it is progressive and related to the duration of the insult." (Gazzard et al., 2003)

Family history of glaucoma (Distelhorst and Hughes, 2003) is correlated with 7-10 fold increase in an individual's chance of developing glaucoma if a first-degree relative has primary open angle glaucoma. (Wolfs et al., 1998) Since the probability of developing glaucoma increases with age, advanced age is also considered a risk factor. A significant increase in the incidence of ocular hypertension and glaucoma is seen in African Americans over 40 years and Caucasians over 65 years of age. (Distelhorst and Hughes, 2003) Race is another risk factor which supports the theory of an underlying

genetic element in the progression of glaucoma. African (Tielsch et al., 1991a)and Hispanic Americans (Broman et al., 2002) have a higher incidence of developing glaucoma than Caucasians. According to population studies published following The Advanced Glaucoma Intervention Study, open angle glaucoma was found to be more frequent and advanced at the time of discovery in African Americans than in Caucasians. (2001) Furthermore, publications following the Baltimore Eye Survey reported prevalence rates among African Americans ranging from 1.23% in ages 40-49 to those 80 years and older with prevalence rates of 11.26%. In contrast, their Caucasian counterparts had glaucoma prevalence rates of 0.92% in ages 40-49 and 2.16% in patents 80 and older. (Tielsch et al., 1991b)

Cupping of the optic nerve describes the appearance of the optic nerve when the viewed through the pupil, it looks like a cup seen from above and has also been described as having a bean-pot appearance. (Daniel Vaughan, 1989) With the loss of nerve fibers from glaucoma, the cup becomes progressively larger since less space is occupied by the remaining nerve fibers. A healthy optic nerve has approximately 1.2 million fibers traveling through it, so there is normally a small cup. As progressively more nerve fibers are damaged, fewer nerve fibers remain and the cup enlarges. Clinically, the amount of cupping is often described as the cup/disc ratio. This ratio calculates the extent of axonal loss by comparing the diameter of the cup to the entire diameter of the optic nerve head. Increases in cupping or nerve fiber loss indicate poorly controlled glaucoma. In primary open angle glaucoma the position of the blood vessels within the optic nerve can shift

with progressive cupping and may lead to hemorrhage. These events can be an important clue that the disease is progressing.

The role of intraocular pressure-dependent displacements of the optic nerve head tissues in the pathogenesis of glaucomatous damage is not entirely known. Several experiments with cadaver (Levy and Crapps, 1984; Zeimer and Ogura, 1989) and monkey eyes (Burgoyne et al., 1994; Coleman et al., 1991) show posterior displacement of the lamina cribrosa after acute elevations in intraocular pressure. This suggests that increased cupping in normal living eyes with acute increments of intraocular pressure is the most likely explanation for the mechanical displacement of the optic nerve head. Normally, an acute increase in intraocular pressure leads to reversible cupping. However, over time, an increased intraocular pressure could compress, rearrange, or displace the optic nerve head to increase cupping permanently. (Hernandez, 2000) The posterior bowing of the lamina cribrosa is probably responsible for part, if not most, of the changes in optic disc topography. (Azuara-Blanco et al., 1998) Jonas and Budde suggested the pathogenesis leading to diffuse damage to the optic nerve head may be differentiated from that leading to a mainly localized pattern of optic nerve fiber loss. They found patients with ocular hypertension had a more diffuse pattern of optic nerve damage with less disc bleeding than observed in their normal tension glaucoma subjects. Of the normal tension subjects, a more localized damage to the optic nerve head, neuroretinal rim notches, disc hemorrhages, and an increase in visual field loss was evident. Jonas and Budde concluded the arrangement of fiber bundles within the lamina

cribrosa may play a role in the pathogenesis of optic nerve fiber loss in normal tension glaucoma. (Jonas and Budde, 2000)

In another study, hypertonic saline injected into the episcleral vein of one eye of Brown Norway rats was followed for one to three months using electroretinography and tonopen intraocular pressure measurements twice a week. Chauhan et al.found cupping in 56% of rats with intraocular pressures elevated above 15mmHg and 65% of these same animals also had abnormal electroretinography readings. Reportedly, cupping was only evident when approximately 55% axonal loss was reached. Therefore, they concluded that structural and functional changes in the rat model are best correlated to peak intraocular pressure and not its duration. The progression of optic disc cupping can occur relatively quickly, suggesting these changes may develop after an intraocular pressure threshold has been reached. (Chauhan et al., 2002) Optic disc cupping is a consequence of a myriad of disorders, therefore, knowledge of the anatomy and vasculature of the disc is essential to the understanding of how, why, when, and what type of optic disc cupping occurs. (Piette and Sergott, 2006)

The Ocular Hypertensive Treatment Study (OHTS) generated convincing evidence that thin central corneal thickness is a strong predictive factor for the conversion from ocular hypertension to primary open angle glaucoma. Therefore, it has been speculated that a thinner central cornea may indicate structural abnormalities within the eye. From data collected during the Ocular Hypertensive Treatment Study, it was concluded that thinner central corneas are associated with normal tension glaucoma but are not correlated with the severity of visual field loss. (Sullivan-Mee et al., 2006)

Reportedly, ocular hypertensive patients with thinner central corneas have a greater risk of developing functional damage over time. (Zeppieri et al., 2005) Although there is general agreement that patients with ocular hypertension have thicker central corneas whereas normal tension glaucoma patients have thinner than average central corneas, the clinical use of corneal thickness as a diagnostic tool remains controversial. Papadia et al. suggested a thinner cornea, causing a systematic underestimation of the true intraocular pressure, could very well lead to a late diagnosis and to greater progression of damage even with well treated intraocular pressure. (Papadia et al., 2006)

Several studies (Chihara et al., 1997; Distelhorst and Hughes, 2003; Jonas et al., 2004; Mayama et al., 2002) have concluded that high myopia represents a threat to the quality of vision in advanced open angle glaucoma with high intraocular pressure but not with low intraocular pressure. Galassi et al. suggested that the decrease of pulsate ocular blood flow in myopic eyes may be caused by low scleral rigidity compared to normal eyes. A clear decrease ocular artery systolic velocity in glaucomatous eyes as well as highly myopic eyes was reported and more interestingly, glaucomatous eyes demonstrated a more reduced ocular artery systolic velocity when associated with high myopia. Although myopic eyes are associated with altered structures which could make optic nerve head more susceptible to pressure-induced damage, Galassi et al. concluded myopia was not affected by glaucoma. Therefore, Galassi et al. suggested myopia may simply serve to exacerbate ischemic ganglion cell damage in glaucomatous eyes.(Galassi et al., 1998)

Traditional diagnosis and treatments focus on high intraocular pressure, considered the most important risk factor even though all glaucoma patients do no have high intraocular pressure. Increasingly, disturbed ocular blood flow has become a risk factor of interest. Studies have demonstrated a weak positive correlation between intraocular pressure and systemic blood pressure. (Flammer et al., 2002; Grieshaber and Flammer, 2005; Tokunaga et al., 2004) Arterial hypertension is slightly more prevalent in patients with primary open-angle glaucoma. Conversely, lower blood pressure or nocturnal hypotension is a known risk factor in the development and progression of glaucomatous optic neuropathy. Tokunagen's long term study reported a lack of physiological dipping in intraocular pressure may be an independent risk factor for progression, indicating an underlying vascular dysregulation. (Tokunaga et al., 2004)

Additionally, risk factors for secondary glaucoma should be considered. Systemic diseases associated with increased risk of open angle glaucoma include diabetes (Distelhorst and Hughes, 2003; Klein et al., 2005) and systemic hypertension. (Fan et al., 2004) Blunt trauma to the eye can result in damage to anterior chamber angle, reducing aqueous outflow and producing high intraocular pressure which may not develop for months or years following the trauma event. Increases in intraocular pressure have been widely noted in patients treated with topical steroids. (Armaly, 1963; Armaly, 1966; Becker and Hahn, 1964) If an eye trauma injury is treated with topical steroids to reduce inflammation, the underlying structural or inflammatory changes in the eye may further contribute to the clinical picture of elevated intraocular pressure. (Bartlett et al., 1993)

Genetics

Since a family history of glaucoma has been shown to increase an individual's risk of also developing primary open angle glaucoma, it is natural to suspect a genetic component is responsible for the manifestation of this disease. Most common forms of glaucoma do not exhibit Mendelian inheritance, but relatives are at an increased risk. (Netland et al., 1993) More than 20 loci have been reported with only three causative genes which account for less than 5% of primary open-angle glaucoma cases. (Fan et al., 2006) There is evidence of a monogenic disorder in which a single gene mutation can lead to primary open-angle glaucoma. But glaucoma is a multifactorial disease, both genetic and environmental factors are implicated in its etiology. Only myocilin (MYOC) is established as directly causative while the roles of optineurin (OPTN) and WD repeat domain 36 (WDR36) remain unclear. (Fan et al., 2006)

Myocilin (MYOC) or trabecular meshwork induced glucocorticoid response gene (TIGR) was the first identified "glaucoma gene" mapped to chromosome 1q by linkage analysis involving large families affected with juvenile onset primary open angle glaucoma. (Alward et al., 2002; Fingert et al., 1999; Stone et al., 1997) Mutations in this gene are associated with most if not all familial cases of juvenile glaucoma as well as approximately 3% of all primary open angle glaucoma. (Fingert et al., 1999) The myocilin clone was isolated based on induction pattern of a glycoprotein in human trabecular meshwork cultures exposed to prolonged treatment with glucocorticoids. Since the induction matched the time course and dose response for glucocorticoid-induced primary open angle glaucoma, myocilin was considered a good candidate gene to further

study biological mechanism responsible for outflow obstruction in glaucoma. (Polansky et al., 1997)

Although its function is unknown, myocilin can be found intracellularly, distributed in vesicles, and secreted as a glycoprotein. (Fingert et al., 2001; Jacobson et al., 2001; Shepard et al., 2001) Found in aqueous humor, trabecular meshwork, ciliary body, and retinal ganglion cells, secreted myocilin is proposed to facilitate outflow through the trabecular meshwork and have a protective roll against stress. (Libby et al., 2005; Tamm, 2002) However, early truncating or deletion of alleles did not produce pathogenic effects and there is no glaucoma phenotype in people or mice with myocilin null alleles. (Libby et al., 2005) Since myocilin-null studies report normal aqueous outflow and over-expression of myocilin does not lead to increased intraocular pressure, it appears that wild-type myocilin is not required to maintain normal outflow or intraocular pressure. Several investigators believe mutant myocilin is necessary to develop of primary open angle glaucoma. (Jacobson et al., 2001; Lam et al., 2000; Wiggs and Vollrath, 2001) Wild-type myocilin is found in human aqueous but the mutant isoform is not. (Jacobson et al., 2001) Mutant myocilin forms insoluble aggregates that are not secreted but accumulate intracellularly. (Gobeil et al., 2004; Jacobson et al., 2001; Liu and Vollrath, 2004) These insoluble aggregates induce stress and death in trabecular meshwork cells cultured at 35°C, however at lower temperatures, mutant myocilin promotes protein folding and secretion leading to cell survival. (Liu and Vollrath, 2004) The idea that secreted myocilin accumulates in the extracellular matrix leading to blockage of outflow and increased intraocular pressure is based on the

observations that glucocorticoid treatment increases intraocular pressure and that myocilin expression and secretion is also induced by glucocorticoid treatment. In 2001, Jacobson et al. proposed secreted myocilin may be required for the maintenance of normal outflow. Since myocilin in the trabecular meshwork is induced by mechanical stretch and therefore may play a role in responding to changes in intraocular pressure, non-secreted mutant myocilin isoforms may suppress the secretion of wild-type myocilin. (Jacobson et al., 2001)

While mutant myocilin has not been found in human steroid glaucoma patients, corticosteroids have been shown to increase the expression of myocilin. (Fingert et al., 2001) Several other factors that induce myocilin in cultured human trabecular meshwork cells, in addition to glucocorticoids, include oxidative insult, TGFβ, and mechanical stretch. One hallmark of myocilin-associated juvenile glaucoma is increased intraocular pressure relatively early and high myocilin expression in the trabecular meshwork. Together, this suggests a possible function of myocilin mutations in the regulation of intraocular pressure. (Libby et al., 2005)

Optineurin is a 577 amino acid protein (assumed secreted) found in the trabecular meshwork, Sclemm's canal, ciliary epithelium, retina, and the optic nerve. Expression in neuronal and glial cells of the retina and optic nerve suggests the ability of optineurin to directly affect the survival of retinal ganglion cells. It appears to interact with proteins that regulate apoptosis and may induce TNF-α, however, few studies have truly tested the functions of optineurin. (Rezaie et al., 2002) Optineurin is the gene most often associated with normal tension glaucoma and has three identified sequence variants. It has been

reported that optineurin is responsible for 16.7% of normal tension glaucoma cases. However, optineurin mutations do not appear to be a common cause of normal or high tension primary open-angle glaucoma. (Rezaie et al., 2002) The roles have definitely not been proven for all implicated alleles, however, a 2003 report found additional evidence for the association of the optineurin sequence variations with familial normal tension glaucoma. However, "because familial normal-tension glaucoma is so rare, this change seems to be responsible for less than 0.1% of all open-angle glaucoma". (Alward, 2003) Libby and Gould have proposed one possibility, that optineurin mutations do not induce primary open-angle glaucoma unless genetic context is permissive. (Libby et al., 2005) This could explain why mutations in the optineurin gene also have been present in the control groups of patient studies.

Even less is known about WD repeat domain 36. Although four mutations have been identified, less than five percent of primary open angle glaucoma cases have been linked to these mutations.(Fan et al., 2006) Presently only a small proportion of primary open-angle glaucoma is genetically accounted for. We have little understanding of the genetics of the disease and even less of an understanding of the underlying cell biology. (Libby et al., 2005)

Ocular anterior segment dysgenesis is a heterogeneous group of maladies also known as Axenfeld's anomaly, Reiger's anomaly, Peter's anomaly, aniridia, iris hypoplasia and iridogoniodysgenesis. (Daniel Vaughan, 1989; Gould et al., 2004; Shields et al., 1985) Mutations in the same gene can cause a range of phenotypes. Developmental abnormalities of the ocular drainage structures are not always clinically

detectable since abnormal development could affect metabolism and function with out changing morphology. Drainage structures, trabecular meshwork and Schlemm's canal, are formed by a coordination of events involving induction and differentiation of three main tissue types. First, the surface ectoderm gives rise to the lens and corneal epithelium, the neural ectoderm from which the retina, iris epithelia, and ciliary epithelia develop and finally, the periocular mesenchyme which forms the ciliary muscle and trabecular meshwork. Schlemm's canal forms by remodeling vasculature and is likely also derived from the periocular mesenchyme. (Gould et al., 2004; Shields et al., 1985) Significant development of anterior chamber structures occurs in the postnatal period. This is especially true for the drainage structures that affect intraocular pressure and glaucoma. To allow aqueous drainage, the mesenchymal mass remodels to form functional trabecular meshwork. Intertrabecular spaces between beams of functioning meshwork, allow aqueous to flow to Schlemm's canal. The aqueous humor passes through endothelial wall of Schlemm's canal and the lumen of Schlemm's canal connects to the venous system through collector channels. Abnormal development of these ample structures can lead to elevated intraocular pressure and glaucoma. (Gould et al., 2004)

Signaling molecules and transcription factors have been the focus of studies whose aim is to identify the genes responsible for this group of malformations of the anterior segment. PAX6 is a transcription factor which is conserved from *Drosophila* to vertebrates and is found in distal optic vesicle, rim of the optic cup, epithelia of the iris and ciliary body, developing iris stroma, ciliary stroma, and trabecular meshwork. FOXC1 and FOXC2 are transcription factors primarily expressed in cells that have

migrated to the eye and may control development by directly or indirectly affecting the synthesis or metabolism of the extracellular matrix. PITX2 is a transcription factor expressed in the angle. PITX2 null mice have unusual corneas that do not develop endothelium or anterior chambers. LIMX1B (LIM homeodomain class transcription factor) is found throughout the periocular mesenchyme. Its absence during postnatal anterior segment development leads to altered morphogenesis of the trabecular meshwork and Schlemm's canal. CYP1B or cytochrome P450 polypeptide is an enzyme of angle development most highly expressed in the ciliary body. Malformations include hypoplastic trabecular meshwork, abnormally located basal lamina in the trabecular meshwork, and iridocorneal adhesions. Developmental malformations in some children with congenital glaucoma resemble those observed in CYP1B null mice. All of these signaling molecules and transcription factors point toward one common theme: that normal development requires cross-talk between networks of interacting pathways that have either synergistic or opposing effects. (Gould et al., 2004)

Nail-Patella Syndrome is a rare autosomal dominant disorder characterized by pleitropic developmental defects of dorsa limb structures such as nails, patella, elbows, bony outgrowths of dorsal ilium (iliac horns) associated with renal abnormalities in some families and open angle glaucoma in others. Loss-of-function mutations in LMX1B were found associated with Nail-Patella Syndrome and open angle glaucoma in three-fourths of families. The putative loss-of-function mutations observed indicate LMX1B can be added to a growing list of homeodomain genes whose haploinsufficiency leads to a variety of developmental defects in humans. (Vollrath et al., 1998)

Known glaucoma genes have been identified in families with relatively early-onset and obvious Mendelian inheritance. Due to the complexity of this group of diseases, it is difficult to link the causal genotype and phenotypic relationships associated with glaucoma. Genetic components will likely prove even more complex and difficult to unravel. (Libby et al., 2005)

Pathogenesis

There are three major regions of the eye involved in the development of glaucoma: the trabecular meshwork, the neuronal layer of retinal ganglion cells, and the optic nerve head or optic disc. In the anterior chamber, the ciliary body continuously manufactures and secretes aqueous humor to provide nutrients to the nonvascular anterior tissues. The majority of aqueous humor exits the eye through the trabecular meshwork, a reticulated network located at the junction of the cornea and iris, (Jacobson et al., 2001) where it collects in Schlemm's canal to exit the eye through venous circulation. In most forms of glaucoma, there is increased aqueous humor outflow resistance which is associated with changes in the trabecular meshwork that usually leads to increased intraocular pressure. Glaucomatous damage to the retina involves a progressive loss of retinal ganglion cells via apoptosis, thinning of the optic disc neural rim, cupping of the optic nerve head, and progressive visual field loss. (Distelhorst and Hughes, 2003) The retinal ganglion cells conduct visual information from the inner retina through the optic nerve to the visual cortex of the brain. The progressive death of retinal ganglion cells is responsible for glaucomatous visual field defects. Finally, the optic nerve head or optic

disc is the point where the unmyelinated axons of retinal ganglion cells converge and join the optic nerve. The characteristic glaucomatous cupping of the optic nerve head is due to the progressive loss of nerve fibers as well as the posterior displacement and collapse of the lamina cribrosa (excavation of the optic nerve head) through which the retinal ganglion cell axons pass to the optic nerve.

Ocular hypertension and primary open angle glaucoma is associated with elevated intraocular pressure and outflow resistance in the trabecular meshwork, located in the anterior segment. The framework of the trabecular meshwork is a series of beams, forming a three-dimensional, interconnected group of oblique fibers of collagen, elastin, fibronectin, and glycosaminoglycans. The trabecular beams are covered by trabecular cells that form an interconnecting syncytium. On the external surface of the trabecular meshwork is a thin layer of spongy tissue called the juxtacanalicular tissue, with pores finer than those of the meshwork. On the external surface of the juxtacanalicular tissue is the single layer of the endothelium of Schlemm's canal. Thus the inner wall of Schlemm's canal is the outer limit of the trabecular meshwork complex. (Robert A. Moses and William M. Hart, 1987) Since the bulk of aqueous humor must traverse the trabecular meshwork in order to exit the eye, structural changes of the trabecular meshwork can directly affect intraocular pressure. For this reason, this tissue is of particular interest to investigators trying to understand the molecular mechanisms responsible for the development of ocular hypertension and primary open angle glaucoma.

There is a progressive loss of trabecular meshwork cells that occurs normally throughout life. This decline represents a loss of approximately 43% of the trabecular cell density between the ages of 20 and 80 or a loss of 58% of cell density from birth to 81 years of age. (Alvarado et al., 1984; Alvarado et al., 1981) There are significantly fewer cells in the trabecular meshwork of aged-matched primary open angle glaucoma patients. (Alvarado et al., 1984) This decrease in the number of cells in the filtration region is due almost entirely to cell loss and a primary degeneration of the collagen and elastic fibers of the trabecular meshwork, without a significant change in the size of the tissue area. (Alvarado et al., 1981; Daniel Vaughan, 1989) Later studies proposed the structural alteration evident during aging is due to thickening and fusion of the trabecular meshwork and detachment and migration of trabecular meshwork cells from the outflow system. Activation of the migration process is associated with phagocytosis, inflammation, injury or a combination of these. Grierson et al. has shown that aqueous humor contains mitogenic factors which stimulate migration and states that "aqueous humor has a complex cocktail of constituents and all of them need not be present to either stimulate migration or be neutral." (Hogg et al., 2000) In addition to this loss of trabecular cells, there is a thickening and fusion of trabecular beams, increased elastic sheath plaques, increased deposition of fibronectin, and changes in the trabecular meshwork glycosaminoglycans.

Transforming growth factor beta (TGF-β) family members have been shown to be involved in inflammation, wound healing, accumulation of extracellular matrix, bone formation, tissue development, cellular differentiation, and tumor progression. There are

three isoforms (β1, β2, β3) each encoded by different genes. Isoforms β1 and β2 have been shown to induce migration and the production of extracellular matrix. TGF-β has direct effects on the trabecular meshwork such as the inhibition of trabecular meshwork cell proliferation. Aqueous humor levels of TGF-β2 are elevated in primary open angle glaucoma patients and it has been reported to elevate intraocular pressure in perfusion culture human anterior segments. Studies using cultured human trabecular meshwork cells have shown they induce several extracellular matrix genes and demonstrate changes in the production of enzymes pro-matrix metalloproteinase 2 (PMMP2) and plasminogen activator inhibitor-1 (PAI-1). In 2006, Fleenor et al. confirmed the ability of TGF-β2 to alter trabecular meshwork expression of fibronectin and PAI-1 and demonstrated of both forms of TGF-β are secreted in human trabecular meshwork cell culture and anterior segments in perfused organ culture models. TGF-β2 increased intraocular pressure by increasing outflow resistance with in twenty-four hours of exposure in perfusion-cultured human anterior segment model. (Fleenor et al., 2006)

Because the trabecular meshwork acts as a "biological filter", changes in its structure or function can directly affect aqueous outflow. For this reason, the trabecular meshwork is considered a key structure in the pathogenesis of primary open angle glaucoma. Changes in the microstructure of the trabecular meshwork are thought to cause a decrease in aqueous outflow leading to an increase in intraocular pressure. Potent steroid analogs such as dexamethasone induce changes in trabecular meshwork cells including an increase in the deposition of extracellular matrix, inhibition of protease activity and inhibition of phagocytosis causing a decrease in the breakdown of materials

in the meshwork, as well as changes in the organization of the cytoskeleton. (Clark, 1995b; Clark et al., 1995; Clark et al., 1994; Wordinger and Clark, 1999)Glucocorticoids have been implicated in the development of primary open angle glaucoma. Exogenously administered glucocorticoids can cause elevated intraocular pressure leading to a secondary form of glaucoma which resembles primary open angle glaucoma. The increase in intraocular pressure seen in this type of secondary glaucoma is often reversible upon removal of the glucocorticoid. (Wordinger and Clark, 1999)

The next region of the eye involved in the development of glaucoma is the ganglion cells of the neural retina. The retina develops from a primitive, pseudostratified neuroepithelium, whose cells run the full retinal thickness, reaching both inner and outer surfaces. The first mature cells to be recognizable are the ganglion cells of the retina, which form the nerve cell layer closest to the vitreous. Eventually the retina develops into its well-known layered appearance as follows: The outer nuclear layer (ONL) containing the photoreceptors; the inner nuclear layer (INL) which contains horizontal cells, bipolar cells, amacrine cells, and Muller cells; and the ganglion cell layer containing the retinal ganglion cells and some astroglial cells. (Daniel Vaughan, 1989; Spaeth, 1993) The synapses between the ONL and INL are called the outer plexiform layer (OPL) and the synapses between the INL and ganglion cell layer is known as the inner plexiform layer (IPL). The optic fibers consisting of the axons of retinal ganglion cells are unmyelinated within the eye, and become myelinated upon entering the optic nerve. It is known that photoreceptors are rich in synaptic vesicles suggesting they communicate with post-synaptic cells via a chemical transmitter. (Robert A. Moses and

William M. Hart, 1987; Spaeth, 1993) Glutamate, acetylcholine esterase, dopamine, glycine, and gamma-aminobutyric acid (GABA) have all been detected in the human retina and there is evidence of the existence of other molecules with possible transmitter action including adenosine, norepinepherine, melatonin, and serotonin. (Robert A. Moses and William M. Hart, 1987)

The initiating events of retinal ganglion cell death remain unclear. Peripheral visual damage occurs first and the disease is typically pain free with no obvious symptoms which means substantial damage can occur before diagnosis. Specific visual field defects and characteristic cupping of the optic nerve head are hallmarks of glaucoma. (Libby et al., 2005) Retinal ganglion cells are subdivided into functional groups that form independent representations of the visual image. Thus the different classes of retinal ganglion cells compromise functional parallel channels from the retina to the brain. (Spaeth, 1993) Glaucoma causes the death of many retinal ganglion cells, and it is possible that at some time during the progression of the disease it may selectively affect one or another class of ganglion cell. There is support for glaucoma-selective damage of retinal ganglion cells where large fibers in the optic nerve are damaged more or are lost earlier than small fibers. (Quigley et al., 1987) In 1999, Quigley et al. reported that the death of retinal ganglion cells occurs by apoptosis. (Quigley, 1999) The events leading up to this programmed cell death have been widely debated. Some of the common hypotheses include elevated hydrostatic pressure, glutamate excitotoxicity, neurotrophic deprivation, autoimmunity, oxidative damage, intracellular calcium toxicity, nitric oxide, and ischemia. (Tan et al., 2006a)

The cellular and molecular mechanisms of excitotoxicity are still not fully understood. It is known that excessive stimulation by excitatory neurotransmitters causes over activation of glutamate receptors. This leads to an excessive increase in intracellular calcium, which can then activate the caspase cascade leading to apoptosis. (Eric R. Kandel, 2000) Human (Honkanen 2003), monkey (Carter-Dawson 2002), and rat (Levkovitch-Verbin 2002) studies have shown neither chronic elevation of intraocular pressure nor optic nerve transsection leads to an increase intravitreal glutamate. However, reduced presence of glutamate transporters were noted in animal models of glaucoma. (Morrison et al., 2005; Quigley, 1999) Decreased uptake of glutamate may cause harmful local concentrations of glutamate which may damage retinal ganglion cells without elevating pressure. Overall, studies of glutamate toxicity in retinal ganglion cells have yielded conflicting results, leaving its role in the pathogenesis of glaucoma open for debate.

Another group of investigators believe the apoptosis of retinal ganglion cells is initiated by an immune response. Glia and immune cells have been associated with glaucomatous neurodegeneration and activated T cells are able to enter the central nervous system as part of immune surveillance. Site-specific recruitment of T cells is initially protective to remove cellular debris, damaged cells, and pathogenic agents. This intervention is thought counteract the toxicity of stress signals. (Tezel and Wax, 2004; Wax, 2000) Tezel and Wax propose the immune system plays a pathogenic role in glaucomatous optic nerve degeneration due to its ability to generate pathology cascades. Heat shock protein antibodies found in the sera of patients with glaucoma have been

identified to induce neuronal apoptosis. Heat shock protein (hsp) 27 and hsp 60 are reportedly up regulated in glaucomatous retina and optic nerve head. (Tezel and Wax, 2004) Over expression of these heat shock proteins has also been suggested to have neuroprotective role in rat retinal models. (Tezel and Wax, 2004)

optic nerve head

The final region of the eye involved in the pathogenesis of glaucoma is the optic nerve head. About one million axons pass from each eye into the optic nerve to conduct partially processed visual information from the retinal ganglion cells to the brain.(Alward, 2000) While coursing through the retinal nerve fiber layer and the optic nerve head, axons are unmyelinated, leaving the retina transparent to allow light to reach the photoreceptors. After passing through the lamina cribrosa to enter the optic nerve, the nerve fibers gain a myelin sheath which protects the axons carrying impulses to the brain. The movement of organelles and proteins along axons occurs by at least two different processes: rapid axonal transport and slow axoplasmic flow. (Robert A. Moses and William M. Hart, 1987) Rapid axonal transport is bidirectional and requires metabolic energy while slow axoplasmic flow describes the slow movement of soluble proteins from the soma toward the axon terminal to replace catabolized materials downstream. (Robert A. Moses and William M. Hart, 1987) Disruption of the axons passing through the optic nerve head has been associated with glaucomatous visual field defects for some time.

Since 1885, the mechanism of glaucomatous damage has centered on whether intraocular pressure (mechanical hypothesis) or optic nerve head blood supply (vascular

hypothesis) is responsible for axonal damage.(Flammer et al., 2002) Axonal transport is compromised at the level of the lamina cribrosa at physiologic levels of pressure and is further impaired following acute or chronic elevations. In addition, ocular blood flow is affected in different ways by chronic and acute elevations in intraocular pressure. (Burgoyne et al., 2005; Flammer et al., 2002)

The hypothesis that mechanical stresses are responsible for optic nerve head cupping is based on studies (Azuara-Blanco et al., 1998; Burgoyne et al., 2005; Burgoyne et al., 1994; Coleman et al., 1991; Spaeth, 1993; Sponsel et al., 2001; Zeimer and Ogura, 1989) of human glaucoma tissues suggesting the appearance of cupping results from posterior bowing of the lamina cribrosa and loss of nerve fibers. These studies also reported significant disorganization of the nerve head cellular structures as well as loss of the normal orderly arrangement of axon bundles, connective tissue laminar beams and supporting astrocytes. (Coleman et al., 1991) The clinical hallmarks of glaucomatous optic neuropathy include a progressive posterior displacement of the surface of the optic nerve head and progressive excavation of the lamina cribrosa region, in addition to visual field loss.

Connective tissue deformation brings about glaucomatous cupping while axonal loss triggers glaucomatous visual field loss, both in a predictable pattern. Optic nerve head hyper compliance occurs early in experimental glaucoma which suggests that damage to the connective tissues occurs early in the pathophysiology of pressure-related damage. The magnitude and distribution of pressure-related stress within a given optic nerve head for a given level of intraocular pressure is primarily influenced by the three-dimensional

anatomy of the eye. The mechanical response of the load-bearing tissues to a given level of intraocular pressure-related stress is determined by their material properties. Tensile stiffness and strength are hypothesized to be manifestations of cross-linking and fiber diameter, as well as interactions of the extracellular matrix. Once load-bearing tissues are damaged, further damage to the connective tissues themselves and damage to the adjacent axons and living cells can be expected to occur, even at low levels of intraocular pressure. (Burgoyne et al., 2005) The principal site of intraocular pressure-related damage to the retinal cell axons is within the lamina cribrosa. The axons within the lamina cribrosa are likely to be more vulnerable to direct effects of pressure than are the cells within the retina. (Burgoyne et al., 2005)

Although studies have yielded partially conflicting results regarding the vascular hypothesis, there is increasing experimental evidence from animal studies and clinical trials to demonstrate the death of retinal ganglion cells and optic nerve head atrophy due to ocular blood flow abnormalities are involved in glaucoma pathogenesis. (Flammer et al., 2002; Fuchsjager-Mayrl et al., 2004) Blood flow decreases as the glaucomatous damage increases in both early and late stages of glaucoma, although it is more pronounced in progressive cases. Indirect signs of compromised flow include changes in conjunctival capillaries, local vasoconstriction of the retina, increased prevalence of optic nerve head hemorrhages, increased prevalence of venous thrombosis, and increased gliosis-like alterations. The evidence to assume orbital blood flow decreases in glaucoma patients includes an increased resistance to flow, a decreased perfusion pressure, and an increase in blood viscosity. (Flammer et al., 2002) A study using fluorescein

angiography to visualize ocular blood flow identified non perfused areas and filling defects around the optic cup. In addition, an intraocular pressure higher than 25mmHg was found to be indicative of a significant decrease in ocular blood flow and a decreased pulse volume than the lower risk ocular hypertensive or normal-tensive groups. In support of the vascular hypothesis, pulsate ocular blood flow was reportedly decreased in conditions associated with choroidal atrophy such as high myopia and retinitis pigmentosa. (Kerr et al., 2003)

In 2004, study of 140 patients with either ocular hypertension or primary open-angle glaucoma, the relation between ocular hemodynamic variables and systemic blood pressure was published by Fuchsjager-Mayrl et al. Few significant differences in age, gender or systemic hemodynamics were observed between the ocular hypertension and primary open-angle glaucoma groups and lower mean perfusion pressures were reported than in normal tensive patients. Since no significant differences in ocular blood flow parameters between patients with primary open-angle glaucoma and ocular hypertension were observed, Fuchsjager-Mayrl et al. have suggested that vascular abnormalities are probably an early event in glaucoma. (Fuchsjager-Mayrl et al., 2004) Also, investigators of the Beaver Dam Eye Study reported changes in intraocular pressure as being directly and significantly associated with changes in systemic blood pressure. Klein et al. claims variations in systemic blood pressure lead to small changes in the formation of aqueous humor possibly related to increased capillary pressure in the ciliary body and therefore may play a role in elevated intraocular pressure as well.(Klein et al., 2005)

Increased resistance to aqueous drainage through the trabecular meshwork is seen in most cases of glaucoma. (Libby et al., 2005) Intraocular pressure is affected by aqueous humor production in the ciliary body and drainage through the trabecular meshwork and uveoscleral pathways. In the normal pathway through the trabecular meshwork, aqueous humor is secreted from the ciliary body and travels around the iris to drain through the trabecular meshwork and into Schlemm's canal to exit the eye via venous circulation. A smaller percentage of aqueous humor normally exits the eye through the uveoscleral path. Typical production and drainage of aqueous humor is important to provide nutrients to structures in the anterior of the eye that do not have access to the vasculature. In addition to the lens and cornea, the aqueous must be transparent and free of debris to maintain the proper phototransmission to allow for useful vision. Aqueous humor outflow is a key determinant of IOP. Outflow facility can be enhanced by altering the actin cytoskeleton and cell-cell adhesions of cells within the trabecular meshwork. (Tan et al., 2006b) Defective uveoscleral drainage also may contribute to increased intraocular pressure in some glaucoma patients; however, most investigators concentrate on the flow of aqueous humor through the trabecular meshwork since it compromises the majority of the outflow path.

Alvarado and Polansky developed a method of trabecular meshwork cell culture grown on filters to allow measurement of the hydraulic conductivity of these cells following a particular type of treatment. In 1988, cytochalasin B was reported to increase flow through trabecular meshwork cells cultured on filters within 30 minutes. (Perkins et al., 1988) Morphological studies of trabecular meshwork cells grown on

filters and treated with dexamethasone for two weeks showed an increase in the number of tight junctions while the number of adherens and gap junctions decreased.(Underwood et al., 1999) Alvarado et al. reported a three to five fold decrease in hydraulic flow in dexamethasone-treated trabecular meshwork cells (Underwood et al., 1999) while beta-adrenergic agonists increased flow. (Alvarado et al., 2004)

Borras has been investigating the possibility that the trabecular meshwork could undergo calcification with age. In support of this theory, genes with high expression levels in cultured human trabecular meshwork cells such as matrix GLA have been shown to inhibit calcification in cartilage and arterial vessels. Borras et al. found that matrix GLA protein in cultured trabecular meshwork cells were able to inhibit alkaline phosphatase activity. (Xue et al., 2006) Alkaline phosphatase activity was used as a marker for osteogenic differentiation and calcification, so the presence of an inhibitor of this marker along with the increase in calcium found as the cells aged, suggests the trabecular meshwork may be susceptible to the calcification process. A less dynamic outflow pathway, due to calcification, would lead to an increase in intraocular pressure.(Xue et al., 2006) The indirect evidence in support of the calcification of the trabecular meshwork with age could also explain the delayed on set of the glaucoma.

In 1994, Clark et al. reported a cytoskeletal rearrangement in cultured trabecular meshwork cells treated with 100n M dexamethasone. Following 7-14 days of treatment, 30-70% of cells lines studied had a conversion from the normal microfilament stress fibers to "three-dimensional geodesic-domes" or cross-linked actin networks (CLANs). (Clark, 1995b; Clark et al., 1995) Additionally, the induction of CLANs appears to be

limited to trabecular meshwork and lamina cribrosa cells in a time and dose dependent manner. (Wordinger and Clark, 1999) Similar to the time course of steroid-induced glaucoma, the appearance of CLANs is delayed and is reversible after the cessation of dexamethasone treatment in both cell culture and perfusion culture models. Since a higher percentage of CLANs were observed in untreated glaucomatous cell lines as well as normal and glaucomatous cell lines treated with dexamethasone, Clark et al. propose these structures are related to an increased resistance to aqueous outflow in trabecular meshwork cells. (Clark, 1995a; Clark, 1995b; Clark et al., 1995; Clark, 2005; Wordinger and Clark, 1999)

Treatments

Two categories of treatment options are available for ocular hypertension and glaucoma patients, medication or surgery. Currently, topical eye drops are the most common with systemic medications prescribed less frequently. Although, beta-blockers (timolol) have been the initial medication prescribed in the recent past, now prostaglandin analogs are more frequently the first-line medication prescribed. Used as systemic treatment for other indications, beta-blockers were accidentally found to lower intraocular pressure by reducing aqueous humor production in ciliary body. Since these medications depress myocardial contractility and excitability, their main side effect is bradycardia. (Katzung, 2001) Alpha adrenergic agonists (dipivefrin) act through a complex series of effects on aqueous inflow and outflow. Alpha- adrenergic stimulation decreased aqueous production but this is short lived and does not contribute much to

epinephrine's long term pressure reduction. Instead, an increase both conventional and uveoscleral outflow occurs due to changes in the metabolism of glycosaminoglycans in the trabecular meshwork. (Alward, 2000) Alpha $_2$ selective agonists (bromonidine) lower intraocular pressure by decreasing the amount of aqueous produced in the ciliary body and may also increase trabecular outflow.

Cholinergic agonists (pilocarpine) enhance or simulate the action of acetyl choline at the neuromuscular junction causing contraction of the longitudinal muscle of the ciliary body, which is attached to the scleral spur. Pulling on the scleral spur opens the trabecular meshwork and increases conventional aqueous outflow. (Alward, 2000) Carbonic anhydrase inhibitors (acetazolamide) are long-standing diuretics now used almost exclusively to treat primary open angle glaucoma. Inhibition of the enzyme, carbonic anhydrase, leads to increased excretion of extracellular bi-carbonate and water to reduce the availability of aqueous fluid components in the ciliary body. (Katzung, 2001; Pollack, 2003) Prostaglandin analogues (lantanoprost) increase uveoscleral outflow through the ciliary body and iris root, either by relaxing muscles of the ciliary body or by the loss of extracellular matrix from between the ciliary body muscle bundles allowing aqueous humor to more readily exit.(Alward, 2000) A frequent side effect of prostaglandin analogues is increased pigmentation in the iris. Lastly, systemic osmotic diuretics are most often used to treat narrow-angle glaucoma before surgery or during eye examinations in the presence of corneal edema. (Pollack, 2003) Hyperosmotic agents (glycerol, mannitol, isosorbide) act primarily by creating an osmotic gradient between the

blood and intraocular contents. The high osmolality of the blood draws fluid from the eye into the blood stream.(Alward, 2000)

These medications are non-invasive and effectively lower intraocular pressure in most patients. However, the cost and poor patient compliance are among the downfalls of topical ocular medications. Because a patient must generally remain on a treatment regimen for the remainder of their life, and with time, it is often necessary to change or include additional medications, the cost of glaucoma treatment can be great. Studies have also demonstrated as the number of doses and/or medications prescribed increases so does the number of missed treatments. (Anderson, 2003; Gordon and Kass, 1999; Leske et al., 2003; Lichter, 2003; Tielsch et al., 1991a; Tielsch et al., 1991b) Missed treatments lead to poor disease management and frequently, a resurgence of intraocular pressure. Therefore, combination medications which reduce the number of applications a patient must remember are becoming increasingly prescribed.

One avenue used to remove the issue of poor patient compliance with topical anti-glaucoma medications as well as the progressive loss of efficacy, are surgical procedures to lower intraocular pressure. Using either traditional surgical tools or focused lasers, clinicians are able to lower intraocular pressure by altering structures in the anterior chamber. To begin, argon laser trabeculoplasty causes thinning and scarring of the trabecular meshwork at the site where the laser energy is absorbed. (Alward, 2000) Lower intraocular pressure occurs either by causing focal contraction of the trabecular meshwork beams and widening of adjacent intertrabecular spaces, or by altering trabecular meshwork cell function. (Pollack, 2003) Initially, it was speculated that a

mechanical tightening of the trabecular ring increased outflow; however, evidence also exists of increased cell division, increased phagocytosis, and alteration of the extracellular matrix, all of which improve trabecular meshwork function. (Alward, 2000) This non-invasive procedure reduces the risk of infection and can increase aqueous outflow up to fifty percent. However, the major disadvantage of argon laser trabeculoplasty is the loss of efficacy, estimated to be ten percent per year following the procedure. (Pollack, 2003)

Next, invasive trabeculectomy filtration surgery involves surgically removing a small piece of tissue where the cornea connects to the sclera and creating a flap to allow fluid to escape the anterior chamber, called a bleb. Fluid can then flow out onto the surface of the eye through the bleb to be absorbed by the conjunctiva. Intraoperative and postoperative metabolites have greatly improved the results of filtration surgery in patients at high risk of failure. 5-Fluorouracil and mitomycin-C are common antimetaboltes that reduce inflammation following surgery by suppressing DNA synthesis in fibroblasts. (Pollack, 2003) Trabeculectomy can be a difficult and unforgiving procedure and is usually only performed on eyes with progressive glaucomatous loss that cannot be controlled with medications or laser trabeculoplasty. It is able to achieve much lower intraocular pressures than medications or trabeculoplasty but is more prone to complications.(Alward, 2000)

Another procedure, cyclophotocoagulation uses a focused laser to deliver sufficient energy to cause thermal necrosis of the ciliary epithelium and stroma. This necrosis results in a fairly prompt reduction in aqueous production, either due to loss of

the ciliary epithelium's secretory function, reduced vascular perfusion in the ciliary body, or both. Another proposed mechanism of reduced intraocular pressure following cyclophotocoagulation is increased outflow through the uveoscleral pathway. The common complications of cyclophotocoagulation include hyperemia, uveitis, and inflammation although routine analgesics are generally sufficient. (Pollack, 2003)

Finally, aqueous drainage devices can be implanted into the eye to shunt aqueous humor into the subconjuctival space. These devices are usually only an option if all other treatments have failed. Aqueous drainage devices often fail due to scarring around the exit point or migration from their intended position. (Alward, 2000) The idea of a single surgical procedure to lower intraocular pressure for an extended period of time is especially attractive for patients in developing countries without access to regular medical care. Surgery can be more cost effective and more convenient than life-long medications and patients do not have the daily burden of treating their disease. (Feiner and Piltz-Seymour, 2003) However, several studies have found variable outcomes for patients undergoing glaucoma surgery, trabeculoectomy, and trabeculoplasty. Factors such as surgeon training and experience, previous medications, and anatomical variations all are able to affect a patient's prognosis following surgery. For this reason, scores of studies have attempted to decipher the most efficacious treatment to lower intraocular pressure(Anderson, 2003; Feiner and Piltz-Seymour, 2003; Gordon and Kass, 1999; Lee and Wilson, 2003; Leske et al., 2003; Lichter, 2003) as well as what criteria to use to diagnose a patient as either ocular hypertensive or glaucomatous. (Anderson, 2003; Lichter, 2003; Wolfs et al., 2000)

Clinical procedures to diagnose primary open angle glaucoma are somewhat ambiguous. Considered the most important risk factor, intraocular pressure is frequently used to diagnose ocular hypertension. However some patients with elevated pressure do not develop cupping or glaucomatous visual field defects while, normal tension glaucoma patients develop glaucomatous symptoms at average levels of intraocular pressure. The Collaborative Normal Tension Glaucoma Study (CNTGS) found migraine, female sex, disc hemorrhage at diagnosis, and racial or genetic heritage to be predictive indicators of developing glaucoma. (Anderson, 2003) The Early Manifest Glaucoma Trial (EMGT) found progression, even in patients with controlled intraocular pressure, may be related to vascular abnormalities (Leske et al., 2003) The Baltimore Eye Survey found a higher prevalence of open angle glaucoma in African Americans and reported approximately half of patients of all races are unaware they are affected by the disease. (Tielsch et al., 1991a; Tielsch et al., 1991b) Clearly, no studies to date have been able to precisely define the criteria to diagnose glaucoma. Clinical trials and observational studies support the need to lower intraocular pressure substantially and maintain low pressure. These studies indicate the method of treatment, whether medication or surgery, is not as important as the treatment being effective and sustained. (Lichter, 2003) Primary open-angle glaucoma appears to be spectrum of diseases that reflect different susceptibilities of individuals to a given pressure level. Complex relationships will not be understood until the precise genetic and biological mechanisms of these disorders are understood. (Libby et al., 2005)

Steroid-Induced Ocular Hypertension

Corticosteroids, also known as adrenocorticosteroids, are either natural or synthetic anti-inflammatory agents modeled after cortisol. Cortisol is a steroid hormone naturally produced and released from the adrenal cortex. Adrenocorticotrophic hormone (ACTH) produced in the pituitary gland stimulates the adrenal glands to produce cortisol in response to stress, emotion, injury, or infection. Both natural and synthetic forms of corticosteroids are useful in treating a variety of conditions ranging from disorders of adrenal function to allergic reactions or arthritis. (Katzung, 2001)

Corticosteroids fall into two main classifications: glucocorticoids which effect intermediary metabolism and mineralocorticoids which possess salt-retaining properties.(Katzung, 2001) Of these, glucocorticoids are of interest due to their superior potency as therapeutic anti-inflammatory agents.(Clark, 1995a) Glucocorticoids are used as potent anti-inflammatory and immunosuppressive agents. In ocular applications, they are useful to decrease inflammation associated with conjunctivitis, uveitis, or following surgery. However, clinicians have noted that the prolonged use of either systemic or topical ocular glucocorticoids often lead to insomnia, peptic ulcers, iatrogenic Cushing's syndrome, increased intraocular pressure, and cataracts. (Katzung, 2001; Rhen and Cidlowski, 2005)

The effects of agents such as cortisol and dexamethasone are mediated through widely distributed glucocorticoid receptors. Once bound to their receptor, glucocorticoids can inhibit inflammation via three paths. First, the hormone-receptor complex shuttles to the nucleus where it binds to glucocorticoid-response elements to

regulate gene transcription. At lower levels of glucocorticoids, the hormone-receptor complex can mediate glucocorticoid-responsive genes through their influence on transcription factors. Signaling through membrane associated receptors and second messengers is the final way glucocorticoids are able to reduce the inflammatory response.(Katzung, 2001; Rhen and Cidlowski, 2005)

Normal production of endogenous steroids follows a diurnal curve similar to the ocular tension curve in most individuals. It usually takes weeks to months for glucocorticoid therapy to increase intraocular pressure, and generally after the cessation of steroid treatment, intraocular pressure returns to normal. Long term administration of systemic steroids produces a small but statistically significant increase in mean pressures and a decrease in ocular rigidity in the general population. Bernstein and Schwartz proposed the mechanism is an effect of corticosteroids on the mucopolysaccharides of the anterior chamber angle and sclera.(Bernstein and Schwartz, 1962)

An individual's response of intraocular pressure to topical corticosteroid administration is determined genetically and genes encoding steroid responsiveness of intraocular pressure and primary open angle glaucoma are considered closely related. There is clinical evidence supporting a significant correlation between intraocular pressure response to corticosteroids and many findings including primary open angle glaucoma, family history, and level of intraocular pressure. Studies (Armaly, 1963; Armaly, 1966; Becker and Ballin, 1966; Becker and Hahn, 1964; Clark, 1995a; Clark et al., 1995; Kitazawa and Horie, 1981; Miller et al., 1965; Wordinger and Clark, 1999; Zhang et al., 2005) suggest the risk of ocular hypertension is higher in steroid responsive

subjects as compared to non-responsive subjects since ocular hypertension developed in approximately 25% of steroid responders with intraocular pressure in the normal range within 10-13 years. Of the corticosteroid responsive normal tensive patients, 54% had glaucomatous visual field defects. (Kitazawa and Horie, 1981) Almost every patient with primary open angle glaucoma and visual field loss responds to steroids with greater elevations in intraocular pressure and has offspring that behave similarly. (Armaly, 1966; Becker and Ballin, 1966; Becker and Hahn, 1964)

Two to eight weeks of treatment with glucocorticoids is sufficient to raise intraocular pressure in some patients. However, this increase is reversible within one to three weeks after cessation of the glucocorticoid treatment. (Bartlett et al., 1993) In a study consisting of relatives of glaucoma patients, increased intraocular pressure and decreased outflow was reported in varying degrees in 80% of subjects in their second or third week of topical ocular glucocorticoid drops. Dexamethasone and prednisolone applied to one eye for six to eight weeks caused an increase in intraocular pressure in all of the treated eyes but none of the contralateral control eyes. The hypertensive effect of dexamethasone is evident at the end of the first week of treatment. (Armaly, 1963) Thickening of the corneas was noted in several subjects, explained by alterations of the corneal endothelium function and might also be associated with cells of the trabecular meshwork. (Miller et al., 1965)

For nearly half a century, investigators have explored the properties of glucocorticoids applied to ocular tissues. The ocular hypertensive effects of glucocorticoids are now a commonly accepted fact. In the mid-1960's, Armaly and

Becker reported approximately 30%-40% could be classified as "steroid responders" and that roughly 5% of the population are considered "high responders". (Armaly, 1963; Armaly, 1966; Becker and Ballin, 1966; Becker and Hahn, 1964) Both systemic and topical ocular routes are able to trigger this "steroid response", however elevated intraocular pressure appears to require longer treatment and involve fewer patients following administration of systemic glucocorticoids as compared to topical ocular treatments.(Clark, 1995a)

"Glucocorticoid-induced glaucoma is an iatrogenic secondary form of open angle glaucoma with very similar clinical characteristics including elevated pressure, a normal-appearing anterior chamber angle, glaucomatous damage to the optic nerve, and glaucomatous visual field loss." (Clark, 1995a) It is possible that previous treatment with glucocorticoids can lead to clinical features of normal tension glaucoma since hypertension is reversible with the removal of glucocorticoids, but the damage to the optic nerve remains. (Clark, 1995a) Ocular hypertension due to the topical ocular administration of a potent glucocorticoid is progressive, generally occurring over the course of weeks or longer. (Clark, 1995a) Normally the ocular hypertension is reversible following the cessation of glucocorticoid treatment, although cases of irreversible pressure elevation have been reported. (Armaly, 1963; Clark, 1995a; Wordinger and Clark, 1999) The length of treatment may be one factor in determining the reversibility of the glucocorticoid-induced ocular hypertension. (Clark, 1995a)

The trabecular meshwork consists of arrays of collagen beams covered by trabecular cells, with extracellular matrix material occupying the spaces between beams.

The outermost juxtacanalicular or cribriform region has no collagenous beams but rather several cell layers in loose extracellular matrix. The adjacent Schlemm's canal is a continuous endothelium-lined channel that drains aqueous to the general venous circulation. With age, resistance to aqueous flow increases, trabecular meshwork cells decrease, and alterations of the extracellular matrix in the trabecular meshwork and the juxtacanalicular region occur. Glaucomatous eyes exhibit fewer trabecular meshwork cells and abnormal-appearing juxtacanalicular extracellular matrix compared to age-matched non-glaucomatous eyes suggesting these cells are critical in the regulation of outflow resistance.(Tian et al., 2000) In the trabecular meshwork, cell number diminishes with age and in primary open-angle glaucoma. (Tan et al., 2006b) Glaucomatous trabecular meshwork cells stain more frequently for senescence-associated-beta-galactosidase (a marker of cell aging) than non-glaucomatous cells. Signs of aging are more marked in older eyes, in which cells have decreased viability, decreased proliferative capacity and less ability to reach confluence after multiple rounds of replication. (Tan et al., 2006b) Biochemical markers of proteosome degradation, protein oxidation and autofluorescence reflecting reduced proteosome activity are also higher with age. (Tan et al., 2006b) These changes may reflect decreased trabecular meshwork cellular function, viability and ability to withstand oxidative stress. Plasma levels of oxidant scavengers glutathione and reduced glutathione are significantly lower in individuals with primary open-angle glaucoma compared with controls."(Tan et al., 2006b)

Many steroid-induced changes in the trabecular meshwork appear to be similar to glaucomatous changes including effects on the extracellular matrix, cytoskeleton, and gene expression. Parallel accounts of increased elastin and fibronectin deposition have been reported in patients with primary open angle glaucoma. Cytoskeletal rearrangements from normal stress fibers to cross-linked actin networks (Clark, 1995b; Clark et al., 1994) are induced by treatment with glucocorticoids and found in untreated glaucomatous trabecular meshwork cells. Finally, changes in expression of genes such as myocilin have been reported in both glaucomatous and glucocorticoid-treated tissues. (Fingert et al., 2001; Jacobson et al., 2001)

An enlargement of nuclei and increased DNA content in trabecular meshwork cells is thought to lead to altered gene expression in both steroid-induced and open angle glaucoma. (Partridge et al., 1989; Tripathi et al., 1989) Exposure of cultured human trabecular meshwork cells to glucocorticoids causes a progressive reorganization in the microfilament structure that appears to alter trabecular meshwork cell function. The cytoskeleton plays a major role in regulating a variety of cellular functions and characteristics including: motility, contractility, proliferation, exocytosis, phagocytosis, cell shape, cell adhesion, and protein synthesis. (Clark, 1995a; Clark et al., 1996; Clark, 1995b; Wordinger and Clark, 1999) Glucocorticoids have a variety of effects on cultured trabecular meshwork cells, causing changes in the extracellular matrix, extracellular proteinase expression, protein expression, and trabecular meshwork cell function. Cross-linked actin networks (Clark, 1995b; Clark et al., 1994) may be responsible for the development of ocular hypertension through a variety of mechanisms such as decreasing

the pore size of the trabecular meshwork, decreasing hydraulic conductivity, altering the interaction between the trabecular meshwork cell and its extracellular matrix, or inhibiting essential trabecular meshwork functions. (Clark, 1995a; Tripathi et al., 1989)

Table 1: Glucocorticoid effects on the Trabecular Meshwork (Wordinger and Clark, 1999)

Location	Glucocorticoid Effects
Trabecular Meshwork	increased cell size decreased phagocytosis decreased cell migration decreased hydraulic flow
Extracellular Matrix	increased fusion vesicles increased chondroitin sulfate decreased hyaluronate progressive increase in fibronectin increased collagen and elastin
Cell Junctions	Re-alignment of gap junction complexes increased expression of tight junction proteins
Gene Expression	increased myocilin
Protein Expression	inhibits several metalloproteinases increased elastin, increased alpha actinin and vinculin
Cytoskeleton	formation of cross-lined actin networks (CLANs) increased microtubule tangles reduced stress fibers

Cytoskeleton

The cytoskeleton is a network of protein filaments extending throughout the cytoplasm of all eukaryotic cells. It provides the structural framework of the cell, determining the shape and size of the cell as well as the organization of the cytoplasm. The cytoskeleton is responsible movements of the entire cell and also the internal transport of organelles. It is much less rigid and permanent than its name implies, rather it is a dynamic, continually reorganized structure allowing cells to move, change shape, migrate, engulf particles, and divide. The cytoskeleton is composed of three types of

protein filaments: actin filaments, intermediate filaments, and microtubules. These filaments are held together and linked to subcellular organelles and the plasma membrane by a variety of binding proteins.

In most cells, the major cytoskeletal protein is actin which polymerizes to form actin filaments. These actin filaments can be organized into higher-order structures to form bundles or three-dimensional networks. Individual actin filaments are assembled into two general types of structures, actin bundles and actin networks. Actin bundles are cross-linked into tightly packed parallel arrays while actin networks are cross-linked into orthogonal arrays to form a three-dimensional structure. All of the actin binding proteins involved in cross-linking contain at least two domains that bind actin, allowing them to bind, and cross-link two different actin filaments. There are two structurally and functionally distinct types of actin-binding proteins each with different linking properties and numbers of spacer sequences which are responsible for their distinct structures. Proteins that link actin filaments into bundles are usually small rigid proteins that force the filaments to align closely with one another. In contrast, proteins that cross-link actin networks tend to be large, flexible proteins that can link perpendicular filaments.

Another cytoskeletal component, intermediate filaments are not directly involved in cellular movements but appear to play a basic structural role by providing mechanical strength. Intermediate filaments are composed of a variety of proteins that are expressed in different cell types. More than 50 intermediate filament proteins have been identified and classified into six groups (I-VI) based on the similarities in amino acid sequences. Intermediate filaments assemble by first forming dimers which then interact to form

tetramers and so on until a filament results. Intermediate filaments are generally more stable than actin filaments or microtubules and do not exhibit dynamic behavior, but are frequently modified by phosphorylation during processes such as cellular division.

The final components of the cytoskeleton are rigid, hollow tubes called microtubules. These dynamic structures function both to determine cell shape and in cellular movements including locomotion, organelle transport and separation of chromosomes during mitosis. Unlike intermediate filaments, microtubules are composed of a single globular protein, tubulin. Tubulin dimers polymerize to form linear filaments around a hollow core. Microtubules, like actin filaments, have polar end to allow for directional movement along their filaments. (Hausman, 2004)

Microfilaments, microtubules, and intermediates make up cytoskeleton systems that differ in their functions and so differ in their involvement in the outflow pathway. Microfilaments assemble into complex bundles and are responsible for cell adhesion, motility, organelle trafficking, and adhesion-mediated signal transduction. They are found in trabecular cells on collagen beams and in the inner wall of Schlemm's canal and are regulated by levels of extracellular calcium, specific G-proteins, mechanical tension, as well as hydrostatic pressure. Microtubules are found at higher density near the nucleus and extend towards the cell's periphery. They are able to affect aqueous outflow by controlling the trafficking of vesicles and organelles driven by motor proteins such as kinesins and dyneins in addition to changing extracellular matrix stability. Intermediate filaments make up the final cytoskeletal system and are composed of a heterogeneous mix of proteins including vimentin. Although it is known that intermediate filaments are

associated with microtubules and are abundant in the cells of the trabecular meshwork and Schlemm's canal, their precise function remains unknown. It is clear that substantial changes to any one of these cytoskeletal systems will also dramatically affect the other two (Tian et al., 2000)

Table 2: Cytoskeletal Functions (Tian et al., 2000)

Cytoskeletal System	Main Component	Diameter	Structure	Function
Microfilaments	filamentous actin	7 nm	bundles and newtworks	cell adhesion cell motility organelle traffic signal transduction
Microtubules	tubulin	25 nm	hollow fibers driven by motor proteins (kinesins and dyneins)	directional cell motility vesicle traffic organelle traffic
Intermediate Filaments	vimentin	10 nm	webs of single filaments or filament bundles	unclear, often found along microtubules

There is evidence that acute and sustained elevated hydrostatic pressure can affect cell morphology and change adenylyl cyclase activity in cell cultures of ciliary epithelium, trabecular meshwork, retina, and lamina cribrosa cells. Wax et al. demonstrated that different cell lines from ocular tissue exhibit morphological and cellular activity responses after acute and sustained elevation of hydrostatic pressure (50 mmHg) for up to six hours.(Wax, 2000) Phalloidin staining of the cytoskeleton showed ocular cells become more rounded and the cytoskeletal structure is altered in response to elevated hydrostatic pressure. Although elevated hydrostatic pressure did not affect cell viability, cellular activity of adenylyl cyclase was increased 50-80% leading to the perturbation of the actin cytoskeleton in ocular cells in vitro. (Wax, 2000)

Much of the current knowledge on the function of different cytoskeletal networks is derived from studies using "cytoskeletal drugs" that destroy or stabilize either microfilaments or microtubules. Cytoskeletal filaments are part of an interactive network, so that affecting one system may have considerable indirect effects on the others.

Cytochalasins are a class of drugs that disrupt the actin cytoskeleton by capping the barbed ends of actin filaments, preventing their elongation. In living monkey and organ-cultured human eyes, cytochalasin decreases outflow resistance by separating the endothelial cells of the beams, the juxtacanalicular region, and the inner wall of Schlemm's canal from their neighboring cells and their surrounding extracellular matrix. (O'Brien et al., 1996) This leads to distension of the meshwork and ruptures the inner wall, enhancing fluid flow and efflux of extracellular matrix.

Ethacrynic acid (ECA) inhibits microtubule assembly in vitro and induces a rapid decrease in phosphorylation of focal adhesion components. (Tian et al., 2000) This blocks signaling pathways normally triggered by integrin-mediated adhesion. In cultured trabecular meshwork cells, ethacryinic acid causes cellular contraction, altering cell shape corresponding with changes in the staining pattern of major cytoskeletal components, including actin, α-actinin, vinculin, and vimentin. Ethacrynic acid reduces outflow resistance in enucleated calf and human eyes and in living monkey eyes and along with reducing intraocular pressure in live rabbit, monkey, and human eyes.(Tian et al., 2000)

H-7, a serine-threonine kinase inhibitor, inhibits actinomyosin-driven contractility and induces general cellular relaxation. Although H-7 does not affect actin

polymerization, the inhibition of contraction leads to deterioration of the actin microfilament bundles and perturbation of its membrane-anchorage cites in the human trabecular meshwork. H-7 is able to dose-dependently and time-dependently decrease outflow resistance and intraocular pressure. (Liu et al., 2001) H-7 appears to decrease outflow resistance by relaxing and expanding the trabecular meshwork and Schlemm's canal, without significantly changing intracellular adhesion.

Latrunculins are microlides derived from the *Latrunculia magnificia* sea sponge which sequesters monomeric actin to disassemble actin filaments and disrupt cell-cell junctions in human trabecular meshwork cells (Liu et al., 2003; Peterson et al., 2000) Since the actomyosin system is present in essentially all cells, cytoskeletal drugs could, in principle, have detrimental effects on other anterior segment tissues, especially the cornea, which would receive a higher drug concentration after topical application. However, differences in tissue architecture and physiological environment between the cornea and trabecular meshwork may allow the cornea to avoid meaningful change at drug concentrations affecting the trabecular meshwork. (Tian et al., 2000) Although, Latrunculin A is less potent than Latrunculin B, both show a reversible, dose-dependent and time-dependent decrease of actin bundles in cultured human trabecular meshwork cells leading to increased outflow. Peters et al. reported intraocular pressure decreases six hours following treatment with Latrunculin A while Latrunculin B was able to decrease intraocular pressure in one hour following treatment. (Peterson et al., 2000) Latrunculin-A has been shown to increase outflow facility in living monkey eyes. (Peterson et al., 2000) Recent studies demonstrate the ability of Latrunculin A to block

dexamethasone-induced cytoskeleton rearrangements. "The degree of actin cross-linking and bundling profoundly influences cellular elasticity and stiffness, and were trabecular meshwork cells to assume such a state, it could be predicted that reduced aqueous outflow would ensue. Dexamethasone treatment in the clinical setting leads the development of elevated intraocular pressure together with a unique configuration of cross-linked actin networks (CLANs) in trabecular meshwork cells." (Tan et al., 2006b)

In vitro studies of cells treated with dexamethasone for seven days demonstrate the ability of potent glucocorticoids to stabilize trabecular meshwork and endothelial cells against chemically induced retraction by ethacrynic acid and ethyleneglycol bis (β-amino-ethylether)-N, N-tetraacetic acid (EGTA). Dexamethasone-pretreated cells showed an increase in f-actin around the cell circumference suggesting it may increase focal adhesions and reduce cytoskeletal movement. The observed dexamethasone effects demonstrated a latency of several days. (O'Brien et al., 1996)

The cytoskeleton in trabecular meshwork cells plays a potentially pathogenic role in ocular hypertension and primary open angle glaucoma. As stated above, pharmaceutical agents that have cytoskeletal altering properties are also able to affect the flow of aqueous humor and thus influence intraocular pressure. Cytoskeletal rearrangements in non-glaucomatous trabecular meshwork cells following glucocorticoid-treatment (Clark, 1995a; Clark et al., 1996; Clark, 1995b; Clark et al., 1995; Clark, 2005; O'Brien et al., 1996; Wordinger and Clark, 1999) are strikingly similar to cytoskeleton structures observed in untreated glaucomatous trabecular meshwork cells. Therefore, in vitro studies of the trabecular meshwork in response to

these "cytoskeletal drugs" may be a useful tool to better understand the pathology underlying primary open angle glaucoma.

Hypothesis and Specific Aims

Hypothesis: Altered expression of actin-associated proteins is responsible for glucocorticoid- and glaucoma-induced actin cytoskeletal rearrangements in trabecular meshwork cells

Specific Aim 1: Identify candidate genes and proteins responsible for CLAN formation in cultured human TM cells. Normal, dexamethasone treated, and glaucomatous human TM cells will be examined to see if there are morphological differences in their actin cytoskeleton. Changes in gene expression of actin-associated protein candidates will be analyzed by quantitative polymerase chain reaction assays of RNA extracted from these TM cells.

Specific Aim 2: Determine whether the proteins identified are associated with the formation of CLANs. Once the first set of experiments has confirmed the expression of actin binding protein candidates, their association with CLANs will be assessed. Direct fluorescence staining of the actin cytoskeleton allows the visualization of the actin cytoskeleton and its reorganization into CLANs in dexamethasone treated cells as well as glaucomatous cells. Indirect immunofluorescence staining of actin-associated candidate proteins will determine the location of these proteins in relation to CLANs.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Normal and glaucomatous human TM cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Gaithersberg, MD) with 10% fetal bovine serum (Atlas, Fort Collins, CO) and 2mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO). Following one week of confluence, cultures were treated with 100 nM dexamethasone (Sigma-Aldrich, St.Louis, MO) in ethanol vehicle or 0.1% ethanol as vehicle control for 14 days.(Clark et al., 1996; Clark et al., 1994; Clark, 2005; Wordinger and Clark, 1999) After chronic treatment with dexamethasone or ethanol, culture media was removed and cells were washed in Dulbecco's Phosphate Buffered Saline (DPBS) (Hyclone Logan, UT) and either harvested for RNA or protein analysis or fixed to cover slips using either 10% formalin (Sigma-Aldrich, St. Louis, MO) or 4 % paraformaldehyde (EM Sciences, Gibbstown, NJ) diluted in phosphate buffered saline.

In addition to chronic treatment with dexamethasone as previously stated, acute treatments were performed on TM cells. Confluent TM cells were dispersed with 0.05% Trypsin-EDTA (Gibco-BRL, Gaithersberg, MD) and plated on cover slips treated with either fibronectin or gelatin, allowed to incubate in either 100 nM dexamethasone

(Sigma-Aldrich, St.Louis, MO), vehicle control (0.1% ethanol), or culture medium containing no treatment for 3 hours prior to being fixed for microscopy studies.

Bioinformatic Search

Gene Spring software was used to analyze Affymetrix gene expression data comparing all human genes in normal and glaucomatous trabecular meshwork cells in the presence or absence of dexamethasone on the U133A genechip. Gene expression information was filtered for: 1) genes that had a 1.5 fold change in expression in trabecular meshwork cells cultured with and without dexamethasone for either 1 or 14 days, 2) genes that showed changes in expression in glaucomatous TM cells when compared to normal TM cells, and 3) cross-referenced to genes coding for proteins that associate with actin filaments or cytoskeleton.

Table 3: Rationale for Choice of Candidate Proteins

Actin Binding Proteins	**Literature References**	**Reported Function of Interest**
Nidogen (NID)	Proteins. 2005 Aug 1;60(2):281-8. Eur J Haematol. 2001 Sep;67(3):176-84.	protein-protein interactions; down-regulated by glucocorticoids
Nebulette (NEBL)	J Mol Biol. 2006 Feb 24;356(3):714-23. J Biol Chem. 2001 Sep 7;276(36):33328-35.	bind to actin; interact with LASP1 and zyxin
Alpha Actin (ACTA2)	Invest Ophthalmol Vis Sci. 1994 Jan;35(1):281-94. Exp Eye Res. 2006 Jun;82(6):945-58. Invest Ophthalmol Vis Sci. 2006 May;47(5):1956-67. Biol Pharm Bull. 2005 Dec;28(12):2189-96.	dynamic component of cytoskeleton; rearrangement occurs following glucocorticoid treatment
Alpha Actinin (ACTN1)	Cell Motil Cytoskeleton. 2005 Feb;60(2):83-95.	associated with CLAN beams and vertices
Coronin-1	Science. 2006 Aug 11;313(5788):839-42.	dynamically regulated

(CORO1c)		component of actin filament networks
LIM and SH3 Protein (LASP1)	J Cell Sci. 2002 Dec 15;115(Pt 24):4787-99.	binds to F-actin; may play a role in cytoskeleton plasticity
Syndecan-4 (SCD4)	Invest Ophthalmol Vis Sci. 2006 May;47(5):1956-67.	associated with CLAN vertices
Vinculin/Metavinculin (VCL)	Exp Eye Res. 2003 Aug;77(2):181-8.	associated with actin cytoskeleton; responsive to dexamethasone
Zyxin (ZYX)	J Biol Chem. 2004 May 7;279(19):20401-10. J Biol Chem. 2001 Sep 7;276(36):33328-35.	component of focal adhesions; interacts with α-actinin, LASP1 and nebulette

Venn diagrams showing the intersection of the three search criteria were used to compile a list of gene candidates for further exploration. Selection rationale for each gene was based on its Venn diagram, PubMed literature searches, as well as functional information obtained from each candidate's Gene Card (http://www.genecards.org/). Once the candidate genes had been selected, GeneScript (https://www.genscript.com/ssl-bin/app/primer) was used to design forward and reverse quantitative PCR primers for each candidate. NCBI BLAST and Vector NTI were used to compare primer sequences to their targets to confirm specificity. Primers were ordered from Integrated DNA Technologies, Inc.

Table 4: Primers used for SYBR Green Assays (QPCR)

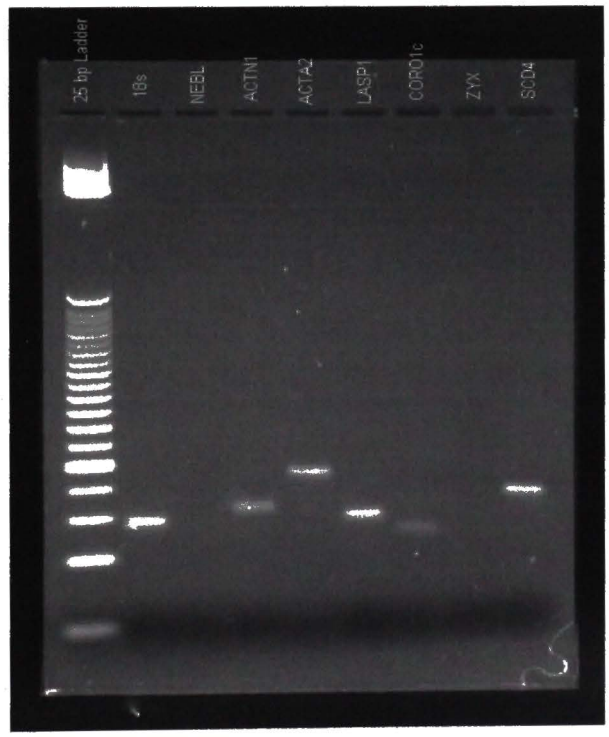
Probeset	Gene Name	GenBank	Description	5' PCR Primer 3' PCR Primer	Product Size
203962_s_at	NEBL	NM_006393	Nebulette	CTACTTGAACCCATAAACTT CCAGGAATATACTGAAGTCA	77bp
203962_s_at	NEBL	NM_006393	Nebulette	CATGCTGCAGTGAGGTAACA AATGGCTTTGCTGATAGGCT	77bp
208636_at	ACTN1	NM_001102	Actinin, alpha 1	TTTGCACCGAAATGTCTTGT GCAGATGGAACAGATAGAAGAAAA	78bp
200974_at	ACTA2	NM_001613	Actin, alpha 2,sm	AAGAGTTACGAGTTGCCTGAT ATGAAGGATGGCTGGAACAG	110bp
200618_at	LASP1	NM_006148	LIM & SH3 protein 1	CTGAGCCCCTGGTCTTCTCTA GATGCCTCTTTCCTGGGATT	72bp
221676_s_at	CORO1c	NM_014325	Coronin 1C	ACCCAATCCTCATCTCCTTG CCACCTTGAGATCCCTGTTT	63bp
202071_at	SCD4	NM_002999	Syndecan 4	CTGCTGCTGTTCTTCGTAGG ATCCTCATCGTCTGGTAGGG	117bp
200931_s_at	VCL	NM_014000	Vinculin or Metavinculin	CCAAAGCCAGAACCAGATGAG GGTGGGAAGGAAGGATGTTCA	63bp
200808_s_at	ZYX	NM_003461	Zyxin	CCCGACTACCACAAGCAGTA CCACTCGCACAGTCTCATCT	91bp
210155_at	MYOC	D88214	Myocilin	GCCCATCTGGCTATCTCAGG CTCAGCGTGAGAGGCTCTCC	82bp

Quantitative Polymerase Chain Reaction Assays (QPCR)

Total RNA was extracted from TM cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) and combined with DNase buffer and random hexamers before adding RT-Master Mix (Taqman Gold RT-PCR kit, Applied Biosystems, Foster City, CA) to generate cDNA. cDNA was then diluted to 0.5 ηg/μl in 50 μg/ml glycogen and stored at -20°C. The SYBR Green Assay was used to analyze cDNAs of candidate genes in 96 well plates containing 50nM of forward and reverse primers, 1X SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 2.5ng cDNA as previously described (Wang et al., 2001). Gene expression analysis was performed using the ABI Prism^{TM} 7700 Sequence Detection System with thermal cycling conditions of 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C

for 20 seconds. A dissociation curve profile consisting of 95°C for 15 seconds, 60°C for 20 seconds, and a 20 minute ramp to 95°C for 15 seconds was added to the end of the thermal cycling conditions. Data analysis was performed with SDS software version 1.9.1, Multicomponent software (Applied Biosystems, Foster City, CA), and Microsoft Excel XP. QPCR products in 10µl aliquots were mixed with 2µl 6X ficolle and 0.5 µl of a molecular weight marker (1Kb DNA ladder, Invitrogen, Carlsbad, CA) and run in parallel on a 3% agarose gel and analyzed by electrophoresis to allow DNA band size estimation. Images of ultraviolet illuminated gels were taken using the Bio-Rad Gel Doc EQ system.

Figure 1: 3% Agarose Gel of QPCR Products from SYBR Green Assay. Lanes consist of (from left to right) 25 base pair ladder, 18S, nebulette, α-actinin, α-actin, LASP1, coronin, zyxin, and syndecan-4. All QPCR products derived from actin binding proteins show one band except for nebulette and zyxin which had no amplification products.



Western Immunoblots

Protein extracts were prepared from cultured human trabecular meshwork cells treated with 100 nM dexamethasone or 0.1% ethanol for 14 days by lysing cell monolayers using Mammalian Protein Extraction Agent (M-PER) per manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The protein concentration of each extract was determined using the Bradford Protein Assay (Pierce Biotechnology, Rockford, IL) in microplates. Absorbance of protein extracts and standards were measured at 595nm using the SpectraMax Plus 384 (Molecular Dynamics, Sunnyvale, CA) plate reader and calculated with Microsoft Excel XP.

Trabecular meshwork protein extract (5 μg) was electrophoresed using NuPAGE Novex Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA) in MOPS SDS running buffer (Invitrogen, Carlsbad, CA). Once preliminary studies determined dilutions for antibodies (table 5) against α-actin, α-actinin, LASP1, coronin, syndecan-4, vinculin, and zyxin were completed, protein extracts of cultured human TM cells were reduced using either β-mercaptoethanol or a more robust sample buffer (2% β-mercaptoethanol, 8M urea, 0.1M tris, and bromophenyl blue) (Stokely et al., 2005) and electrophoresed for 50 minutes at 200V. (Shepard et al., 2001) Proteins were then transferred from gels onto polyvinyldifluoride (PVDF) membranes for 160 minutes at 28 V (Invitrogen, Carlsbad, CA), blocked in 2.7% gelatin, and incubated with primary antibodies (Table 4) diluted in phosphate buffered saline containing 1.0% gelatin and 0.1% Tween 20 at room temperature in a hybridization oven overnight. The next morning, membranes were washed with phosphate buffered saline and placed back in the hybridization oven at room

temperature for one hour with the appropriate secondary antibody also diluted in phosphate buffered saline containing 1% gelatin and Tween 20. Following additional washes in phosphate buffered saline, membranes were treated with the chemiluminescent detection reagent ECL Plus (GE Healthcare, Piscataway, NJ) and developed using a Konica SRX-101A Processor (Minolta, Tokyo, Japan). PVDF membranes were then stripped using Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) and reprobed using anti-GAPDH antibodies (Table 5) as a loading control.

Table 5: Antibodies used for Western Immunoblots

Protein	MW (kDa)	Primary Antibodies	Secondary Antibodies
α-Actin	42	Monoclonal anti-α-actin A5228 Sigma, St. Louis, MO 1:10,000 dilution	sheep anti-mouse IgG-HRP (Amersham NA931V) 1:25000
α-Actinin	100	monoclonal anti-α-actinin A5044 Sigma, St. Louis, MO 1:20,000 dilution	sheep anti-mouse IgG-HRP (Amersham NA931V) 1:25000
LASP1	35	Monoclonal anti-LASP1 MAB8991 Chemicon, Billerica, MA 1:2000 dilution	sheep anti-mouse IgG-HRP (Amersham NA931V) 1:25000
Coronin	53-57	goat polyclonal anti-coronin 1 IMG-3094 Imgenex, San Diego, CA 1:500 dilution	rabbit anti-goat IgG-HRP (Invitrogen 61-1620) 1:25000
Zyxin	82-84	rabbit polycolonal anti-zyxin Z4751 Sigma, St. Louis, MO 1:20,000 dilution	donkey anti-rabbit IgG-HRP (Amersham NA934V) 1:25000
Syndecan-4	22-24	rabbit polycolonal anti-syndecan-4 ab24511 Abcam, Cambridge, MA 1:100 dilution	donkey anti-rabbit IgG-HRP (Amersham NA934V) 1:25000
Myocilin	57	goat polyclonal anti-myocilin sc-21245 Santa Cruz , Santa Cruz, CA 1:5000	rabbit anti-goat IgG-HRP (Invitrogen 61-1620) 1:25000

Vinculin	124	Monoclonal anti-vinculin V4505 Sigma, St. Louis, MO 1:2000	sheep anti-mouse IgG-HRP (Amersham NA931V) 1:25000
GAPDH	36	Monoclonal anti-GAPDH ab9484 Abcam, Cambridge, MA 1:10,000	sheep anti-mouse IgG-HRP (Amersham NA931V) 1:25000

Fluorescence Microscopy

To visualize actin filaments and nuclei, cells fixed to cover slips were treated with of 0.1% Triton X-100 (Molecular Biology Cert., Shelton, CT) and gently mixed for 5 minutes. Following two rinses with Dulbecco's Phosphate Buffered Saline (DPBS) (Hyclone Logan, UT), cells were treated with 2.5% Alexa Fluor 488-Phalloidin (Invitrogen, Carlsbad, CA) for 20 minutes protected from light and rinsed twice before applying 0.5% Hoechst 33342 (Invitrogen, Carlsbad, CA) as a nuclear, counter stain for 10 minutes protected from light. Stained TM cells on cover slips were then placed on ethanol cleaned slides using Flurotech mounting media (Accurate Chemicals, Westbury, NY) and allowed to air dry overnight. Stained TM cells were examined using an Olympus BX51 fluorescence microscope with phase contrast capabilities to calculate the percentage of cross-linked actin network (CLAN) formation based upon the average of subjective observations of three visual fields.

For indirect immunofluorescence studies, trabecular meshwork cells fixed on cover slips were treated with of 0.1% Triton X-100 (Molecular Biology Cert., Shelton, CT) for 2-5 minutes, rinsed twice in DPBS, and blocked at room temperature for 30 minutes with either 2% serum from either bovine or the secondary antibody species (Table 6). After rinsing three times with DPBS containing calcium and magnesium

(Hyclone, Logan, UT), primary antibodies (Table 6) were added and allowed to incubate for one hour at room temperature. Following another three rinses, the secondary antibodies (Table 6) were applied and incubated for 45 minutes at room temperature. To visualize actin filaments, 0.25% Alexa Fluor 488-Phalloidin (Invitrogen, Carlsbad, CA) was added and allowed to incubate for 20 minutes at room temperature. (Note the necessity to further dilute direct stains in order to achieve optimal antibody staining.) Cells underwent three additional rinses prior to the addition of the nuclear stain, 0.05% Hoechst 33342 (Invitrogen, Carlsbad, CA) which was allowed to incubate 5-10 minutes at room temperature. All antibodies and direct stains were incubated on an orbital shaker protected from light. Stained cells on cover slips were then placed on ethanol cleaned slides using Fluortech mounting media (Accurate Chemicals, Westbury, NY) and allowed to air dry overnight. Cells were examined using an Olympus BX51 fluorescence microscope using Microfire software (Leeds Instruments, Inc., Irving,TX).

Table 6: Antibodies used in Indirect Immunofluorescence Studies

Protein	Blocking Serum	Primary Antibodies	Secondary Antibodies
α-Actin	2% goat serum G9023 Sigma, St. Louis, MO	Monoclonal anti-α-actin A5228 Sigma, St. Louis, MO 1:800	Alexa Fluor 555 goat anti-mouse IgG [2mg/ml] A21424 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:200 or 1:2000]
α-Actinin	2% goat serum G9023 Sigma, St. Louis, MO	monoclonal anti-α-actinin A5044 Sigma, St. Louis, MO 1:800	Alexa Fluor 555 goat anti-mouse IgG [2mg/ml] A21424 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:200 or 1:2000]
LASP1	2% goat serum G9023 Sigma, St. Louis, MO	Monoclonal anti-LASP1 MAB8991 Chemicon, Billerica, MA 1:800	Alexa Fluor 555 goat anti-mouse IgG [2mg/ml] A21424 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:200 or 1:2000]
Vinculin	2% goat serum G9023 Sigma, St. Louis, MO	Monoclonal anti-vinculin V4505 Sigma, St. Louis, MO 1:800	Alexa Fluor 555 goat anti-mouse IgG [2mg/ml] A21424 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:200 or 1:2000]
Coronin	2% donkey serum D9663 Sigma, St. Louis, MO	goat polyclonal anti-coronin 1 IMG-3094 Imgenex, San Diego, CA 1:500	Alexa Fluor 546 donkey anti-goat IgG [2mg/ml] A11056 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:200 or 1:2000]
Zyxin	2% goat serum G9023 Sigma, St. Louis, MO	rabbit polycolonal anti-zyxin Z4751 Sigma, St. Louis, MO 1:100	Alexa Fluor 594 goat anti-rabbit IgG [1mg/ml] A11012 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:100 or 1:1000]
Syndecan-4	2% goat serum G9023 Sigma, St. Louis, MO	rabbit polycolonal anti-syndecan-4 ab24511 Abcam, Cambridge, MA 1:100	Alexa Fluor 594 goat anti-rabbit IgG [1mg/ml] A11012 Invitrogen, Carlsbad, CA 1-10 ug/ml [1:100 or 1:1000]

CHAPTER III

RESULTS

Cell Culture Results

Human TM cell lines were allowed to become confluent prior to treatment to observe baseline or ethanol treated morphology as well as to see if any cell lines responded to dexamethasone by forming CLANs. The percentage of CLANs formed by each cell line was calculated as an average of three visual fields per cover slip (table 4). A higher base-level of CLANs are present in glaucomatous cultured TM cells as compared to normal TM cells (Clark et al., 1996). Similarly, when both normal and glaucomatous TM cells are chronically treated with dexamethasone, a higher percentage of CLANs are present in glaucomatous TM cells even though both cell types show an increase in CLANs over baseline. Of the human TM cell lines examined for the development of CLANs in response to dexamethasone, two normal TM cell lines and one glaucomatous TM cell line produced CLANs and were included in subsequent experiments. However, further use of one normal TM cell line (NTM 24B) was discontinued due to contamination.

Table 7: CLAN Screen of Human TM Cell Lines. Cells from each cell line were grown on cover slips and 3 each treated with 0.1% ethanol and 100nM dexamethasone for 2 weeks prior to fixation. The actin cytoskeleton was stained with Alexa 488 phalloidin and the nuclei were stained with Hoechst 33342 so that the number of cells and CLANs could be counted. For each cell line, 3 representative visual fields from each cover slip were studied and the CLANs per visual field were divided by the number of cells in the same visual field to obtain the % CLAN per visual field. The Mean % CLANs was calculated by dividing the average of % CLANs per visual field by the three cover slips containing TM cells from the same line and same treatment.

Visual Field	2 Week Treatment	Cell Line	% CLANs in Visual Field	Mean % CLANs
1	0.1 % Ethanol	NTM 24B	12.50	4.17
2			0.0	
3			0.0	
1	100 nM Dexamethasone		6.06	15.92
2			0.0	
3			41.7	
1	0.1 % Ethanol	GTM 730	4.00	7.77
2			16.67	
3			2.63	
1	100 nM Dexamethasone		31.37	52.57
2			51.35	
3			75.00	
1	0.1 % Ethanol	NTM 486	43.33	30.00
2			24.44	
3			22.22	
1	100 nM Dexamethasone		31.15	49.24
2			52.17	
3			64.41	

QPCR Results

Total RNA was extracted from TM 70, NTM 24B and GTM 730 cells cultured with or without dexamethasone for 14 days. The effect of dexamethasone on NTM 24B and TM 70 gene expression was determined using Affymetrix gene chips (U133A and U133 Plus 2). Altered gene expression was confirmed by reverse transcriptase quantitative polymerase chain reaction (RT-QPCR).

Amplification of nebulette, α-actin, α-actinin, coronin, LASP1, syndecan-4, meta-vinculin, and zyxin were determined using SYBR Green assays run on cDNA from TM70 and GTM730 cell lines. In addition to the candidate genes, human TM cell cDNA was analyzed for the induction of myocilin following chronic glucocorticoid treatment to confirm their steroid responsiveness. QPCR data indicates chronic treatment with dexamethasone induces a 13.1-fold increase in myocilin in NTM 24B cells and 1.03-fold increase in GTM 730 cells.

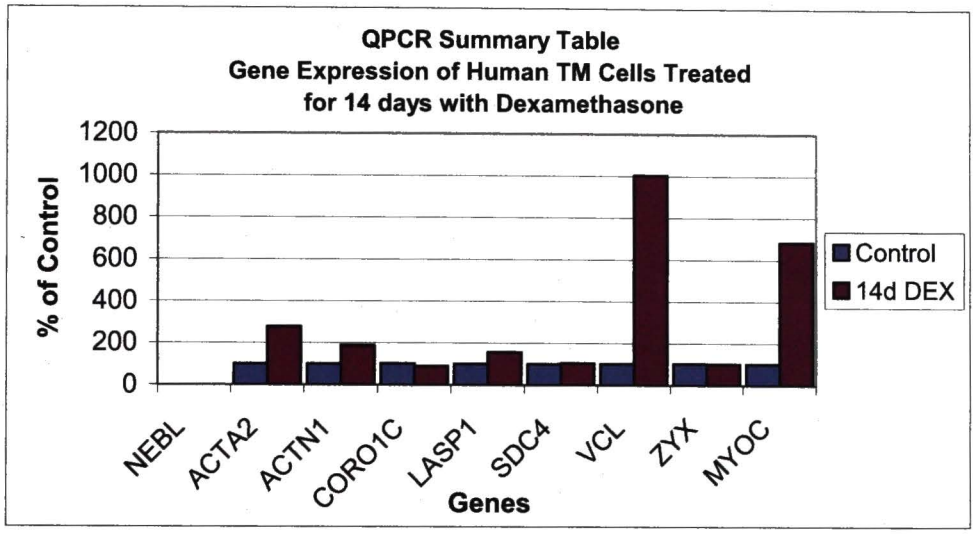
Nebulette has actin-binding properties and interacts with two other candidates, LASP1 and zyxin. Unfortunately, QPCR experiments yielded no amplification of nebulette in both TM cell lines in either of two primer sets. Since published reports have indicated up to a 12-fold increase in nebulette following chronic treatment with dexamethasone (Rozsa et al., 2006), additional testing of primers was conducted. According to www.genecards.org, nebulette is abundantly expressed in cardiac myocytes, therefore the amplification of nebulette by QPCR analysis of cDNA of cardiac myocytes was run in parallel with cDNA of control and glucocorticoid-treated TM cells to confirm primer quality. Since efficient amplification of nebulette was seen in cDNA of cardiac myocytes but not in that of TM cells, it was concluded that the cDNA of the TM cell lines assayed did not encode nebulette.

Of the isoforms of actin, α-actin has been shown to be a dynamic component of cytoskeleton which rearranges following glucocorticoid treatment. Analysis of cDNA of TM70 and GTM 730 cell lines treated with dexamethasone for 2 weeks resulted in a 2-fold increase in α-actin. Non-muscle α-actinin, associated with beams and vertices of

cross-linked actin networks (Clark et al., 1994; Clark, 2005), showed dexamethasone induced increases slightly above 1-fold by QPCR. Coronin, a dynamically regulated component of actin networks, had 0.8 to 2-fold increase in RNA expression following 2 weeks of dexamethasone treatment. LASP1, or LIM and SH3 protein, binds to filamentous actin and may play a role in cytoskeleton plasticity. QPCR analysis of cDNA from TM cells chronically treated with dexamethasone produced an approximate 1-fold induction in LASP1 expression. Syndecan-4 reportedly co-localizes with actin at CLAN vertices (Filla et al., 2006) following treatment with glucocorticoids and produced a slight increase in expression of 0.6 to greater than 1-fold in cDNA of TM cells treated with dexamethasone for two weeks.

QPCR analysis of meta-vinculin, associated with the actin cytoskeleton and responsive to dexamethasone, shows dexamethasone induced increases in gene expression of 3 to 9-fold, significantly higher than indicated by Affimetrix gene expression data. Zyxin, a component of focal adhesions, interacts with α-actinin, LASP1 and nebulette. No increase of zyxin expression was detected regardless of treatment or cell line. This agrees with Affimetrix gene expression data which indicates there is no change in gene expression with chronic glucocorticoid treatment.

Table 8: Quantitative PCR analysis of mRNA levels of actin binding protein candidates from TM 70, NTM 24B, and GTM 730 cells normalized to 18S rRNA levels. Summary table depicts the gene expression as an average of 3 separate QPCR runs.



Western Immunoblot Results

Initially, western blots were intended be an indicator of the protein response of TM cell lines treated with dexamethasone. However, due to the inability to consistently and accurately quantify the amount of protein, western blot information was used to confirm the specificity of primary antibodies which were also used in indirect immunofluorescence experiments. One band at the expected molecular weight was achieved for α-actin (figure 8), α-actinin (figure 9), meta-vinculin (figure 13), and zyxin (figure 14). Although a single band at the expected molecular weight was achieved for syndecan-4 (figure 12), a reducing agent containing urea was necessary due to the SDS-resistant properties of syndecan-4. Similarly when using β-mercaptoethanol as a reducing agent, four bands were visible on LASP1 blots (figure 11). However, when the reducing agent containing urea was used, only two bands were visible, one at the

expected molecular weight of LASP1 and another approximately twice the expected molecular weight. For coronin (figure 10), multiple non-specific bands were visible regardless of reducing agent. In addition to TM protein extract, a Jurkat cell lysate recommended by the manufacturer of the primary antibody (Imgenex, San Diego, CA) also did not produce a band at the expected molecular weight when electrophoresed and probed for coronin. Western blot analysis of nebulette protein expression was not attempted due to the lack of commercially available antibodies.

Figure 2: Western Blot of α-Smooth Muscle Actin. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. α-Actin labeled bands can be seen near its expected molecular weight of 42 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

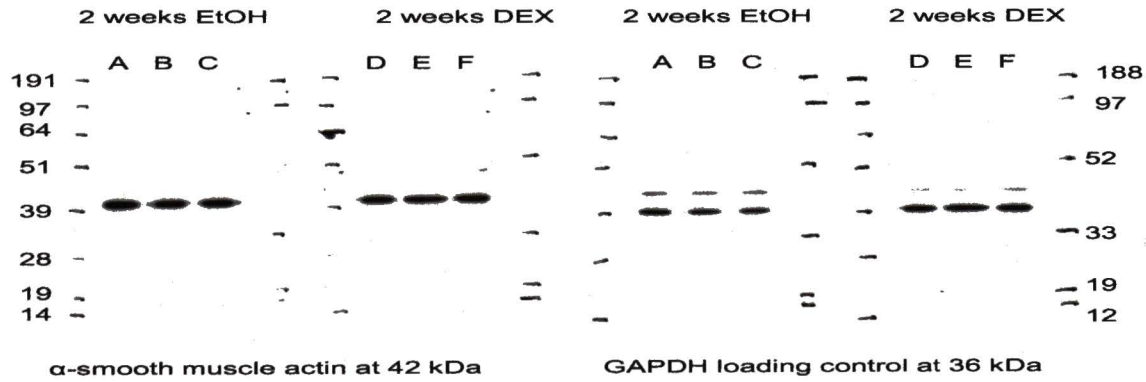


Figure 3: Western Blot of α-Non-Muscle Actinin. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. α-Actinin labeled bands can be seen near its expected molecular weight of 100 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

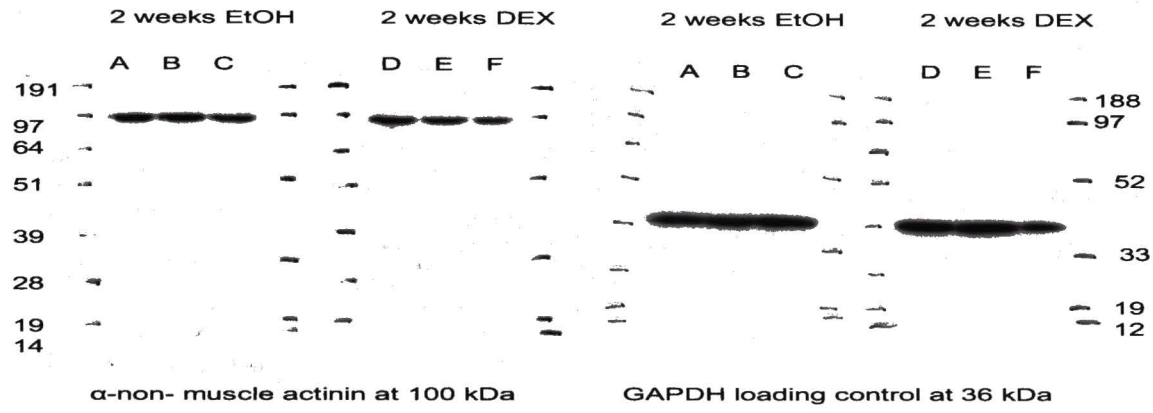


Figure 4: Western blot of Coronin 1c. Lanes A and B contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D and E and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. Lanes C and F contain Jurkat cell lysate recommended as a positive control from the manufacturer of the primary antibody (Imgenex, San Diego, CA). No coronin labeled bands can be seen near its expected molecular weight of 55 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

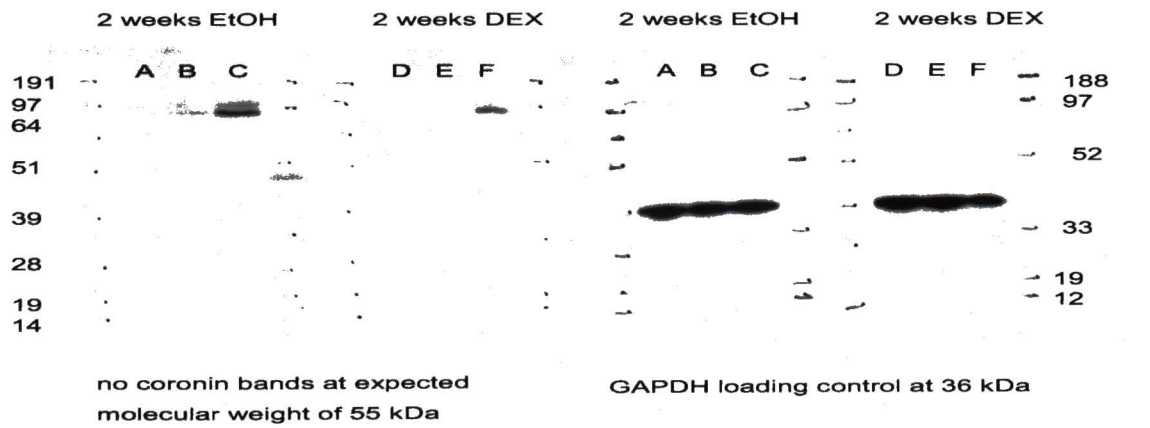


Figure 5: Western Blot of LASP1. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. LASP1 labeled bands can be seen near its expected molecular weight of 35 kDa and a doublet at 63 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

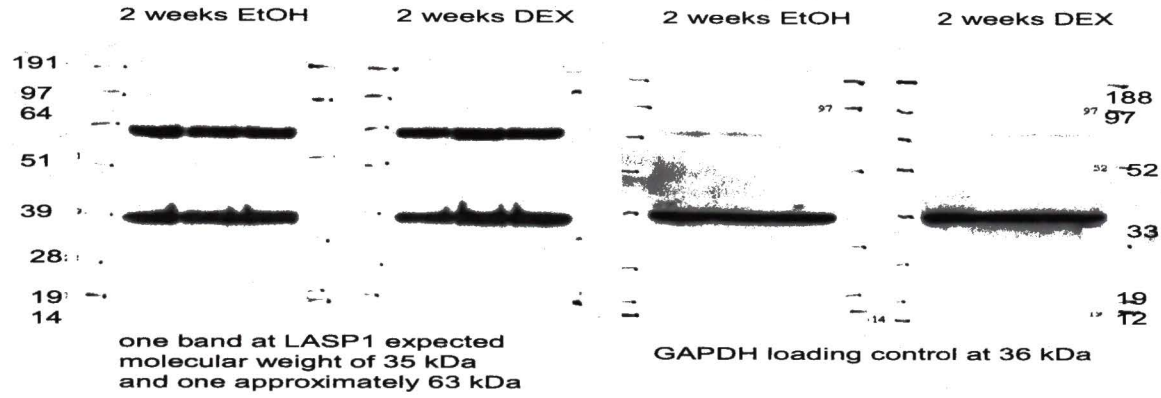


Figure 6: Western Blot of Syndecan-4. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. Syndecan-4 labeled bands (D, E, and F) can be seen near its expected molecular weight of 23 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

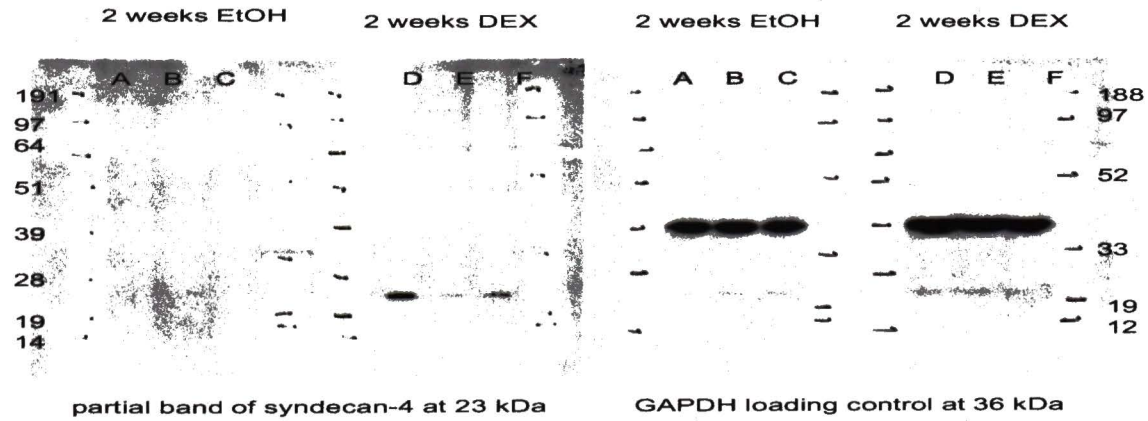


Figure 7: Western Blot of Meta-Vinculin. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. Meta-vinculin labeled bands can be seen near its expected molecular weight of 124 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.

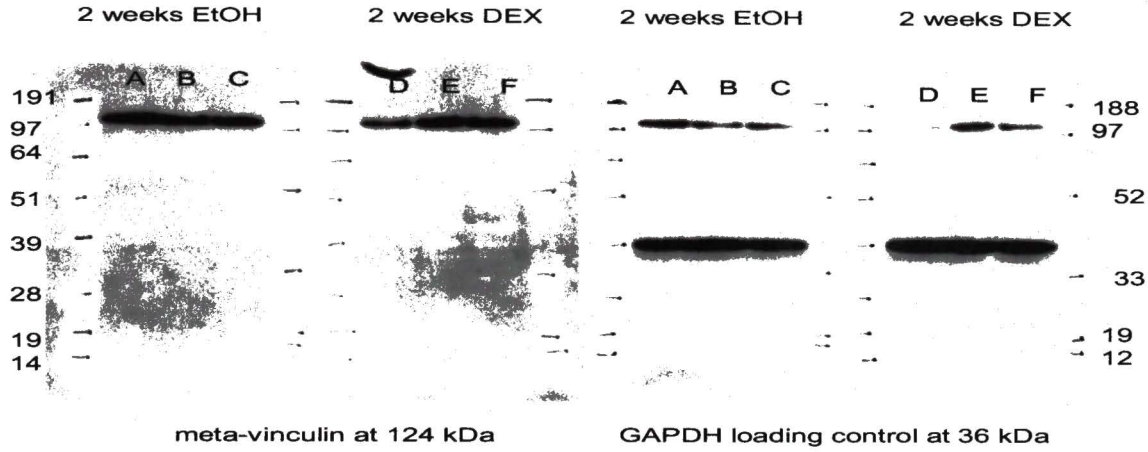
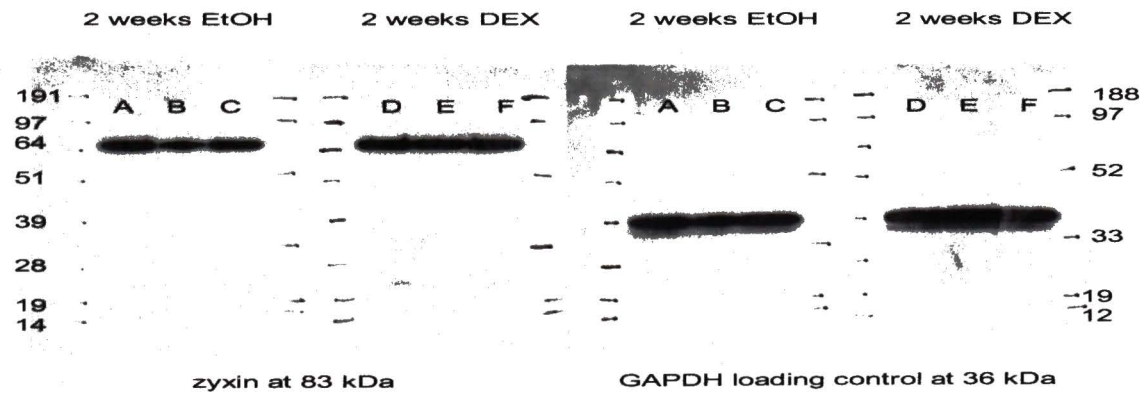


Figure 8: Western Blot of Zyxin. Lanes A, B, and C contain protein extracted from human TM cells cultured in the presence of 0.1% ethanol for 2 weeks. Lanes D, E, and F contain protein extracted from TM cells cultured in the presence of 100nM dexamethasone for 2 weeks. Zyxin labeled bands can be seen near its expected molecular weight of 38 kDa (left) and GAPDH at its expected molecular weight of 36 kDa (right) on the same membrane after stripping and being reprobed to visualize protein loading.



Fluorescence Microscopy Results

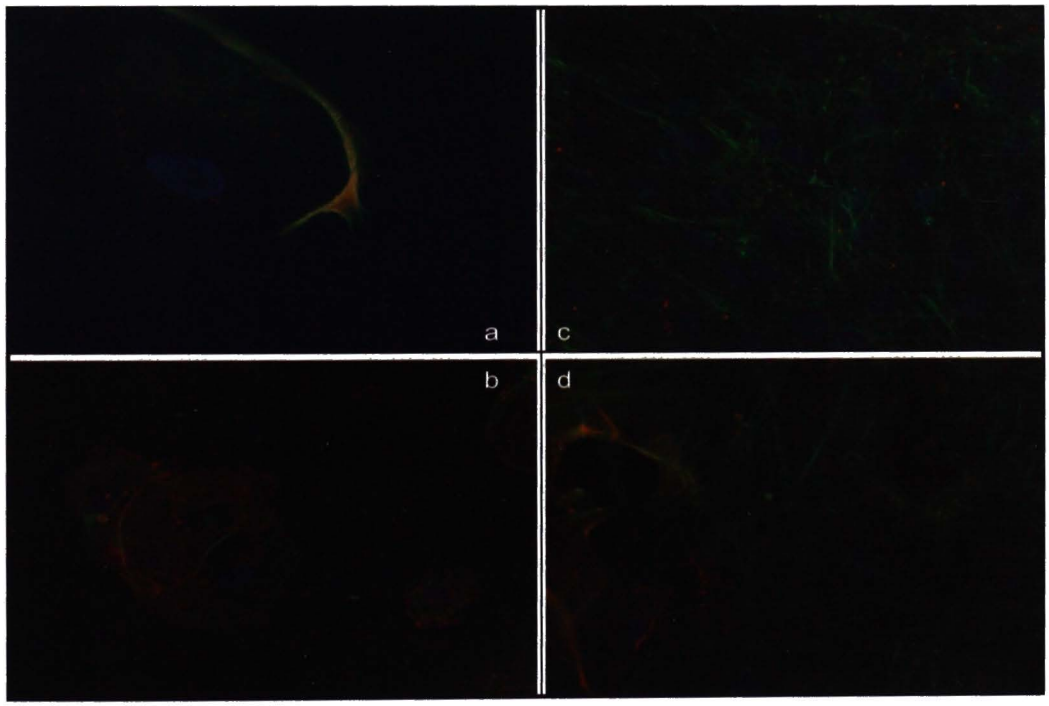
The original intent of this study was to visualize the actin binding protein candidates previously studied by QPCR and Western blot to assess their location in relation to the actin cytoskeleton and especially CLAN structures. These studies were to be conducted on human TM cells cultured in the presence or absence of chronic dexamethasone to determine whether or not glucocorticoids had any affect on the expression of these actin binding proteins. However, during the course of my studies, a report was published which claimed chronic glucocorticoid treatment was unnecessary to produce CLANs. (Filla et al., 2006) To ascertain whether or not the make up of CLANs seen following brief exposures to dexamethasone were similar to those induced by chronic glucocorticoid treatments, immunofluorescence studies include comparisons of TM cells treated with dexamethasone or ethanol control for 2 weeks, chronic treatments, and also those treated for only 3 hours with either dexamethasone or ethanol, acute treatments. This allows for the side-by-side comparison of CLAN structures produced by both acute and chronic treatment with dexamethasone. Each actin binding protein candidate was subjected to immunofluorescence studies following both acute and chronic exposures to dexamethasone as well as ethanol vehicle. Photo-micrographs of these experiments were combined into figures to more easily examine the effects of these treatments on the staining pattern for each actin binding protein candidate.

As expected, abundant staining of smooth muscle α-actin was seen decorating the microfilaments throughout the TM cells regardless of the treatment or length of treatment. However, acute treatments of ethanol and dexamethasone lead to transient CLAN-like structures in the periphery of the cytoskeleton while chronic treatments of dexamethasone induced actin rearrangements to more static CLAN structures through out the cell. Direct staining and immunofluorescence staining of α-actin show intense labeling in all forms of the cytoskeleton, including stress fibers and CLANs.

Figure 9: Immunofluorescence Staning of α-Actin (red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.

The "beads on a string" appearance of α-actinin along the actin stress fibers as well as cases of co-localization with CLAN vertices was observed, although, α-actinin was not present in every CLAN or along all actin stress fibers. Of the cells treated with dexamethasone, those which underwent chronic treatments had more α-actinin decorating the actin fibers and CLAN vertices than did TM cells with acute treatments.

Figure 10: Immunofluorescence Staining of α-Actinin (red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.



Immunofluorescence staining of LASP1 indicates it is located just outside the nuclear membrane in ethanol treated TM cells and is more widely distributed in cells treated with dexamethasone, labeling the entire cytoplasm. However LASP1 was not seen along the actin filaments or within the structure of CLANs. TM cells treated with ethanol showed similar distribution of LASP1 regardless of length of treatment. There were slight differences observed in cells treated with dexamethasone. Those treated with 100nM dexamethasone for 3 hours were similar in appearance to ethanol treated cells while cells that underwent chronic dexamethasone treatment have an increase in the amount of LASP1 labeling within the cytoplasm.

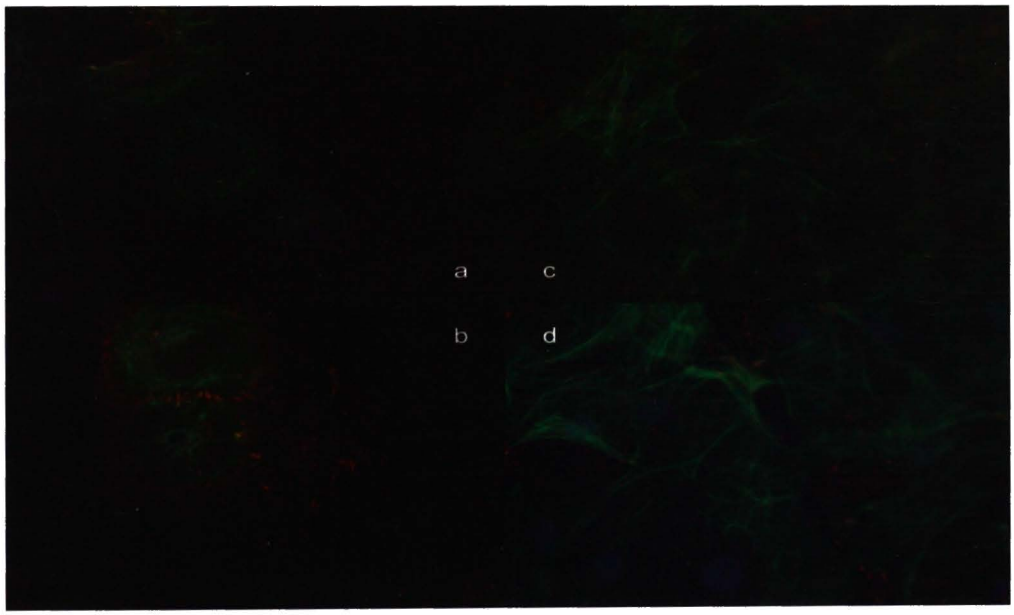
Figure 11: Immunofluorescence Staining of LASP1 (red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.

Although a previous publication has reported the presence of syndecan-4 at CLAN vertices (Filla et al., 2006), I have been unsuccessful in duplicating these results. Immunofluorescence staining of syndecan-4 has consistently been located in the perinuclear cytoplasm and not within the actin cytoskeleton or any CLAN structure. Assays to determine the location of syndecan-4 in TM cells cultured in the presence of both acute and chronic exposures to dexamethasone on fibronectin-coated cover slips (Filla et al., 2006) and on gelatin-coated cover slips (Clark et al., 1996) showed syndecan-4 in similar locations within the cell regardless of the structure of the actin cytoskeleton. No matter the treatment or its length, syndecan-4 is consistently seen in the perinuclear spaces of cultured TM cells.

Figure 12: Immunofluorescence Staining of Syndecan-4(red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.

The location of meta-vinculin in cultured TM cells was consistently in the lower plane of focus regardless of the type or length of treatment. In TM cells subjected to 3 hour treatments of either ethanol or dexamethasone, meta-vinculin can be seen concentrated around the cells' periphery. Conversely, in TM cells that underwent chronic treatment with either ethanol or dexamethasone, meta-vinculin can be seen anchoring cells to the substrate through out the lower plane of focus.

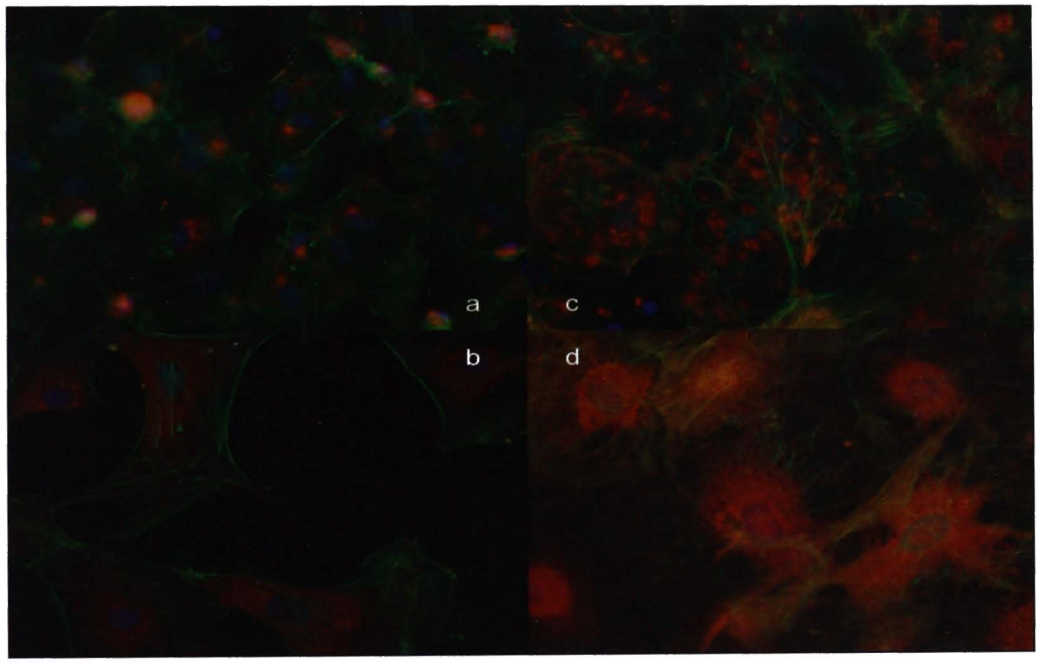
Figure 13: Immunofluorescence Staining of Meta-Vinculin (red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.



Immunofluorescence staining of zyxin shows that regardless of length of treatment, ethanol treated TM cells have concentrated pockets of zyxin while TM cells treated with dexamethasone have more even dispersal of zyxin through out the cytoplasm. However, none of the acute or chronic treatments led to the co-localization of

zyxin with the actin cytoskeleton or CLAN structures. Rather, zyxin appears to be localized around the nuclear envelope.

Figure 14: Immunofluorescence Staining of Zyxin (red), F-actin (green), and Nuclei (blue) of TM Cells. (a): cells cultured for 3 hours in media containing 0.1% ethanol on a fibronectin treated cover slip, (b): cells cultured for 3 hours in media containg 100nM dexamethasone on a fibronectin treated cover slip, (c): cells cultured for 2 weeks in media containing 0.1% ethanol on a gelatin treated cover slip, (d): cells cultured for 2 weeks in 100nM dexamethasone on a gelatin treated cover slip.



Since the western immunoblots indicate the labeling of the coronin antibody to be non-specific, further attempts to utilize this antibody for immunofluorescence staining were abandoned.

To summarize the findings of the immunofluorescence studies, the morphology of trabecular meshwork cells which were allowed to become confluent prior to chronic treatment with either dexamethasone or ethanol was different from that of cells only

allowed three hours exposure. Chronically treated TM cells contained many more cells per cover slip, often with two nuclei per cell while acutely treated cells had fewer cells per cover slip. In addition, acutely treated cells had long processes around their periphery illustrating their attempt to attach to the cover slip while confluent TM cells were more normal in appearance with fewer long processes. Although both acute treatments and chronic treatments with dexamethasone produced CLAN structures, there are differences. Trypsin-treated TM cells cultured for 3 hours in the presence of dexamethasone look similar to control treated cells. The CLANs produced in these acute glucocorticoid-experiments appear to be elongated net-like structures around the cells' periphery. Additionally, much of the actin cytoskeleton is localized at the cells' periphery. Conversely, chronic glucocorticoid-experiments produce CLANs located not only at the cells' periphery but throughout the widely distributed cytoskeleton. Since many cells types in culture will undergo a cytoskeletal rearrangement (Glukhova and Thiery, 1993; Nag et al., 1986) similar to that seen in the acute glucocorticoid-experiments, I have concluded that these structures are the same type of transient cytoskeletal rearrangement and due to the process of cells attaching to the substrate instead of being a steroid response.

Figure 15: Comparison of Acute and Chronic CLANs. Human TM cells cultured in media containing no treatment for 3 hours on a fibronectin-coated cover slip (left) and human TM cells cultured in media containing 100nM dexamethasone for 2 weeks on a gelatin-coated cover slip (right).

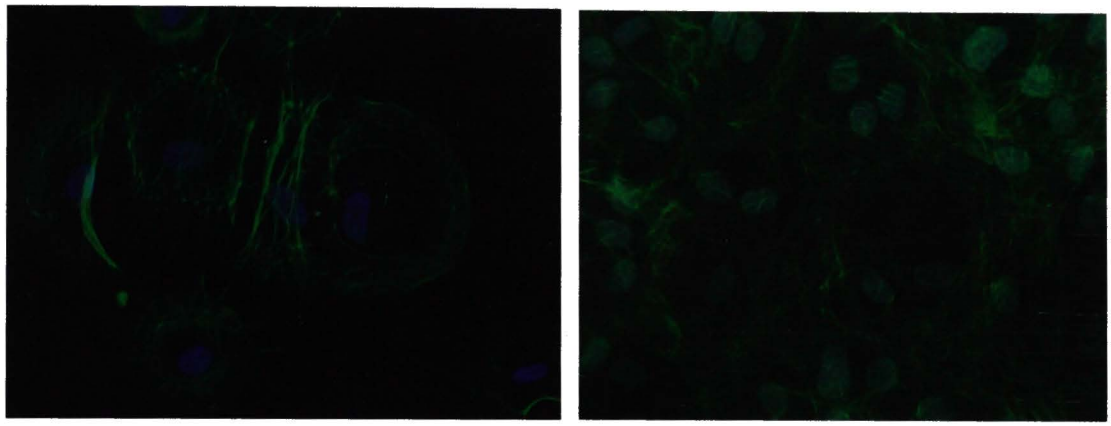


Table 9: Results summary of the evaluation of 8 different actin binding proteins for dexamethasone-induction of gene expression (QPCR), protein expression (western immunoblot), and for cellular distribution (immunofluorescence).

Protein Candidates	QPCR Expression	Western Immunoblot	Immunofluorescence
Nebulette (NEBL)	no amplification	N/A	N/A
α-actin (ACTA2)	1.5 fold increase	42 kDa band	along actin fibers
α-actinin (ACTN1)	1 fold increase	100 kDa band	beads along actin fibers and at CLAN vertices
Coronin 1c (COR1c)	no change	multiple, non-specific bands	non-specific labeling
LIM & SH3 Protein (LASP1)	0.5 fold increase	35 kDa band 63 kDa band	cytoplasmic staining, does not co-localize with CLANs
Syndecan-4 (SCD4)	no change	23 kDa band	cytoplasmic staining, does not co-localize with CLANs
Meta-Vinculin (VCL)	9 fold increase	124 kDa band	anchor protein in lower plane of focus (F-actin and CLANs)
Zyxin (ZYX)	no change	83 kDa band	DEX response but not co-localized with CLANs

CHAPTER IV

DISCUSSION

Using the cell culture model of steroid-induced ocular hypertension, specific proteins may be identified that associate with cross-linked actin networks (CLANs). Since CLANs are proposed to play a role in the development of elevated intraocular pressure (IOP), the identification of elements leading to the formation of CLANs could explain molecular mechanisms involved in the formation of glaucoma. The relative abundance of these CLAN-forming proteins in normal vs. glaucomatous cell lines could also provide insight into the significance of such proteins in the disease process. My hypothesis stated the altered expression of actin-associated proteins is responsible for glucocorticoid- and glaucoma-induced actin cytoskeletal rearrangements in trabecular meshwork cells. More specifically, the rearrangement to form cross-linked actin networks (CLANs) leads to a more static cytoskeleton, altered cell shape and function, which contribute to increased outflow resistance and ocular hypertension.

Although altered gene expression was not confirmed in all actin binding protein candidates, chronic glucocorticoid treatment did lead to increases in gene expression of myocilin, α-actin, α-actinin, LASP1 and meta-vinculin. Immunofluorescence studies show that α-actin is a component found throughout the actin cytoskeleton as well as in the beams and vertices of cross-linked actin networks (CLANs). Similarly, α-actinin was found in clusters or "beads" along the fibers of the actin cytoskeleton and within the vertices of some CLANs. However, its expression was significantly less than that of α-

actin and the role of α-actinin remains unclear. Apparently α-actinin is not required for the cytoskeleton of TM cells to form CLANs since not all CLAN-structures stained for α-actinin. Also meta-vinculin staining suggests its main function is as a focal adhesion anchor protein. Immunofluorescence staining of meta-vinculin demonstrates the ability of this protein to bind to the actin cytoskeleton regardless of the presence of CLAN-structures.

Even though steroid-induced changes in the gene expression of zyxin were not confirmed, immunofluorescence staining of zyxin in cultured TM cells suggests there may be a response to chronic dexamethasone treatment. A change in the distribution of zyxin from cytoplasmic pockets of zyxin in control cells to a more even scattering of zyxin in dexamethasone-treated cells was observed in several immunofluorescence staining experiments. I think it is important to note that at no time was zyxin staining co-localized with any structure of the actin cytoskeleton. Similarly, LASP1 staining remained within the cytoplasmic spaces and apart from the actin cytoskeleton. Since functional database information (www.genecards.org) suggests a connection between nebulette, LASP1, and zyxin, it is tempting to speculate on their role in cytoskeletal regulation in human TM cells.

Candidate proteins were selected by data available through Affymatrix gene expression database searches, but it is also likely that proteins are involved that do not exhibit changes in gene expression. Pathways other than gene expression could influence the location as well as the function of proteins regulating the actin cytoskeleton. The current group of experiments does not take into account post-translational modifications,

phosphorylation for example. It is also possible that mechanical stress or glucocorticoids are part of an unknown signal transductions system that leads to a reorganization of the cytoskeleton and its associated proteins. The actin binding proteins included in these experiments are merely a sampling of possible candidates and there could be other endpoints which remain unknown.

Previous publications show alpha-actinin (Clark et al., 1996; Clark, 1995b; Clark et al., 1995; Clark et al., 1994; Clark, 2005) and meta-vinculin are associated with cytoskeletal rearrangements to form CLANs following dexamethasone treatment. Although I was able to reproduce these results, smooth muscle α-actinin was not always visible along the actin stress fibers nor was it required for CLAN formation. In addition, meta-vinculin was consistently observed in the lower plane of focus anchoring both the actin stress fibers and CLANs to the cover slip. Meta-vinculin was not observed in CLANs located in the upper planes of focus nor was it localized within CLAN vertices. Therefore, the role of meta-vinculin in a more static cytoskeleton could be assumed to be limited to areas such as basement membranes to which layers of trabeculae would adhere.

In early 2006, Rozsa et al. published (Rozsa et al., 2006; Wordinger and Clark, 1999) an extensive list of genes, including some acting binding proteins and cytoskeletal elements, in which changes in gene expression are reported following treatment with dexamethasone for 21 days. Of the more than 18,000 genes studied, Rozsa et al. found 23 up regulated and 18 down regulated genes with greater than 3-fold changes in three human TM cell lines. Interestingly, Rozsa et al. were able to show a 12-fold increase in the expression of nebulette, an 8-fold increase in α-actin, and 2.9-fold increase in filamin-

B by QPCR. The large increase in nebulette expression was surprising since I was unable to amplify nebulette cDNA in any of my human TM cell lines regardless of treatment. Also the 8-fold increase in α-actin was more than double the increase seen in my QPCR experiments. I believe these differences may be attributed to differences in the levels of steroid-responsiveness between TM cell lines. Since I did not perform any experiments including 21 day treatments with dexamethasone, I am not certain of the effects of a longer treatment on gene expression.

In May of 2006, a report was published stating syndecan-4 is localized at the vertices of actin filaments which make up CLAN structures and argue that brief exposures to glucocorticoids are sufficient to produce CLANs in cultured TM cells. (Filla et al., 2006) However, the treatment of TM cells forming these structures is quite different from the treatments used to induce CLANs by Clark et al. Peters et al. cultured human TM cells for 2-10 days in the presence of dexamethasone, then trypsin-treated the cells, and plated them onto cover slips coated in 100nM fibronectin. After incubation in cell culture media containing 500nM dexamethasone for 3 hours, TM cells were fixed to the cover slips and stained for syndecan-4. Conversely, Clark et al. cultured human TM cells on cover slips until they were confluent for one week, treated cells with 100nM dexamethasone for 2 weeks, and fixed the cells for staining. A comparison of these methods has shown both are able to produce CLANs, however, the structure of the CLANs and cells are dissimilar and, more importantly, syndecan-4 was neither found within the actin cytoskeleton nor within CLAN structures. The acute treatments with glucocorticoids favored by Peters et al. lead to elongated-transient CLANs at the

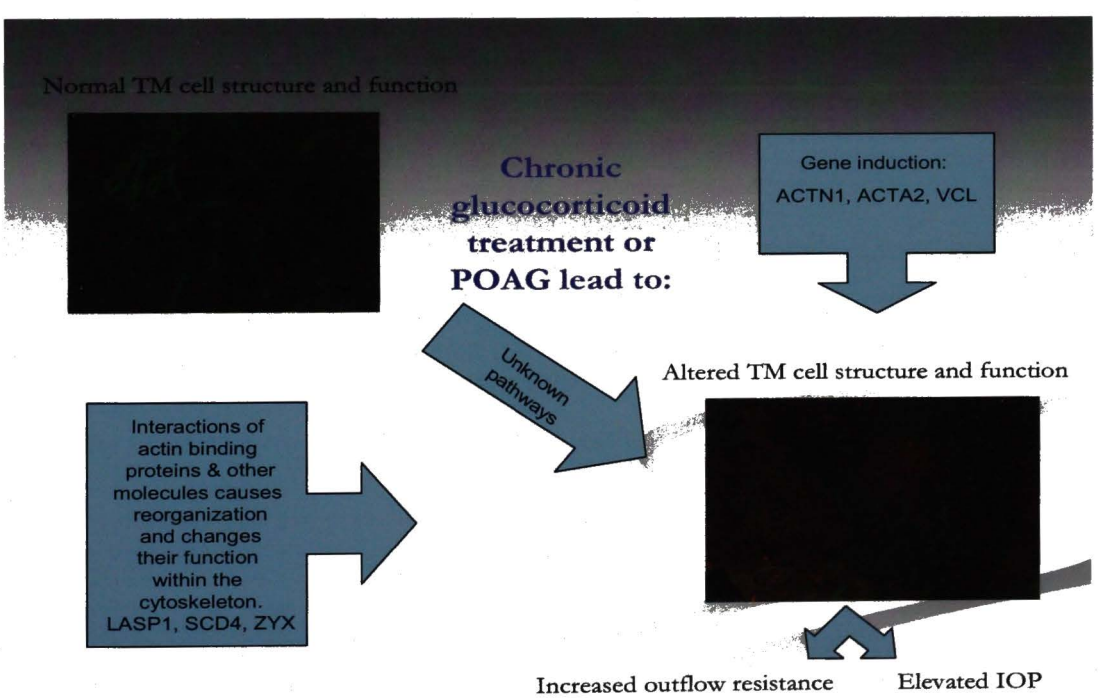
periphery of cells and many of these cells have a sun-burst appearance. I believe these structures are a result of the cell attachment process rather than any response to a brief encounter with dexamethasone. On the other hand, the chronic treatments with glucocorticoids favored by Clark et al. lead to several different cross-linked actin structures throughout the cytoskeleton of cultured TM cells. CLANs produced in a confluent TM cell culture by chronic dexamethasone treatment include a continuum of structures ranging from geodesic domes to cross-linking structures not only at the cells' periphery but potentially anywhere within the actin cytoskeleton.

To supplement the current group of experiments, 1D or 2D gels could be used to visualize differences in proteins associated with the TM cytoskeleton, and the protein bands removed for further analysis. Each protein band could then be digested using Trypsin and protein fragments could be sequenced by liquid chromatography mass spectrophotometry (LC/MS). Finally, the sequence of protein fragments could be compared to each antibody's target peptide to confirm target specificity.

To enhance immunofluorescence studies, either a deconvolution microscope or a confocal microscope could be utilized to see the spatial relationship of actin binding proteins in relation to the actin cytoskeleton and CLANs. Perhaps some of these protein candidates, such as nebulette, LASP1, or zyxin, interact with other actin binding proteins to influence the shape and function of the TM cell's cytoskeleton. Since actin microfilaments are involved in organelle trafficking and signal transduction, any disruption in the normal functions of cytoskeletal systems could have significant impact on the function of TM cells and the entire mesh. It is widely accepted that chronic

glucocorticoid treatment leads to a more static TM cytoskeleton. Although there are numerous theories of the mechanism leading to this stasis, many investigators agree that chronic glucocorticoid treatment leads to an increase in outflow resistance and elevated intraocular pressure in human TM cells. Therefore, it is useful to strive to better understand the mechanisms and signaling processes which affect the TM actin cytoskeleton in order to gain insight into the causes which lead to the complex group of diseases known as glaucoma.

Figure 16: Summary figure depicting the change from normal TM cell structure and function due to chronic glucocorticoid treatment or glaucoma to altered TM cell structure and function, for example CLANs. Three possible pathways could lead to this change. First, glucocorticoid treatement or the glaucomatous disease process could lead to changes in gene expression of particular actin binding proteins which are associated with CLANs. Second, actin binding proteins already present in the cell could interact with other molecules and lead to cytoskeletal rearrangements. Finally, unknown pathways could influence cellular processes and lead to a more static TM cytoskeleton.



CHAPTER VI

REFERENCES

http://www.genecards.org/.

2001. The Advanced Glaucoma Intervention Study (AGIS): 9. Comparison of glaucoma outcomes in black and white patients within treatment groups. *Am J Ophthalmol.* 132:311-20.

Alvarado, J., C. Murphy, and R. Juster. 1984. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. *Ophthalmology.* 91:564-79.

Alvarado, J., C. Murphy, J. Polansky, and R. Juster. 1981. Age-related changes in trabecular meshwork cellularity. *Invest Ophthalmol Vis Sci.* 21:714-27.

Alvarado, J.A., A. Betanzos, L. Franse-Carman, J. Chen, and L. Gonzalez-Mariscal. 2004. Endothelia of Schlemm's canal and trabecular meshwork: distinct molecular, functional, and anatomic features. *Am J Physiol Cell Physiol.* 286:C621-34.

Alward, W.L. 2003. Evaluation of optineurin sequence variations in 1,048 patients with open-angle glaucoma. *Am J Ophthalmol.* 136:904-910.

Alward, W.L., Y.H. Kwon, C.L. Khanna, A.T. Johnson, S.S. Hayreh, M.B. Zimmerman, J. Narkiewicz, J.L. Andorf, P.A. Moore, J.H. Fingert, V.C. Sheffield, and E.M. Stone. 2002. Variations in the myocilin gene in patients with open-angle glaucoma. *Arch Ophthalmol.* 120:1189-97.

Alward, W.L.M. 2000. Glaucoma. The Requisites in Ophthalmology. *In* The Requisites in Ophthalmology. J.H. Krachmer, editor. Mosby, St. Louis, MO.

American Academy of Ophthalmology, G.P. 2000. Primary Open-Angle Glaucoma. Preferred Practice Pattern. . *In* American Academy of Ophthalmology, San Francisco, CA. 1-36.

Anderson, D.R. 2003. Collaborative normal tension glaucoma study. *Curr Opin Ophthalmol.* 14:86-90.

Armaly, M.F. 1963. Effect of Corticosteroids on Intraocular Pressure and Fluid Dynamics. I. the Effect of Dexamethasone in the Normal Eye. *Arch Ophthalmol.* 70:482-91.

Armaly, M.F. 1966. The heritable nature of dexamethasone-induced ocular hypertension. *Arch Ophthalmol.* 75:32-5.

Azuara-Blanco, A., A. Harris, L.B. Cantor, M.M. Abreu, and M. Weinland. 1998. Effects of short term increase of intraocular pressure on optic disc cupping. *Br J Ophthalmol.* 82:880-3.

Bartlett, J.D., T.W. Woolley, and C.M. Adams. 1993. Identification of high intraocular pressure responders to topical ophthalmic corticosteroids. *J Ocul Pharmacol.* 9:35-45.

Becker, B., and N. Ballin. 1966. Glaucoma. *Annu Rev Med.* 17:235-42.

Becker, B., and K.A. Hahn. 1964. Topical Corticosteroids and Heredity in Primary Open-Angle Glaucoma. *Am J Ophthalmol.* 57:543-51.

Bernstein, H.N., and B. Schwartz. 1962. Effects of long-term systemic steroids on ocular pressure and tonographic values. *Arch Ophthalmol.* 68:742-53.

Broman, A.T., B. Munoz, J. Rodriguez, R. Sanchez, H.A. Quigley, R. Klein, R. Snyder, and S.K. West. 2002. The impact of visual impairment and eye disease on vision-related quality of life in a Mexican-American population: proyecto VER. *Invest Ophthalmol Vis Sci.* 43:3393-8.

Burgoyne, C.F., J.C. Downs, A.J. Bellezza, J.K. Suh, and R.T. Hart. 2005. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Prog Retin Eye Res.* 24:39-73.

Burgoyne, C.F., R. Varma, H.A. Quigley, S. Vitale, M.E. Pease, and P.L. Lenane. 1994. Global and regional detection of induced optic disc change by digitized image analysis. *Arch Ophthalmol.* 112:261-8.

Chauhan, B.C., J. Pan, M.L. Archibald, T.L. LeVatte, M.E. Kelly, and F. Tremblay. 2002. Effect of intraocular pressure on optic disc topography, electroretinography, and axonal loss in a chronic pressure-induced rat model of optic nerve damage. *Invest Ophthalmol Vis Sci.* 43:2969-76.

Chihara, E., X. Liu, J. Dong, Y. Takashima, M. Akimoto, M. Hangai, S. Kuriyama, H. Tanihara, M. Hosoda, and S. Tsukahara. 1997. Severe myopia as a risk factor for progressive visual field loss in primary open-angle glaucoma. *Ophthalmologica.* 211:66-71.

Clark, A. 1995a. Steroids, Ocular Hypertension, and Glaucoma. *Journal of Glaucoma.* 4:354-369.

Clark, A.F., D. Lane, K. Wilson, S.T. Miggans, and M.D. McCartney. 1996. Inhibition of dexamethasone-induced cytoskeletal changes in cultured human trabecular meshwork cells by tetrahydrocortisol. *Invest Ophthalmol Vis Sci.* 37:805-13.

Clark, A.F., S.T. Miggans, K. Wilson, S. Browder, M.D. McCartney. 1995b. Cytoskeletal Changes in Cultured Human Glaucoma Trabecular Meshwork Cells. *Journal of Glaucoma.* 4:183-188.

Clark, A.F., K. Wilson, A.W. de Kater, R.R. Allingham, and M.D. McCartney. 1995. Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci.* 36:478-89.

Clark, A.F., K. Wilson, M.D. McCartney, S.T. Miggans, M. Kunkle, and W. Howe. 1994. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 35:281-94.

Clark, A.F.B., Daniel. Read, A Thomas. Hellberg, Peggy. English-Wright, Sherry. Pang, Iok-Hou. Ethier, C Ross. Grierson, Ian. 2005. Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. *Cell Motility & the Cytoskeleton. 60(2):83-95, 2005 Feb.* 60:83-95.

Coleman, A.L., H.A. Quigley, S. Vitale, and G. Dunkelberger. 1991. Displacement of the optic nerve head by acute changes in intraocular pressure in monkey eyes. *Ophthalmology.* 98:35-40.

Daniel Vaughan, T.A., Khalid F. Tabbara. 1989. General Ophthalmology. Prentice Hall, Englewood Cliffs, New Jersey.

Distelhorst, J.S., and G.M. Hughes. 2003. Open-angle glaucoma. *Am Fam Physician.* 67:1937-44.

Eric R. Kandel, J.H.S., Thomas M. Jessell. 2000. Principles of Neural Science. J.H.S. Eric R. Kandel, Thomas M. Jessell, editor. McGraw-Hill, St. Louis, MO.

Fan, B.J., Y.F. Leung, N. Wang, S.C. Lam, Y. Liu, O.S. Tam, and C.P. Pang. 2004. Genetic and environmental risk factors for primary open-angle glaucoma. *Chin Med J (Engl).* 117:706-10.

Fan, B.J., D.Y. Wang, D.S. Lam, and C.P. Pang. 2006. Gene mapping for primary open angle glaucoma. *Clin Biochem.* 39:249-58.

Feiner, L., and J.R. Piltz-Seymour. 2003. Collaborative Initial Glaucoma Treatment Study: a summary of results to date. *Curr Opin Ophthalmol.* 14:106-11.

Filla, M.S., A. Woods, P.L. Kaufman, and D.M. Peters. 2006. Beta1 and beta3 integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 47:1956-67.

Fingert, J.H., A.F. Clark, J.E. Craig, W.L. Alward, G.R. Snibson, M. McLaughlin, L. Tuttle, D.A. Mackey, V.C. Sheffield, and E.M. Stone. 2001. Evaluation of the myocilin (MYOC) glaucoma gene in monkey and human steroid-induced ocular hypertension. *Invest Ophthalmol Vis Sci.* 42:145-52.

Fingert, J.H., E. Heon, J.M. Liebmann, T. Yamamoto, J.E. Craig, J. Rait, K. Kawase, S.T. Hoh, Y.M. Buys, J. Dickinson, R.R. Hockey, D. Williams-Lyn, G. Trope, Y. Kitazawa, R. Ritch, D.A. Mackey, W.L. Alward, V.C. Sheffield, and E.M. Stone. 1999. Analysis of myocilin mutations in 1703 glaucoma patients from five different populations. *Hum Mol Genet.* 8:899-905.

Flammer, J., S. Orgul, V.P. Costa, N. Orzalesi, G.K. Krieglstein, L.M. Serra, J.P. Renard, and E. Stefansson. 2002. The impact of ocular blood flow in glaucoma. *Prog Retin Eye Res.* 21:359-93.

Fleenor, D.L., A.R. Shepard, P.E. Hellberg, N. Jacobson, I.H. Pang, and A.F. Clark. 2006. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci.* 47:226-34.

Fuchsjager-Mayrl, G., B. Wally, M. Georgopoulos, G. Rainer, K. Kircher, W. Buehl, T. Amoako-Mensah, H.G. Eichler, C. Vass, and L. Schmetterer. 2004. Ocular blood flow and systemic blood pressure in patients with primary open-angle glaucoma and ocular hypertension. *Invest Ophthalmol Vis Sci.* 45:834-9.

Galassi, F., A. Sodi, F. Ucci, A. Harris, and H.S. Chung. 1998. Ocular haemodynamics in glaucoma associated with high myopia. *Int Ophthalmol.* 22:299-305.

Gazzard, G., P.J. Foster, J.G. Devereux, F. Oen, P. Chew, P.T. Khaw, and S. Seah. 2003. Intraocular pressure and visual field loss in primary angle closure and primary open angle glaucomas. *Br J Ophthalmol.* 87:720-5.

Glukhova, M.A., and J.P. Thiery. 1993. Fibronectin and integrins in development. *Semin Cancer Biol.* 4:241-9.

Gobeil, S., M.A. Rodrigue, S. Moisan, T.D. Nguyen, J.R. Polansky, J. Morissette, and V. Raymond. 2004. Intracellular sequestration of hetero-oligomers formed by wild-type and glaucoma-causing myocilin mutants. *Invest Ophthalmol Vis Sci.* 45:3560-7.

Gordon, M.O., and M.A. Kass. 1999. The Ocular Hypertension Treatment Study: design and baseline description of the participants. *Arch Ophthalmol.* 117:573-83.

Gould, D.B., R.S. Smith, and S.W. John. 2004. Anterior segment development relevant to glaucoma. *Int J Dev Biol.* 48:1015-29.

Grieshaber, M.C., and J. Flammer. 2005. Blood flow in glaucoma. *Curr Opin Ophthalmol.* 16:79-83.

Hausman, G.M.C.a.R.E. 2004. The Cell: A Molecular Approach. ASM Press (The American Society for Microbiology), Washington DC.

Hernandez, M.R. 2000. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. *Prog Retin Eye Res.* 19:297-321.

Hitchings, R.A. 1997. Glaucoma. *Eye.* 11 (Pt 6):900-3.

Hogg, P., M. Calthorpe, M. Batterbury, and I. Grierson. 2000. Aqueous humor stimulates the migration of human trabecular meshwork cells in vitro. *Invest Ophthalmol Vis Sci.* 41:1091-8.

Jacobson, N., M. Andrews, A.R. Shepard, D. Nishimura, C. Searby, J.H. Fingert, G. Hageman, R. Mullins, B.L. Davidson, Y.H. Kwon, W.L. Alward, E.M. Stone, A.F. Clark, and V.C. Sheffield. 2001. Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor. *Hum Mol Genet.* 10:117-25.

Jonas, J.B., E. Berenshtein, and L. Holbach. 2004. Lamina cribrosa thickness and spatial relationships between intraocular space and cerebrospinal fluid space in highly myopic eyes. *Invest Ophthalmol Vis Sci.* 45:2660-5.

Jonas, J.B., and W.M. Budde. 2000. Optic nerve head appearance in juvenile-onset chronic high-pressure glaucoma and normal-pressure glaucoma. *Ophthalmology.* 107:704-11.

Kamal, D., and R. Hitchings. 1998. Normal tension glaucoma--a practical approach. *Br J Ophthalmol.* 82:835-40.

Katzung, B.G. 2001. Basic & Clinical Pharmacology. Lange Medical Books/McGraw-Hill, St, Louis, MO. 1217 pp.

Kerr, J., P. Nelson, and C. O'Brien. 2003. Pulsatile ocular blood flow in primary open-angle glaucoma and ocular hypertension. *Am J Ophthalmol.* 136:1106-13.

Kitazawa, Y., and T. Horie. 1981. The prognosis of corticosteroid-responsive individuals. *Arch Ophthalmol.* 99:819-23.

Klein, B.E., R. Klein, and M.D. Knudtson. 2005. Intraocular pressure and systemic blood pressure: longitudinal perspective: the Beaver Dam Eye Study. *Br J Ophthalmol.* 89:284-7.

Lam, D.S., Y.F. Leung, J.K. Chua, L. Baum, D.S. Fan, K.W. Choy, and C.P. Pang. 2000. Truncations in the TIGR gene in individuals with and without primary open-angle glaucoma. *Invest Ophthalmol Vis Sci.* 41:1386-91.

Lee, B.L., and M.R. Wilson. 2003. Ocular Hypertension Treatment Study (OHTS) commentary. *Curr Opin Ophthalmol.* 14:74-7.

Leske, M.C., A. Heijl, M. Hussein, B. Bengtsson, L. Hyman, and E. Komaroff. 2003. Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. *Arch Ophthalmol.* 121:48-56.

Levy, N.S., and E.E. Crapps. 1984. Displacement of optic nerve head in response to short-term intraocular pressure elevation in human eyes. *Arch Ophthalmol.* 102:782-6.

Libby, R.T., D.B. Gould, M.G. Anderson, and S.W. John. 2005. Complex genetics of glaucoma susceptibility. *Annu Rev Genomics Hum Genet.* 6:15-44.

Lichter, P.R. 2003. Glaucoma clinical trials and what they mean for our patients. *Am J Ophthalmol.* 136:136-45.

Liu, X., S. Cai, A. Glasser, T. Volberg, J.R. Polansky, D.J. Fauss, C.R. Brandt, B. Geiger, and P.L. Kaufman. 2001. Effect of H-7 on cultured human trabecular meshwork cells. *Mol Vis.* 7:145-53.

Liu, X., Z. Wu, N. Sheibani, C.R. Brandt, J.R. Polansky, and P.L. Kaufman. 2003. Low dose latrunculin-A inhibits dexamethasone-induced changes in the actin cytoskeleton and alters extracellular matrix protein expression in cultured human trabecular meshwork cells. *Exp Eye Res.* 77:181-8.

Liu, Y., and D. Vollrath. 2004. Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. *Hum Mol Genet.* 13:1193-204.

Mayama, C., Y. Suzuki, M. Araie, K. Ishida, T. Akira, T. Yamamoto, Y. Kitazawa, S. Funaki, M. Shirakashi, H. Abe, H. Tsukamoto, K. Okada, and H.K. Mishima. 2002. Myopia and advanced-stage open-angle glaucoma. *Ophthalmology.* 109:2072-7.

Miller, D., J.D. Peczon, and C.G. Whitworth. 1965. Corticosteroids and Functions in the Anterior Segment of the Eye. *Am J Ophthalmol.* 59:31-4.

Morrison, J.C., E.C. Johnson, W. Cepurna, and L. Jia. 2005. Understanding mechanisms of pressure-induced optic nerve damage. *Prog Retin Eye Res.* 24:217-40.

Nag, A.C., W. Krehel, and M. Cheng. 1986. Distributions of vimentin and desmin filaments in embryonic cardiac muscle cells in culture. *Cytobios.* 45:195-209.

Netland, P.A., J.L. Wiggs, and E.B. Dreyer. 1993. Inheritance of glaucoma and genetic counseling of glaucoma patients. *Int Ophthalmol Clin.* 33:101-20.

O'Brien, E.T., S.L. Perkins, B.C. Roberts, and D.L. Epstein. 1996. Dexamethasone inhibits trabecular cell retraction. *Exp Eye Res.* 62:675-88.

Papadia, M., C. Sofianos, M. Iester, G. Bricola, M. Mete, and C.E. Traverso. 2006. Corneal thickness and visual field damage in glaucoma patients. *Eye.*

Partridge, C.A., B.I. Weinstein, A.L. Southren, and M.E. Gerritsen. 1989. Dexamethasone induces specific proteins in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 30:1843-7.

Perkins, T.W., J.A. Alvarado, J.R. Polansky, L. Stilwell, M. Maglio, and R. Juster. 1988. Trabecular meshwork cells grown on filters. Conductivity and cytochalasin effects. *Invest Ophthalmol Vis Sci.* 29:1836-46.

Peterson, J.A., B. Tian, J.W. McLaren, W.C. Hubbard, B. Geiger, and P.L. Kaufman. 2000. Latrunculins' effects on intraocular pressure, aqueous humor flow, and corneal endothelium. *Invest Ophthalmol Vis Sci.* 41:1749-58.

Piette, S.D., and R.C. Sergott. 2006. Pathological optic-disc cupping. *Curr Opin Ophthalmol.* 17:1-6.

Polansky, J.R., D.J. Fauss, P. Chen, H. Chen, E. Lutjen-Drecoll, D. Johnson, R.M. Kurtz, Z.D. Ma, E. Bloom, and T.D. Nguyen. 1997. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica.* 211:126-39.

Pollack, J.C.M.a.I.P. 2003. Glaucoma: Science and Practice. *In* Glaucoma: Science and Practice. J.C.M.a.I.P. Pollack, editor. Thieme, New York.

Quigley, H.A. 1999. Neuronal death in glaucoma. *Prog Retin Eye Res.* 18:39-57.

Quigley, H.A. 2005. New paradigms in the mechanisms and management of glaucoma. *Eye.* 19:1241-8.

Quigley, H.A., and A.T. Broman. 2006. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol.* 90:262-7.

Quigley, H.A., R.M. Sanchez, G.R. Dunkelberger, N.L. L'Hernault, and T.A. Baginski. 1987. Chronic glaucoma selectively damages large optic nerve fibers. *Invest Ophthalmol Vis Sci.* 28:913-20.

Rezaie, T., A. Child, R. Hitchings, G. Brice, L. Miller, M. Coca-Prados, E. Heon, T. Krupin, R. Ritch, D. Kreutzer, R.P. Crick, and M. Sarfarazi. 2002. Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science.* 295:1077-9.

Rhen, T., and J.A. Cidlowski. 2005. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med.* 353:1711-23.

Robert A. Moses and William M. Hart, J. 1987. Alder's Physiology of the Eye. Clinical Application. *In* Alder's Physiology of the Eye. E.A. Klein, editor. The C. V. Mosby Company, St. Louis. 709.

Rotchford, A. 2005. What is practical in glaucoma management? *Eye.* 19:1125-32.

Rozsa, F.W., D.M. Reed, K.M. Scott, H. Pawar, S.E. Moroi, T.G. Kijek, C.M. Krafchak, M.I. Othman, D. Vollrath, V.M. Elner, and J.E. Richards. 2006. Gene expression profile of human trabecular meshwork cells in response to long-term dexamethasone exposure. *Mol Vis.* 12:125-41.

Shepard, A.R., N. Jacobson, J.H. Fingert, E.M. Stone, V.C. Sheffield, and A.F. Clark. 2001. Delayed secondary glucocorticoid responsiveness of MYOC in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 42:3173-81.

Shields, M.B., E. Buckley, G.K. Klintworth, and R. Thresher. 1985. Axenfeld-Rieger syndrome. A spectrum of developmental disorders. *Surv Ophthalmol.* 29:387-409.

Spaeth, R.V.a.G.L. 1993. The Optic Nerve in Glaucoma. R. Varma, editor. J.B. Lippincott Company, Philadelphia, PA. 354.

Sponsel, W.E., J. Shoemaker, Y. Trigo, J. Mensah, R. Rugwani, and B. Garrett. 2001. Frequency of sustained glaucomatous-type visual field loss and associated optic nerve cupping in Beaver Dam, Wisconsin. *Clin Experiment Ophthalmol.* 29:352-8.

Stokely, M.E., T. Yorio, and M.A. King. 2005. Endothelin-1 modulates anterograde fast axonal transport in the central nervous system. *J Neurosci Res.* 79:598-607.

Stone, E.M., J.H. Fingert, W.L. Alward, T.D. Nguyen, J.R. Polansky, S.L. Sunden, D. Nishimura, A.F. Clark, A. Nystuen, B.E. Nichols, D.A. Mackey, R. Ritch, J.W. Kalenak, E.R. Craven, and V.C. Sheffield. 1997. Identification of a gene that causes primary open angle glaucoma. *Science.* 275:668-70.

Sullivan-Mee, M., K.D. Halverson, M.C. Saxon, G.B. Saxon, and C. Qualls. 2006. Central corneal thickness and normal tension glaucoma: a cross-sectional study. *Optometry*. 77:134-40.

Tamm, E.R. 2002. Myocilin and glaucoma: facts and ideas. *Prog Retin Eye Res*. 21:395-428.

Tan, J.C., F.B. Kalapesi, and M.T. Coroneo. 2006a. Mechanosensitivity and the eye: cells coping with the pressure. *Br J Ophthalmol*. 90:383-8.

Tan, J.C., D.M. Peters, and P.L. Kaufman. 2006b. Recent developments in understanding the pathophysiology of elevated intraocular pressure. *Curr Opin Ophthalmol*. 17:168-74.

Tezel, G., and M.B. Wax. 2004. The immune system and glaucoma. *Curr Opin Ophthalmol*. 15:80-4.

Tian, B., B. Geiger, D.L. Epstein, and P.L. Kaufman. 2000. Cytoskeletal involvement in the regulation of aqueous humor outflow. *Invest Ophthalmol Vis Sci*. 41:619-23.

Tielsch, J.M., J. Katz, K. Singh, H.A. Quigley, J.D. Gottsch, J. Javitt, and A. Sommer. 1991a. A population-based evaluation of glaucoma screening: the Baltimore Eye Survey. *Am J Epidemiol*. 134:1102-10.

Tielsch, J.M., A. Sommer, J. Katz, R.M. Royall, H.A. Quigley, and J. Javitt. 1991b. Racial variations in the prevalence of primary open-angle glaucoma. The Baltimore Eye Survey. *Jama*. 266:369-74.

Tokunaga, T., K. Kashiwagi, T. Tsumura, K. Taguchi, and S. Tsukahara. 2004. Association between nocturnal blood pressure reduction and progression of visual field defect in patients with primary open-angle glaucoma or normal-tension glaucoma. *Jpn J Ophthalmol*. 48:380-5.

Tripathi, B.J., R.C. Tripathi, and H.H. Swift. 1989. Hydrocortisone-induced DNA endoreplication in human trabecular cells in vitro. *Exp Eye Res*. 49:259-70.

Underwood, J.L., C.G. Murphy, J. Chen, L. Franse-Carman, I. Wood, D.L. Epstein, and J.A. Alvarado. 1999. Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. *Am J Physiol*. 277:C330-42.

Vesti, E., and T. Kivel a. 2000. Exfoliation syndrome and exfoliation glaucoma. *Prog Retin Eye Res*. 19:345-68.

Vollrath, D., V.L. Jaramillo-Babb, M.V. Clough, I. McIntosh, K.M. Scott, P.R. Lichter, and J.E. Richards. 1998. Loss-of-function mutations in the LIM-homeodomain gene, LMX1B, in nail-patella syndrome. *Hum Mol Genet.* 7:1091-8.

Wang, W.H., L.G. McNatt, A.R. Shepard, N. Jacobson, D.Y. Nishimura, E.M. Stone, V.C. Sheffield, and A.F. Clark. 2001. Optimal procedure for extracting RNA from human ocular tissues and expression profiling of the congenital glaucoma gene FOXC1 using quantitative RT-PCR. *Mol Vis.* 7:89-94.

Wax, M.B.T., Gulgun; Kobayashi, Shigeki; Hernandez, M Rosario. 2000. Responses of different cell lines from ocular tissues to elevated hydrostatic pressure. *British Journal of Ophthalmology.* 84:423-428.

Wiggs, J.L., K.F. Damji, J.L. Haines, M.A. Pericak-Vance, and R.R. Allingham. 1996. The distinction between juvenile and adult-onset primary open-angle glaucoma. *Am J Hum Genet.* 58:243-4.

Wiggs, J.L., E.A. Del Bono, J.S. Schuman, B.T. Hutchinson, and D.S. Walton. 1995. Clinical features of five pedigrees genetically linked to the juvenile glaucoma locus on chromosome 1q21-q31. *Ophthalmology.* 102:1782-9.

Wiggs, J.L., and D. Vollrath. 2001. Molecular and clinical evaluation of a patient hemizygous for TIGR/MYOC. *Arch Ophthalmol.* 119:1674-8.

Wolfs, R.C., P.H. Borger, R.S. Ramrattan, C.C. Klaver, C.A. Hulsman, A. Hofman, J.R. Vingerling, R.A. Hitchings, and P.T. de Jong. 2000. Changing views on open-angle glaucoma: definitions and prevalences--The Rotterdam Study. *Invest Ophthalmol Vis Sci.* 41:3309-21.

Wolfs, R.C., C.C. Klaver, R.S. Ramrattan, C.M. van Duijn, A. Hofman, and P.T. de Jong. 1998. Genetic risk of primary open-angle glaucoma. Population-based familial aggregation study. *Arch Ophthalmol.* 116:1640-5.

Wordinger, R.J., and A.F. Clark. 1999. Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. *Prog Retin Eye Res.* 18:629-67.

Xue, W., R. Wallin, E.A. Olmsted-Davis, and T. Borras. 2006. Matrix GLA protein function in human trabecular meshwork cells: inhibition of BMP2-induced calcification process. *Invest Ophthalmol Vis Sci.* 47:997-1007.

Zeimer, R.C., and Y. Ogura. 1989. The relation between glaucomatous damage and optic nerve head mechanical compliance. *Arch Ophthalmol.* 107:1232-4.

Zeppieri, M., P. Brusini, and S. Miglior. 2005. Corneal thickness and functional damage in patients with ocular hypertension. *Eur J Ophthalmol.* 15:196-201.

Zhang, X., A.F. Clark, and T. Yorio. 2005. Regulation of glucocorticoid responsiveness in glaucomatous trabecular meshwork cells by glucocorticoid receptor-beta. *Invest Ophthalmol Vis Sci.* 46:4607-16.