Published in final edited form as:

Prog Retin Eye Res. 2007 September ; 26(5): 453–469.

Cochlin in the eye: functional implications

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Abstract

Aqueous humor is actively produced in the ciliary epithelium of the anterior chamber and has important functions for the eye. Under normal physiological conditions, the inflow and outflow of the aqueous humor are tightly regulated, but in the pathologic state this balance is lost. Aqueous outflow involves structures of the anterior chamber and experiences most resistance at the level of the trabecular meshwork (TM) that acts as a filter. The modulation of the TM structure regulates the filter and its mechanism remains poorly understood. Proteomic analyses have identified cochlin, a protein of poorly understood function, in the glaucomatous TM but not in healthy control TM from human cadaver eyes. The presence of cochlin has subsequently been confirmed by Western and immunohistochemical analyses. Functionally, cochlin undergoes multimerization induced by shear stress and other changes in the microenvironment. Cochlin along with mucopolysaccharide deposits have been found in the TM of glaucoma patients and in the inner ear of subjects affected by the hearing disorder DNFA9, a late onset, progressive disease that also involves alterations in fluid shear regimes. *In vitro*, cochlin induces aggregation of primary TM cells suggesting a role in cell adhesion, possibly in mechanosensation, and in modulation of the TM filter.

1. Introduction

1.1 Introduction to aqueous humor outflow, glaucoma and cochlin

The aqueous humor flows within the anterior chamber and plays important functions in the eye, such as providing nutrition, removing the excretory products and contributing to the regulation of the homeostasis of the anterior eye segment (Ling et al., 2005). It is actively secreted by the ciliary epithelium and, after bathing the anterior chamber, exits through structures in the anterior chamber angle (Morrison and Acott, 2003). Normally, the majority of the aqueous humor leaves the eye by filtering through the trabecular meshwork (TM) into Schlemm's canal (SC) and into the aqueous veins. An alternative route to the aqueous outflow encompasses all pathways which do not involve the TM and is known as uveoscleral outflow (Bill, 1965; Bill, 1989). The contribution of this unconventional route to total outflow is dependent on age and varies between individuals, suggesting that it can play a more important role than TM outflow in some eyes (Toris et al., 1999).

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In humans, the TM is constituted of collagen beams covered by endothelial-like cells working as a filter. The space between the collagen beams is filled with extracellular matrix (ECM). Under normal physiological conditions, the aqueous inflow and outflow are tightly regulated and balanced. The modulation of the meshwork structures, resulting in regulation of the filter to meet the requirements for normal conditions, remains poorly understood. A decrease in the outflow that leads to elevated intraocular pressure (IOP) is an aberration from the normal state. Increased resistance to aqueous outflow at the TM can be caused by known mechanisms, such as accumulation of cells, changes in fluid viscosity and activation of coagulation factors. However, the central molecular and cellular mechanisms which underlie increased TM resistance remain unknown.

The TM endothelial cells produce mucopolysaccharides (MPS) that are important constituents of the ECM and play an essential role in regulating migration, proliferation and adhesion of macrophages (Laurent and Fraser, 1992; Knudson and Knudson, 1993) for phagocytic clearance of particulate material that enters the outflow system (Sherwood and Richardson, 1988; Johnson and Johnson, 2001). Abnormal MPS levels disrupt the self-cleaning process resulting in large changes in aqueous humor outflow and, subsequently, in elevation of IOP (Nickla et al., 2002).

Glaucoma refers to a group of late onset and progressive eye diseases that can cause damage to the optic nerve and result in vision loss. It is one of the leading causes of blindness worldwide (Bucher and Ijsselmuiden, 1988; Goldberg, 2000). The number of people affected by primary glaucoma varies in different reports. It is estimated that approximately 66.8 million people are affected (Quigley, 1996). One of the most prevalent forms is primary open-angle glaucoma (POAG), with approximately 33.1 million individuals affected around the world (Quigley, 1996; Goldberg, 2000). In the United States alone, it is estimated that 2.47 million people are affected by POAG, 5% of whom have become bilaterally blind (Quigley and Vitale, 1997). Glaucoma has a critical impact on the quality of life of a significant number of people (Alward, 2000). In spite of many advances in diagnosis and treatment of glaucoma, the fundamental causes remain unknown (Coleman, 2003). At present, elevated IOP is a major recognized risk factor for the development of glaucoma and it continues to be the most modifiable one.

Cochlin, a secretory ECM protein was identified in human POAG glaucomatous TM samples by classical proteomic comparisons of diseased and normal tissues, which was subsequently confirmed by Western and immunohistochemical analyses. Western analyses revealed that cochlin was present in the TM from POAG subjects and absent in the TM from normal, control donors. Similarly, cochlin containing deposits were found exclusively in glaucomatous but not in normal TM tissue (Bhattacharya et al., 2005b; Bhattacharya et al., 2005c).

Cochlin comprises the major non-collagen component of the ECM of the inner ear (Robertson et al., 1998; Ikezono et al., 2001; Ikezono et al., 2004). It is the product of the *COCH* (coagulation factor C homology) gene (Robertson et al., 1994; Robertson et al., 1997) which is mutated in individuals with the autosomal dominant deafness and vestibular disorder designated as DFNA9 (Robertson et al., 1998; de Kok et al., 1999; Kamarinos et al., 2001). Most cochlin mutations associated with hearing disorders have been found in the N-terminal regions and have been shown to cause protein misfolding (Liepinsh et al., 2001; Robertson et al., 2003). Microfibrillar deposits containing MPS and cochlin have been observed in DFNA9 (Khetarpal, 1993; Robertson et al., 1998; Robertson et al., 2003). Such deposits are dense, highly branched and contain disarrayed nonparallel microfibrils and granules of glycosaminoglycans (Khetarpal, 2000). Misfolding of mutated cochlin has been implicated in formation of such deposits (Robertson et al., 2003). However, the mechanism of pathogenesis in DFNA9 is still under investigation. Cochlin mutations have also been implicated in cases of Menière's disease and presbycusis (age-related hearing loss), having features of hearing loss and vertigo (de

Kok et al., 1999;Fransen et al., 1999). These diseases are late-onset and progressive in nature, and therefore parallel the clinical characteristics of POAG.

2. Cochlin domain organization

In the human, *COCH* gene is located on chromosome 14 in bands q12–13 (Robertson et al., 1994;Robertson et al., 1997;Robertson et al., 2003) and contains 12 exons. Cochlin mRNA is expressed in the cochlea and at very low levels in the cerebellum and in the eye (Robertson et al., 1994;Robertson et al., 1997). The NCBI UniGene database reports presence of cochlin mRNA in cochlea, placenta, whole brain, colon, liver, spleen, kidney, TM, B-cells and many tumor cell lines, including retinoblastomas. In the mouse, cochlin mRNA is localized in spleen, eye, lung, brain and thymus (Robertson et al., 1994;Robertson et al., 1997).

Cochlin protein sequence is highly conserved in human, mouse and chicken, with 94% and 79% amino acid identity of human to mouse and chicken sequences, respectively (Robertson et al., 1998). Structurally (Figure 1), cochlin contains a short predicted signal peptide (SP), an N-terminal factor C homology (FCH) and two von Willebrand factor A-like (vWFA1 and vWFA2) domains (Robertson et al., 2003). Three isoforms of cochlin, (~40, ~46 and ~60 kDa) have been reported in the cochlea (Robertson et al., 1997;Ikezono et al., 2004), the smaller isoforms lacking the FCH domain (Ikezono et al., 2004). Cochlin contains two potential N-linked glycosylation sites at the amino acid positions 100 and 221 of its primary amino acid sequence. Cochlin isoforms are secreted by cells in the cochlea as glycosylated proteins (Robertson et al., 2003).

2.1 The FCH (or LCCL) domain

The FCH domain, present in the N-terminal region of cochlin, was first described in the horseshoe crab *Limulus* factor C clotting protein (Muta et al., 1991) and later referred to as LCCL domain from the three proteins in which it was identified: *Limulus* factor C, *Cochlin*, and *Late gestation lung protein* (*Lgl1*). More, recently, the FCH domain has been identified in other proteins such as *CocoaCrisp*, *vitrin* (Mayne et al., 1999), human bone morphogenetic protein (*BMP1*) (Hartigan et al., 2003;Leighton and Kadler, 2003), and *akhrin* (Ahsan et al., 2005) (Table 1). Several of these proteins comprise many family members and some of them (for example, *BMP1*) have many isoforms. The function of the FCH domain remains to be elucidated. Amino acid sequence comparisons, circular dichroism (Trexler et al., 2000) and NMR (Liepinsh et al., 2001) analyses have identified FCH as an autonomous folding domain. The recombinant FCH domain alone, however, fails to show binding with lipopolysaccharides (LPS) or other immunogenic carbohydrates, such as glucose, mannose, cellobiose and 2-N-acetyl-glucosamine (Liepinsh et al., 2001). Evidence suggests that FCH domains are involved in antibody-independent host defense. For example, *Lgl1* is expressed in fetal lungs coincident with the production of pulmonary surfactant and *Lgl1*-like surfactant proteins, SP-A and SP-D, that may serve to protect the lung against pathogens (Kaplan et al., 1999). *Limulus* factor C is a trypsin-type serine protease, activated upon binding to LPS in its zymogen state. This activation initiates the *Limulus* coagulation cascade, which participates in hemostasis and in defense against infection (Nakamura et al., 1988a;Nakamura et al., 1988b;Liepinsh et al., 2001).

The FCH domain is the site of at least seven known mutations in *COCH* gene (Usami et al., 2003;Nagy et al., 2004;Street et al., 2005;Robertson et al., 2006), all located within exons 4 or 5. These mutations were observed in patients carrying the diagnosis of DFNA9 (Grabski et al., 2003;Robertson et al., 2006), Meniere's disease and presbycusis (Fransen et al., 1999). The functional role of mutations remains to be elucidated, however, it has been shown that proteins with mutated FCH fail to fold properly (Liepinsh et al., 2001;Robertson et al., 2003). FCH mutants of cochlin are processed and secreted in transfected COS-7 or 293T cells, suggesting

that extracellular events and not secretory defects underlie DFNA9 pathology (Grabski et al., 2003;Robertson et al., 2003). In parallel, it is likely that the presence of cochlin alters extracellular events in glaucomatous TM.

2.2 The von Willebrand factor A (vWFA) domains

Cochlin contains two vWFA domains encoded by exons 8–10 and 11–12, respectively (Robertson et al., 1997;Robertson et al., 1998). The von Willebrand A (vWFA) is a well studied domain participating in shear-induced self-aggregation and in cell adhesion (Ruggeri et al., 1983;Tuckwell, 1999). A number of human diseases are associated with mutations in vWFA domains (Whittaker and Hynes, 2002). Many vWFA domains bind metal ions via a noncontiguous sequence motif called metal ion-dependent adhesion site (MIDAS). Frequently, vWFA domain-containing proteins participate functionally in multi-protein complexes. The eponymous domains of von Willebrand factor play key roles in the linkage of platelets to collagen (Whittaker and Hynes, 2002). The vWFA domains are present in a variety of ECM components, including cartilage matrix protein and collagen types II, VI, VII, XII and XIV. The vWFA domains have also been implicated in host defense mechanisms and in hemostasis as complement factor C, factor B, integrins LFA-1, Mac-1, VLA-1 and 2, p150 and p95 are part of the immune system that harbor vWF domains (Colombatti and Bonaldo, 1991;Colombatti et al., 1993). A number of proteins such as collagen, GpIb α , and integrin α IIb β 3 are known to interact with vWF domains (Ruggeri et al., 1983;Eble and Tuckwell, 2003;Vanhoorelbeke et al., 2003). Many of these interactions play a role in different regimes of fluid dynamics (Shankaran et al., 2003) and have been implicated in adherence to platelets and possibly also to macrophages. High fluid shear induces platelet aggregation that involves binding of von Willebrand factor (vWF) to the platelet membrane GpIb/IX/V complex (O'Brien and Salmon, 1987;Kroll et al., 1996;Ikeda et al., 1997). The von Willebrand factor protein plays a critical role in blood clotting and mutations within the gene can lead to bleeding disorders (Colombatti and Bonaldo, 1991;Colombatti et al., 1993). Hydrodynamic forces induce vWF aggregation in suspension (Figure 2a) and have been suggested as one of the factors regulating cell adhesion rates in the blood stream (Dong et al., 2003;Shankaran et al., 2003). Purified cochlin also undergoes similar shear induced aggregation (Figure 2b–d). The implications of the observed shear induced aggregation in cochlin (Bhattacharya et al., 2005c) remain to be understood.

Formation of multimeric aggregates *in vivo* with physiological relevance has been shown in a number of proteins such as apoptin (Leliveld et al., 2003), frataxin (Gakh et al., 2002) and basic fibroblast growth factor (bFGF) (Astafieva et al., 1996). In the case of apoptin, semi-random aggregation rather than stoichiometric complex formation is sufficient for physiological activity (Leliveld et al., 2003). Often the presence of ions or oxidative conditions are sufficient to induce multimerization or aggregation (Astafieva et al., 1996). Function of the C-terminal vWFA domain of cochlin that possesses a highly conserved MIDAS motif is yet to be understood (Whittaker and Hynes, 2002). The MIDAS motif may mediate *in vivo* multimerization of cochlin induced by changes in ion concentration and oxidative environment in addition to changes in fluid shear. Protein and cell aggregation is a common theme in the pathogenesis of diverse diseases, including atherosclerosis, Parkinson's disease and Alzheimer's (Kourie and Henry, 2001;Masliah and Hashimoto, 2002). Previous studies suggest that the glaucomas may share some features of this disease group (McKinnon, 2003;Parisi, 2003).

In POAG, the altered fluid shear and the hydrodynamic forces in the TM are associated with the elevation of IOP (Bhattacharya et al., 2005b). The vWFA domains of cochlin may have roles in the response to different fluid flow regimes (for example, increased diurnal fluctuation in IOP) or that of altered local environment such as changes in ions or oxidative conditions in

TM present in glaucoma. In response to one or all of these changes cochlin may undergo multimeric aggregation (Figures 2b–d). However, it remains to be investigated whether multimeric aggregates of wild-type full-length cochlin are biologically active and play a physiological role at the cellular and tissue levels.

3. Glaucoma patients and cochlin mutations

Screening of 190 glaucoma patients detected four sequence changes in *COCH* (IVS4-8AG, Thr352Ser, Phe389Phe, Asp423Asp) (Pertz et al., 2006). Thr352Ser had a minor allele frequency of 34.4% and is a non-pathogenic polymorphism. IVS4-8AG was identified heterozygously in one patient with glaucoma yielding a minor allele frequency of 0.28%. Phe389Phe had a minor allele frequency of 0.56% in patients, while Asp423Asp had a minor allele frequency of 1.1% in patients. Additional SSCP variant bands were identified in exons 7, 9, and 10 and are currently being sequenced (Pertz et al., 2006). However, so far these sequence changes have not been found associated with disease. An evaluation of the other SSCP variant bands identified in these patients and an evaluation of all exons in additional patients are in progress (Pertz et al., 2006).

4. Cochlin homologs in the eye

Three proteins that share common structural motifs, akhirin, vitrin and cochlin have been detected in the eye (Mayne et al., 1999; Ahsan et al., 2005; Bhattacharya et al., 2005c). Akhirin, a protein of 90 kDa possessing FCH and two vWFA domains, has been detected in the lens and retina in chicken. Akhirin is involved in cell adhesion (Ahsan et al., 2005). Vitirin, which also has a FCH and two vWFA domains, was originally found in the vitreous of the bovine eye (hence the name vitrin) and its function remains unknown (Mayne et al., 1999). Both cochlin and vitrin have at least three isoforms and possess two vWFA-like domain with ion binding MIDAS motif (Whittaker and Hynes, 2002). In addition, other proteins that have homology to cochlin, for example, bone morphogenic protein 1 (BMP1) and CocoaCrisp (at mRNA level) are also found in the eye (Table 1; Figure 3, Figure 4).

5. Cochlin and Extracellular matrix

POAG is associated with aberrant and decreased aqueous outflow leading to elevated intraocular pressure. Abnormal aqueous outflow is attributed to increased resistance at the level of the TM, presumably due to changes in the ECM. In DFNA9, elevated intravestibular pressure is associated with up-regulation of cochlin and the presence of cochlin deposits. Similarly, accumulation of cochlin deposits in the ECM has been found in glaucomatous TM, but not in control TM (Figures 5a, b). Two types of cochlin deposits have been observed in the glaucomatous TM, small granular deposits and large tracts of deposits (Bhattacharya et al., 2005b; Bhattacharya et al., 2005c). It is unclear whether small granular deposits coalesce to form large deposits *in vivo*. Studies *in vitro* support the possibility that small deposits may merge forming larger deposits (data not shown).

The non-radioactive in situ hybridization (ISH) demonstrated the presence of cochlin message in the TM of glaucoma patients (Figure 5c) suggesting that cochlin is formed in the TM and could be deposited locally. It is unclear whether the observed accumulation of cochlin correlates with up-regulation of cochlin mRNA in the glaucomatous TM tissue.

The human glaucomatous TM tissue received from donors either from TM surgery or cadavers undergoes changes in expression profile compared to that from normal individuals. Drug treatments are known to cause changes at cellular and tissue levels. Because of long term glaucoma medication of the donors prior to tissue collection it is not possible to retrospectively associate changes in expression with the pathology alone. Conflicting results have been found

in our own studies, either using Northern blot or real-time PCR analysis with respect to cochlin mRNA level using either surgical or cadaver tissue that have undergone different medication. In other studies, cochlin mRNA has been found down-regulated in glaucomatous TM (Liton et al., 2006). Proteomic and Western analyses, however, consistently have shown elevated levels of cochlin in glaucomatous TM compared to control (Bhattacharya et al., 2005c). Variation in modulation of COCH expression has been detected in human TM cultures subjected to dexamethasone, a pharmacological agent used in treatment of glaucoma. Prolonged treatment with dexamethasone is also known to result in glaucoma *in vivo* and to induce expression of myocilin in cultured human TM cells (Polansky et al., 1997; Johnson, 2000; Baulmann et al., 2002). In the absence of a model system that accurately recapitulates the POAG and mimics the aqueous dynamics in the human TM, mRNA expression studies will either require understanding of gene expression changes that occur with each type of medication *in vitro* or a comparison with surgical donor tissue that has not undergone medication. A corollary as to whether accumulation of cochlin in the ECM of glaucomatous TM is due to increased and continued expression or due to lack of commensurate degradation also remains to be addressed. Notably, microfibrillary deposits containing mucopolysaccharide (MPS) and cochlin are found in the cochlea of patients presenting with DFNA9 (Khetarpal, 1993; Robertson et al., 2003). Similarly, MPS were found associated with cochlin deposits in glaucomatous TM as well (Figure 6), but were absent in normal tissue (Bhattacharya et al., 2005c). Whether cochlin deposition precedes MPS deposition or vice versa and the elucidation of the mechanisms leading to the formation of such deposits awaits further investigation.

Full-length cochlin expressed in COS-7 or HeLa cells as HA-tagged or myc-tagged proteins fail to show aggregation or degradation, respectively (Grabski et al., 2003; Robertson et al., 2003). Aggregation of primary TM cells *in vitro* in response to the presence of exogenous cochlin suggests that this may be mediated by extracellular protein-protein interactions. Three cochlin isoforms (~40, ~46 and ~60 kDa) have been described in the human cochlea (Robertson et al., 2001), indicating molecular heterogeneity within the inner ear *in vivo*. It was initially postulated that size heterogeneity of cochlin may be due to alternative mRNA splicing, exon skipping or posttranslational modifications (Robertson et al., 1997). Smaller cochlin isoforms lack the FCH domain and only the larger isoform could be influenced by mutations in this domain. There are two N-linked glycosylation sites in human cochlin [at positions 100 (NYS) and 221 (NFT)]. Treatment of bovine cochlin with PNGase leads to the collapsing of the ~63 kDa band into ~61 kDa and 60 kDa (Ikezono et al., 2001; Ikezono et al., 2004); a similar observation has been made with human cochlin (Robertson et al., 2003). Glycosylation and posttranslational proteolytic processing could be responsible for the observed charge/size heterogeneity. The functional consequence of glycosylation in cochlin as evident from PNGase treatment remains unknown. There is a marked decrease in the density of cells that normally express cochlin in patients with DFNA9 and an increase in accumulation of MPS deposits that obstruct the cochlear and vestibular nerve channels (Robertson et al., 1998; Khetarpal, 2000; Merchant et al., 2000). When wild-type or mutant cochlins are expressed in HeLa cells they accumulate in extracellular deposits that closely parallel the ECM component fibronectin. Expressed cochlin or its mutants do not co-localize with known focal adhesion markers such as actinin, vinculin or paxilin, hence cochlin deposits in HeLa cells are not at focal adhesion sites (Grabski et al., 2003). It has been concluded that DFNA9 is not caused by significant defects in cochlin synthesis, glycosylation or secretion, but that the observed acidophilic deposits in DFNA9 may be linked to ECM related events (Grabski et al., 2003). Multiple protein-protein interactions mediated by FCH and/or vWFA domains may underlie cochlin localization and function *in vivo* (Grabski et al., 2003). Interacting partners for the FCH domain are not known, but several proteins are known to interact with the vWFA domain. Shear-induced platelet aggregation (SIPA) initiated by binding of vWF with platelet membrane GpIb complex plays a role in vascular diseases such as cerebral ischemia and acute myocardial infarction (Uchiyama et al., 1994; Konstantopoulos et al., 1997; Goto et al., 1999; Tanigawa et

al., 2000). Glaucoma patients, like the patients with diseases mentioned above, display enhanced SIPA *in vitro* (Matsumoto et al., 2001). A role for cochlin in host defense is also possible. Loss-of-function mutations in DFNA9 cause increased susceptibility to infection (de Kok et al., 1999; Baek et al., 2006). Furthermore, a number of DFNA9 patients tend to develop cardiovascular disease (Bom et al., 1999). In view of these findings and the association of cochlin with the TM in glaucoma, we anticipate a broader functional role for cochlin. Studies using anterior segment organ culture with transforming growth factor-beta2 treated monkey eyes indicates overexpression of cochlin, among events that is concomitant or precedes elevation of IOP (Gabelt et al., 2007). Kidneys are also the tissues that require tight regulation of fluid flow and experience changes in fluid flow regimes somewhat similar to that experienced in aqueous outflow and in the inner ear. In polycystic kidney disease, polycystin 1 (PC1) has been demonstrated to have the property of mechanosensation and contributes to molecular events that subsequently renders transcriptional activation (Low et al., 2006). The glaucomatous TM cells may experience diurnal fluctuations of greater amplitude in intraocular pressure prior to the onset of a pathologic phase and cochlin expression may be a physiological response for the course correction. Increased expression and multimerization in response to greater diurnal IOP amplitude or high IOP may be a step in that direction. Cells may sense and transduce a wide range of mechanical stimuli into distinct biochemical signals that help in regulating several fundamental cellular processes such as adhesion, proliferation and differentiation. It is important to understand the design principles of these sensory systems at the molecular level. The insight into the integration of these design principles at molecular/protein levels is key to understanding normal cell responses and their alteration in pathological states. While several mechanosensory units are membrane based proteins, nevertheless several such proteins belong to the soluble repertoire as well (Vogel, 2006; Vogel and Sheetz, 2006). Loss or aberrant mechanosensing and transduction of mechanosensory signal may also be part of pathology in progressive hearing loss (Knoll et al., 2003; Ricci et al., 2006). Dysfunction or absence of any of the mechanosensing transmembrane molecules results in disruption of the network causing sensorineural degeneration in the inner ear and in the retina in Usher syndrome, another disease that leads to retinal degeneration and progressive hearing loss (Reiners et al., 2006), and other forms of progressive hearing losses such as DFNA4 and DFNB6 (Bearer et al., 2000; Mitchem et al., 2002). The extracellular matrix of apical epithelia of the inner ear plays a role in mechanosensing, and type II collagen constitutes one of the critical molecular components of ECM of apical epithelia (Goodyear and Richardson, 2002). Although TM under mechanical stress is known to induce cytokines and interleukins (Liton et al., 2005) as well as other signaling molecules and matrix metalloproteinases (Bradley et al., 1998; Bradley et al., 2003), little is known of mechanosensory molecules, transducers and effectors in the TM. Trabecular meshwork cells form a beam-like filter. The activity of the filter can be regulated by the local ionic and oxidative microenvironment as well as by changes in fluid shear (Alvarado et al., 2005; Chudgar et al., 2006; Tan et al., 2006). However, it remains to be investigated how the cells undergo mechanosensation and the potential role of cochlin as one of the proteins involved in this function. Environmental factors such as pH and ionic strength and fluid shear are known to affect mechanical stability and homophilic oligomerization of either free or secreted proteins which have been shown to be effectors of mechanosensory systems (Vogel, 2006; Vogel and Sheetz, 2006). The first step in modulation of cells and the local tissue environment appears to be mechanosensing or force-induced conformational changes such as multimerization. Mechanosensing and multimerization may result in the initiation of cascade of subsequent biochemical reactions. The latter causes mechanotransduction that activates intracellular signaling pathways resulting in amplification of the signal, which in turn activates mechanoresponsive cell functions through spatiotemporal signal integration (Vogel, 2006).

5.1 Exogenous cochlin and TM cell aggregation

Aggregation of primary TM cells in response to exogenous cochlin (Figure 7a), but lack of aggregation with control Notch or mock purified media, (Figures 7b, c) suggests that cochlin is likely the factor that mediate the observed aggregation. The prior incubation of cochlin with anti-cochlin inhibits this primary TM cell aggregation, further suggesting that the cell aggregation is mediated through cochlin (Figure 7d). In chicken, akhirin has been shown to mediate cell adhesion (Ahsan et al., 2005). Future studies will confirm whether the observed cell aggregation *in vitro* with cochlin occurs *in vivo* and is, therefore, physiologically relevant. The factors and mechanistic steps mediating such aggregation also remain to be elucidated. It is important to note that aggregation has also been observed with bone morphogenic factor on primary TM cells, however, with a much longer exposure time span compared to cochlin (Xue et al., 2006). The BMP family members, like cochlin, share a vWFA domain (Table 1). The implication of aggregation for TM cell physiology remains to be understood. Whether multimerized forms of cochlin show any differences in aggregation and whether mutant cochlin renders process of multimerization or aggregation distinctively different than the wild type has also not been investigated.

5.2 Cochlin in the TM of a mouse model of glaucoma

An animal model that resembles features of POAG is lacking. However, a mouse model of hereditary glaucoma, the DBA/2J, was identified (John et al., 1998). The DBA/2J mouse line develops, besides progressive hearing loss, many of the characteristic features of human glaucoma, including elevated IOP around 6–8 months, optic nerve damage and ganglion cell degeneration (John et al., 1998; Chang et al., 1999; Danias et al., 2003). DBA/2J mice develop glaucoma due to iris stromal atrophy and pigmentary dispersion. However, despite having a similar genetic background, not all mice in a given cohort develop characteristic glaucoma at a given age and they show wide variation. A number of mice develop elevated IOP prior to the demonstration of proper pigmentary dispersion. Based on findings from human TM, cochlin expression was probed in the DBA/2J mouse (Bhattacharya et al., 2005a; Bhattacharya et al., 2005c). If cochlin is involved in mechanosensing and in some stage of ECM remodeling that precedes significant elevation of IOP, it will be expected that a model that demonstrates elevation of IOP will also demonstrate expression of cochlin at very initial stages much before the manifestation of pathology. Western analyses of TM extracts from 10 week-old DBA/2J mice and from age-matched C57BL/6J, CD1 or BALBc/ByJ control mice, which do not develop increased IOP, revealed not only that cochlin is expressed only in TM from the DBA/2J mouse as early as 2–3 weeks of age, but also that this expression progressively increases between 3 to 16 weeks of age (Bhattacharya et al., 2005c). Future studies with DBA/2J mice may provide clues to molecular events involving cochlin that renders elevation of IOP.

5.3 Overexpression of cochlin and degradation of collagen type II

The space between the TM beams is filled with ECM. TM endothelial cells produce MPS that are part of the ECM and function as regulators of migration, proliferation and adhesion of macrophages (Laurent and Fraser, 1992; Knudson and Knudson, 1993) for phagocytic clearance of particles that penetrate the outflow system (Sherwood and Richardson, 1988; Johnson and Johnson, 2001). Abnormal MPS levels can interfere with the regulation of the aqueous outflow and disrupt the cleaning process, resulting in large changes in IOP (Nickla et al., 2002). Sheath-derived plaques and microfibrillar deposits have been reported in the TM of POAG donors (Lutjen-Drecoll et al., 1986b; Lutjen-Drecoll et al., 1986a; Ueda et al., 2002). Histochemical analyses of human TM revealed that cochlin is associated with MPS and forms acidophilic deposits within glaucomatous TM, but not in control TM. These deposits were observed around the Schlemm's canal and in pseudoendothelial cells (Bhattacharya et al., 2005c). The two vWFA domains of cochlin have been implicated in adherence and

aggregation for platelets, macrophages and leukocytes (Shankaran et al., 2003) through interaction with a number of proteins such as collagen, GpIb α , and integrin α Iib β 3 (Eble and Tuckwell, 2003; Vanhoorelbeke et al., 2003).

In DFNA9, acidophilic deposits may be linked to ECM-related events and multiple protein interactions mediated by cochlin domains may underlie these events (Grabski et al., 2003). Cochlin vWFA has a MIDAS domain that may render it responsive to local ion environment *in vivo*. About 16 collagen types contain a vWFA like domain, collagen type II being one prominent member (Whittaker and Hynes, 2002). The role of collagen type II, a relatively minor component of TM, is not understood.

An age-dependent increase in cochlin expression was observed in human glaucomatous TM, with a simultaneous decrease in type II collagen (Bhattacharya et al., 2005c). In normal human TM, no significant change in type II collagen was detected. Similarly, glaucomatous DBA/2J mice showed a progressive, age-related decline in type II collagen expression in TM, while it remained stable in older C57BL/6J mice (Bhattacharya et al., 2005a). Altered interactions between fibrillar collagens and other ECM components have been suggested to trigger collagen degradation (Pareti et al., 1987). Perturbations in collagenous fibrillar assembly due to changes in collagen levels are known to result in loss of tissue-specific morphology (Marchant et al., 1996). These findings suggest that extracellular and prolonged presence of cochlin may disrupt the interaction of surrounding collagens with ECM protein components, compromising the integrity of the TM extracellular matrix, rendering it vulnerable to proteolytic degradation and debris deposition, thus, leading to the obstruction of the aqueous humor outflow (Bhattacharya et al., 2005a; Bhattacharya et al., 2005c).

While decreased collagen biosynthesis cannot be ruled out, increased cochlin may help dissociate collagen from other ECM proteins, rendering ECM components more susceptible to proteolytic degradation and the TM more liable to collapse and deposition of debris. The large cochlin deposits observed in glaucomatous TM could obstruct aqueous outflow across a wide region and thus have the potential to increase IOP. In the ECM, presence or overexpression of proteins is poised to alter interaction of normal protein partners. The vWFA domains of cochlin may act as competitors for some proteins and collagen. In particular, type II collagen is a likely candidate due to the similarity of its vWFA-like domain. Western (Figure 8) and immunohistochemical results (Figure 9) suggest degradation of collagen type II and possibly other collagens in glaucomatous TM. Early reports could not detect type II collagen in normal human TM by immunohistochemistry (Marshall et al., 1991). However, more recently, degradation of collagen type II has been shown in both human glaucomatous TM (Bhattacharya et al., 2005c) and in the TM of DBA/2J mice (Bhattacharya et al., 2005a). Furthermore, a weak signal for collagen type II message could be detected in the TM by ISH, using a type II collagen-specific probe (Figure 10). Finally, collagen type II message was also detected by RT-PCR analysis of total RNA isolated from carefully dissected or laser captured glaucomatous TM tissue (data not shown). This corroborates the detection of collagen type II in TM by Western and immunohistochemical methods and likely demonstrates its actual expression rather than the presence of an artifact. It is important to note that a few commercially available antibodies (for example, goat polyclonal antibody Abcam 19752; mouse monoclonal antibody Abcam 3092 and antibody C2C1 from Developmental Studies Hybridoma Bank, University of Iowa) fail to detect native collagen type II by immunohistochemistry but they can detect the denatured protein by Western blot analysis. These and reported immunohistochemical results (Marshall et al., 1991) likely represent some epitope masking or failure of the antibody to recognize the immunogenic antigen in the TM.

5.4 Cochlin interacting proteins in the glaucomatous TM

If the presence of cochlin in the TM disrupts normal collagen interactions in the ECM, can the potential cochlin interacting partners be identified? Immunoprecipitation (IP) studies utilizing human glaucomatous TM tissue and cochlin or control antibodies and mass spectrometry have been used to address this question. Human glaucomatous cadaver eyes were procured from the Cleveland Eye Bank. The TM was excised and protein extract was prepared in phosphate buffered saline (PBS) pH 7.4 with 0.1% genapol. The IP was performed using three different methods: 1. IP of hemagglutinin tagged cochlin (HA-cochlin) bait with HA-antibody agarose (Pierce Biotechnology Inc., IL); 2. IP of full length cochlin with chicken polyclonal anti-cochlin antibody (Robertson et al., 2003) and PrecipHen agarose beads (Aves Labs Inc., Portland, OR), following suitable modification of previously published protocols (Harlow and Lane, 1988); and, 3. IP of full length cochlin with rabbit polyclonal anti-cochlin antibody and commercially available Catch and Release™ (Upstate Biotechnology, NY) reagents (Figure 11). Each individual IP experiment was performed in triplicate. HA-tagged recombinant pCDNA 3.0 plasmid (for cochlin and Notch) prepared in *E. coli* plysS was purified using a Qiagen kit. Plasmids were used to transfect mammalian COS-7 cells. Secreted proteins (HA-cochlin or HA-Notch) were purified using previously published protocols (Bhattacharya et al., 2005c) and used as bait for immunoprecipitation.

For the protein identification, of IP products, the samples were analyzed by mass spectrometry and bioinformatics. Database search results were tabulated and visually inspected using Scaffold (Proteome Software, Portland, OR). These methods are routinely used in our laboratory. A combined list of all identified proteins was prepared. The interacting proteins that replicated in all IP assays at least twice (out of three independent experiments for each IP) were considered as true interacting proteins (Table 2 and 3). Annexin A2, an ECM protein, appeared with high frequency and was further tested for its interaction with cochlin by reciprocal IP. For identification of cochlin interacting partners, custom peptide antibodies against cochlin peptides (KR LKK TPE KKT GNK DC from cochlin coding region 147–162 designated as hCochlin#1; ZCZ TYD QRT EFS FTD YST KEN from cochlin coding region 412–429 designated as hCochlin#2 and, CZ DDL KDM ASK PKE SH from cochlin coding region 358–371 designated as hCochlin#3) were prepared using the commercial services of Aves Lab Inc., Portland, OR.

6. Glaucoma and progressive hearing loss

Although a number of cochlin mutations are associated with DFNA9 and accumulation of cochlin in the inner ear has been observed, whether these patients also suffer from glaucoma remains unknown. Abnormal central oculomotor functions have been reported in family members with the c.1625G > T COCH alteration implying a possible central nervous system change due to cochlin mutation (Street et al., 2005). Geographical distribution of patients, lack of comprehensive medical record and other regulatory statutes have prohibited an efficient retrospective study to assess the frequency and severity of glaucoma in DFNA9 patients. A prospective study for sensorineural hearing loss in glaucoma patients is in progress.

7. Summary and conclusions

Proteomic analyses have identified a variety of proteins from the TM of human cadaver POAG and normal tissue donors. Cochlin, a secreted protein, whose function in the TM is still unknown, has been confirmed to be present exclusively in the glaucomatous tissues and appears likely to contribute to glaucoma pathology. It is implicated in late onset, progressive diseases involving altered fluid flow through ECM. Current results suggest that cochlin contributes to elevated IOP in POAG by altering physiological mechanisms within the TM that involve cell aggregation, MPS associated deposition and obstruction of the aqueous humor circulation.

Aggregation of primary TM cells upon exogenous cochlin addition suggests that the presence of cochlin in ECM may abnormally reduce aqueous outflow. Cochlin may be part of a local regulatory network of proteins that respond to fluid shear and/or local microenvironmental changes and, thus, play a role in very early events that eventually leads to POAG.

8. Future directions

Ongoing research utilizing DBA/2J and monkey models will contribute to determine how cochlin expression and deposition in the TM is related to IOP. Future research will address whether cochlin accumulation is due to over-expression or decreased degradation or both. In the inner ear as well as in glaucomatous TM, cochlin deposits occur in association with mucopolysaccharides. The molecules responsible for initiating the cascade of events and the mechanistic steps involved in the initial formation of such deposits remain unknown. In the glaucomatous TM, the relationship between small deposit spots and large deposits remains to be investigated. Fluid shear induced multimerization of cochlin has been shown *in vitro*. It remains to be determined whether cochlin multimerization occurs *in vivo* in response to fluid shear and/or other environmental factors. Similarly, it is still unknown what the biological role of multimerized cochlin species is. The questions that still need to be addressed are whether cochlin mediates mechanosensation and, if so, what are the downstream effectors, and how mechanotransduction leads to remodeling of the TM. The establishment of cochlin-protein interactions in the glaucomatous TM will provide valuable insights. It is also unclear whether exogenous cochlin-induced primary TM cell aggregation is a different function of this protein than that of multimerization and its possible role in mechanotransduction. Defining the role of cochlin will help in the advancement of the understanding of the biology of the TM filter. Elucidation of the role of cochlin and its involvement in the early stages of the pathology will enable cochlin to be used as a target of intervention.

Acknowledgements

This work was partly supported by grants from American Health Assistance Foundation (Thomas Lee Award to SKB), Hope for Vision Foundation (SKB) and NIH grants EY16112, EY15266 (SKB), P30 EY014801 (BPEI center core grant), and by an unrestricted grant to the University of Miami from Research to Prevent Blindness. We are grateful to all our colleagues who participated in the original studies. We thank Drs. Neal S. Peachey and Nahid G. Robertson for critical reading of the manuscript. We thank Dominique Rose, Claudia Garcia and Mabel Algeciras for their assistance in experiments.

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Figure 1. Schematic representation of cochlin protein

A predicted signal peptide (SP) is followed by the FCH domain (also known as LCCL domain) and two von Willebrand factor A-like domains (vWFA1 and vWFA2). Mutations in FCH and vWFA2 domains have been detected in patients with the hearing and vestibular disorder DFNA9 (shown in blue). The polymorphisms detected in glaucoma patients in the vWFA2 domain (shown in grey) are presented. The individual motifs (FCH, vWFA1 and vWFA2) and their boundary positions with the convention of the first amino acid corresponding to the cochlin precursor have been identified.

Figure 2. Hemodynamics and multimerization of von Willebrand factor (vWF) and cochlin
 (a) Effect of fluid shear on VWF size. Purified VWF (125 μ L; 0.1 mg/mL) was diluted 8-fold in HEPES buffer and immediately subjected to Static light scattering (SLS), molecular weight (MW) and radius of gyration (R_g) were determined from this plot. MW equals $1/(\text{y-axis intercept})$. An increase in vWF homotypic aggregate size and MW was observed with shear and time. Data are mean $\pm$ SEM for two shear runs with a single batch of VWF; the side panel shows Western blot analysis of unsheared and sheared samples demonstrating qualitative increases in vWF size. Lane 1 indicates unsheared vWF; lane 2, vWF sheared at 2155/s for 30 seconds; lane 3, vWF sheared at 6000/s for 120 seconds. Equal amounts of protein were loaded in each lane (Adopted from Shankaran et al., 2003 with permission). (b) Coomassie Blue-

stained SDS-PAGE of HA-tagged recombinant cochlin and Notch. (c) Purified recombinant HA-cochlin and HA-Notch detected using anti-HA rabbit polyclonal antibody and (d) Purified HA-cochlin probed with anti-cochlin antibody analyzed on a non-denaturing PAGE plus and minus representing cochlin subjected to shear or without being subjected to shear, respectively.

Figure 3. Schematic representation of the domain structures of cochlin homologs: akhirin, vitrin, Late gestation lung protein (Lgl1) and CocoaCrisp

The numbers represent the amino acid position in the protein sequence. The individual motifs (FCH, vWFA1 and vWFA2) and their boundary position in the amino acid sequence have been identified. The convention of first amino acid corresponding to this position in the precursor protein sequence has been used and the N- and C- terminal of the protein have been depicted for each protein.

a

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Akhirin aa 37 IDCDUKAGKII---NPE---FIACPPGCGQDUK---YRVYGTDIYASFSSACSAAINHGA 87
Vitrin aa 44 INCDDKAGKII---DPE---FIUKCPAGCQDPK---YRVYGTDIYASYSSUCGAAWHSGV 94
Cochlin aa 32 ITCITRGLD IR---KEK---ADULCPGGCPLLE---FSUYGNIVYASVSSICGAAWHRGV 82
Lg1l aa 389 LDCYTTVAQLCPFEKPATHCPRIHCPAHCKDEFSYQAPVFGTNIIYADTSSICKTAUWAGV 448
CocoaCrisp aa 394 UTCETTUEQLCPFHKPASHCPRVYCPRMCMQANPHYARVIGTRVYSDLSICRAAWHAGV 453
Lg1l aa 288 URCDTKMKDRC---KGSTCNRYQCPAGCLNHH---AKIFGTLFYESSSSICRAAIHYGI 340
CocoaCrisp aa 293 USCEVRLRDQC---KGTTCNRYECPAGCLDSK---AKVIGSVHYEMQSSICRAAIHYGI 345
: * . . ** * : * . * ** * ; * ; *

Akhirin aa 88 IDN-TGGKILVQKVAGHAGYRGSTFSGVRSLSLPRMRESF 126
Vitrin aa 95 LDN-SGGKILVRKVAGQSGYKGSYSNGVQSLSLPRMRESF 133
Cochlin aa 83 ISN-SGGPVRUYSLPGRENTYSUDANGIQSQMLSRWSASF 121
Lg1l aa 449 ISNESGGDUDUMPUDKKKTYUGSLRNGVQSSLSLGTFRDGK 488
CocoaCrisp aa 454 URN-HGGYUDUMPUDKKKTYIASFQNGIFSSLSLQNPFGGK 492
Lg1l aa 341 LDD-KGGLUDITRNGKUPFFUKSERHGVQSLSKYKPSSSF 379
CocoaCrisp aa 345 IDN-DGGWUDITRQGRKHYFIKSNRNGIQTIGKYQSANSF 384
: : ** : : : : * : :

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b

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Akhirin aa 363 DLPFLMDGWSIGKRRFQLQKQLINVAHALGINNAGPLMGIVUYGDDPSTEFNLKTYAS 422
Vitrin aa 293 DLSFLIDGSTSIGKRRFRIGKQLLADVAQALDIGPAGPLMGUUYGDMPTATHFNLKTHN 352
Cochlin aa 165 DIAFLIDGSTFNIQRRFNLQKQFUGKVALMLGIGTEGPHUGLVQASEHPKIEFYLNKFTS 224
* : ** : *** : ** : *** : ** : : ** * . * . ** : ** : ** : * . * . * .

Akhirin aa 423 PKELRNAIEKIPQKGLSNUGKALSFUNKNFFSDANGNRGGAPNVAUVMUDGWPTDRUEE 482
Vitrin aa 353 SRDLKTAIEKITQRGLSNUGRAISFUTKNFFSKANGNRSGAPNVAUVMUDGWPTDKUEE 412
Cochlin aa 225 AKDULFAIKEUGFRGNSNTGKALKHTAQKFFTVDAQVRKGIPKVAUUVIDGWPSDDIEE 284
: : : ** : : ** ** : * : : : : ** : * * * : * : * : * : * : * : * :

Akhirin aa 483 ASRLARESGINIFFITIEGAAQNEKQNVIEPNFUDKAUCRTNGFYITVPSWFLHKAUQPL- 544
Vitrin aa 413 ASRLARESGINIFFITIEGAAQNEKQNVIEPNFANKAUCRTNGFYSLHUQSWFGLHKTLPV 475
Cochlin aa 285 AGIAREFGUUVFIVSUAKPIPEEL----- 309
* . : *** * : * : : : . : *

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c

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Akhirin aa 565 DIGFVIDGSSSUGTSNFRVLQFVANISKEFEISDTRIGAIQYTYEQRLFSFDKYST 624
Vitrin aa 495 DIGFVIDGSSSUGTGFRVLQFUTNLTKFEISDTRIGAVQYTYEQRLFGFDKYSS 554
Cochlin aa 367 NIAFLIDGSSSUGDSNFRMLLEFVSNIAKTFEISDIGAKIAAVQFTYDQRTFSTFDYST 426
: * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

Akhirin aa 525 KQDULSAIKRINYWSGGTSTGAAISYASEQLFT--KSKPMKRMILITDGRSYDDVRMP 582
Vitrin aa 555 EPDILNAIKRUGYWSGGTSTGAAINFALQLFK--KSKPMKRMILITDGRSYDDVRIP 612
Cochlin aa 427 KENULAVIRNIRYWSGGTATGDAISFTURNVFCPIRESNPK-NFLVIUTDGGSYDDVQGP 485
* : : * . * : : * : * : * : * : * : * : * : * : * : * : * : * : * :

Akhirin aa 583 ALTAHQNGUIAYSIGVAAWAAQDELEAIATDPDKHSFFUDEFDMLYQFUMPL 734
Vitrin aa 513 AMAAHLKGUIITYAIGVAAWAAQEELEVIATHPARDHSFFUDEFDMLHQYUPRI 564
Cochlin aa 486 AAAANDAGITIFSUGVAAWAPLDDLKDMASKPKESHAFPTREFTGLEPIVSDU 537
* : ** * : : : * : * : * : * : * : * : * : * : * : * : * : * :

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Figure 4. Alignment of peptide sequences of cochlin homologs

Alignments were performed using the ClustalW program (EMBL-EBI), utilizing multiple alignment option. Comparisons (FCH domain) were made with chicken protein akhirin (Q5NTW9) and human proteins vitrin (Q6UXI7), cochlin (O43405), late gestation lung protein 1 (Lg1l; Q9H0B8) and CocoaCrisp (Q9H336). (a) Alignment of the FCH domain amino-acid sequences of cochlin homologs. (b) Alignment of the vWFA-1 domain amino-acid sequences of cochlin homologs. (c) Alignment of the vWFA-2 domain amino-acid sequences for homolog proteins. The degree of conservation in each sequence has been indicated: “*”, depicts the residues identical in all sequences; “:”, represents conserved substitutions and “.”, indicates semi-conserved substitutions. Gaps, required for optimal alignment are represented by dashes.

Figure 5. Localization of cochlin and cochlin message in the human TM

(a–b) Representative microphotographs of human TM sections immunoreacted with chicken polyclonal anti-cochlin antibody (hcochlin#3). Upper and lower panels represent bright field and fluorescence images, respectively. (a) Anterior segment from a non-glaucomatous 36 year-old male control donor, and (b) anterior segment from a 34 year-old male glaucomatous donor. (c) *In situ* hybridization for localization of cochlin message in human glaucoma TM sections. Human cochlin fragment (nucleotides 1482–2026) was used as specific probe. Upper and lower panels in (c) show sense negative control and anti-sense probe hybridization, respectively. * and ** represent Schlemm's canal and TM region, respectively.

Figure 6. Light microscopy of Movat's pentachrome-stained anterior segment tissue
 (a) Normal TM from a 75 year-old Caucasian female and (b) glaucomatous TM from a 77 year-old Caucasian female. * indicates Schlemm's canal. Arrow indicates MPS deposit around Schlemm's canal. Bar = 100 μ m.

Figure 7. Aggregation of TM cells by exogenous cochlin

Primary TM cell cultures were established following published protocols and treated (a) with purified recombinant cochlin (5 μ g in 10 μ l of PBS), (b) purified recombinant Notch (5 μ g in 10 μ l of PBS); (c) medium (10 μ l) from empty vector transfected COS-7 cells and (d) purified recombinant cochlin (5 μ g in 10 μ l of PBS) pre-incubated with about 40 μ g of anti-cochlin antibody for 15 minutes prior to addition to the culture.

Figure 8. Western analyses for presence of cochlin and collagen

(a) Human TM extracts probed with rabbit polyclonal antibodies to collagen type I, (b) probed with rabbit polyclonal antibodies to collagen type II, (c) probed with antibodies to collagen type III, (d) probed with chicken polyclonal antibodies to cochlin (hcochlin#1), and (e) Coomassie Blue-stained SDS-PAGE after Western transfer of the proteins on to PVDF membrane. Age of donors and their gender are as indicated.

Figure 9. Immunohistochemical analysis for collagen in the human TM
 (a, a') antibodies against collagen type I, (b, b') antibodies against collagen type II and (c, c') antibodies against collagen type III. (a–c) normal human TM and (a'–c') glaucomatous TM. Bottom panels represent DAPI staining for nuclei. Bar = 50μm.

Figure 10. *In situ* hybridization for localization of collagen type II message in human glaucoma TM Microphotographs of human glaucomatous TM sections are shown. Human collagen type II specific probe encompassed nucleotides 1341–1717. This probe detected collagen type II message on control human liver tissues (data not shown). Sense control and anti-sense probe have been presented in upper and lower panel as indicated. (a) normal and (b) glaucomatous eye. * denotes the TM region

Figure 11. Immunoprecipitation to determine cochlin-interacting proteins in human TM tissue
Immunoprecipitation (IP) was carried out using three different methods. 100 μ g of glaucomatous TM were extracted and prepared in buffer containing 0.1% genapol. In IP utilizing anti-HA Sepharose (rabbit polyclonal antibody against HA; Pierce Biotechnology Inc., IL) HA- tagged cochlin was used and HA-Notch served as negative control. In IP protocol using anti-chicken IgG coupled to agarose (PrecipHen; Aves Lab Inc., OR) chicken polyclonal antibody against cochlin was used and a non-specific chicken IgY served as negative control. Catch and ReleaseTM reagent from Upstate Biotechnology Inc, NY was used in combination with anti-cochlin or anti- HA antibody or a control nonspecific antibody (MPC 21). All protein bands from all IP lanes were excised from top to bottom and subjected to mass spectrometry in an LTQ device for protein identification (Table 1 and 2).

Table 1.

The FCH and vWFA domain containing proteins

Accession number	GenBank Accession Number	Protein Name	Known isoforms	Expression
Q01528 O43405	M96983 AF006740	Limulus factor C ^a Cochlin	At least 3	cochlea, brain, uncharacterized tissue, whole brain, testis, lung, blood, mixed, liver, small intestine, eye, embryonic tissue, lymph node, mouth, pituitary gland, adrenal gland, kidney, pancreas, prostate, placenta, colon, larynx, bone, thymus, mammary gland
Q9H0B8	AL136861	Late gestation lung protein	5	placenta, uterus, prostate, mammary gland, lung, larynx, whole brain, bladder, colon, testis, uncharacterized tissue, small intestine, cochlea, trachea, esophagus, blood, connective tissue, stomach, eye, ovary, mixed, mouth, cranial nerve, brain, embryonic tissue
Q9H336	AF142573	CocoaCrisp ^c		brain, embryonic tissue, cochlea, testis, connective tissue, bone, vascular, uterus, placenta, pancreas, mammary gland, prostate, kidney, eye, bone marrow, mouth, trachea, uncharacterized tissue, ovary, whole brain, liver, mixed, whole body, esophagus, thymus
P13497	U50330	Bone morphogenetic protein (BMP1) ^c	7	placenta, brain, testis, cervix, mammary gland, uterus, uncharacterized tissue, mixed, colon, prostate, larynx, stomach, skin, lung, kidney, embryonic tissue, eye, esophagus, whole brain, pancreas, connective tissue, adrenal gland, mouth, bladder, small intestine
Q5NTW9 Q6UXI7	AB185956 AF063833	Akhirin ^b Vitrin	3	embryonic tissue, mammary gland, larynx, connective tissue, prostate, eye, placenta, ovary, skin, colon, vascular, mixed, liver, heart, brain, bone, cranial nerve, uncharacterized tissue, nerve, whole body, mouth, uterus, thymus, trachea, thyroid, muscle, whole brain

All Swiss-Prot accession numbers are for human proteins except

^aHorseshoe crab coagulation factor (Limulus factor C) and

^bChicken protein Akhirin.

^cCocoaCrisp and Bone morphogenetic protein have several family members and each member have isoforms, for example, bone morphogenetic protein 1 (BMP1) has seven isoforms.

Table 2.

Identification of cochlin interacting proteins by mass spectrometry

Accession number	Match	Description	IP-HA antibody	Detection in immunoprecipitation (IP) experiments	IP-Catch and Release
P60709	3	Actin, cytoplasmic 1	+		
P63261	4	Actin, cytoplasmic 2	+		
P07355	2	Annexin A1	+		
P04083	15	Annexin A2	+		+
P02647	2	Apolipoprotein A-I	+		
P06576	2	ATP synthase subunit beta, mitochondrial	+		
P21926	1	CD9 antigen	+		
P01024	1	Complement C3	+		+
O81WJ2	3	GRIP and coiled-coil domain-containing protein 2	+		
Q1HDT5	1	Hemoglobin alpha 1-2 hybrid	+		+
P68871	6	Hemoglobin subunit beta	+		+
P02790	11	Hemopexin	+		+
Q0VAC5	4	HP protein	+		+
Q0JTD4	1	Hypothetical protein HBB	+		+
P01859	11	Ig gamma-2 chain C region	+		+
P08727	3	Keratin, type I cytoskeletal 19	+		+
P04264	25	Keratin, type II cytoskeletal 1	+		+
P13647	3	Keratin, type II cytoskeletal 5	+		+
P02538	11	Keratin, type II cytoskeletal 6A	+		+
P48668	10	Keratin, type II cytoskeletal 6E	+		+
P05787	2	Keratin, type II cytoskeletal 8	+		+
P00338	2	L-lactate dehydrogenase A chain	+		
P61626	2	Lysozyme C	+		
Q549N7	6	Mutant beta-globin	+		+
P62834	1	Ras-related protein Rap-1A	+		
P02743	1	Serum amyloid P-component	+		

Swiss-Prot database accession numbers are shown. Proteins identified in at least two of the three independent IP experiments have been shown with (+) for a particular IP method and presented in alphabetical order.

Table 3.

Proteins identified in control immunoprecipitations by mass spectrometry

Accession number	Peptide Matches	Description	Detection in immunoprecipitation (IP) IP-Notch	Detection in immunoprecipitation (IP) IP-NS-IgY	experiments IP-Catch and Release (HA)
P30838	2	Aldehyde dehydrogenase, dimeric NADP-prefering	+	+	+
O75976	2	Carboxypeptidase D	+		+
P00450	2	Ceruloplasmin	+		+
Q9BY41	1	Histone deacetylase 8	+		+
P13646	4	Keratin, type I cytoskeletal 13	+		+
P02533	7	Keratin, type I cytoskeletal 14	+		+
P08779	6	Keratin, type I cytoskeletal 16	+		+
Q04695	4	Keratin, type I cytoskeletal 17	+		+
P35527	17	Keratin, type I cytoskeletal 9	+		+
P19013	6	Keratin, type II cytoskeletal 4	+		+
P13647	6	Keratin, type II cytoskeletal 5	+		+
Q9ULV0	1	Myosin-5B	+		+
P32119	2	Peroxioredoxin 2	+	+	
Q15084	2	Protein disulfide-isomerase A6	+	+	
Q92583	1	Small inducible cytokine A17	+		+
Q75DA4	2	Nucleolar GTP-binding protein 2 ^A	+		+
O97592	2	Dystrophin ^B	+		+
Q69422	2	Genome polyprotein ^C	+		+
Q27684	2	Phosphoglycerate kinase, cytosolic ^D	+		+
P0A679	2	Histidinol-phosphate aminotransferase ^E	+		+
Q9F219	2	Methionyl-tRNA synthetase ^F	+		+
O07853	2	Signal recognition 54 kDa protein ^G	+		+
Q97CQ3	2	V-type ATP synthase subunit E ^H	+		+
P38822	2	Protein BZZ1 ^I	+		+
P07863	2	Pectinesterase A ^J	+		+
P87498	3	Vitellogenin Y ^K	+	+	+

Swiss-Prot database accession numbers are shown. Proteins identified in at least two of the three independent IP experiments have been shown with (+) for a particular IP method and presented in alphabetical order. Superscripted letters designate identifications based on homology with other species:

- ^A *Erenothecium gossypii*
- ^B *Canis familiaris*
- ^C *Hepatitis GB virus B*
- ^D *Leishmania mexicana*
- ^E *Mycobacterium bovis*
- ^F *Streptomyces coelicolor*
- ^G *Sulfolobus acidocaldarius*
- ^H *Thermoplasma volcanium*

J Saccharomyces cerevisiae

J Erwinia chrysanthemi

K Gallus gallus.