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# Extracellular Matrix in the Trabecular Meshwork

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## Abstract

The extracellular matrix (ECM) of the trabecular meshwork (TM) is thought to be important in regulating intraocular pressure (IOP) in both normal and glaucomatous eyes. IOP is regulated primarily by a fluid resistance to aqueous humor outflow. However, neither the exact site nor the identity of the normal resistance to aqueous humor outflow has been established. Whether the site and nature of the increased outflow resistance, which is associated with open-angle glaucoma, is the same or different from the normal resistance is also unclear.

The ECMs of the TM beams, juxtacanalicular region (JCT) and Schlemm's canal (SC) inner wall are comprised of fibrillar and non-fibrillar collagens, elastin-containing microfibrils, matricellular and structural organizing proteins, glycosaminoglycans (GAGs) and proteoglycans. Both basement membranes and stromal ECM are present in the TM beams and JCT region. Cell adhesion proteins, cell surface ECM receptors and associated binding proteins are also present in the beams, JCT and SC inner wall region.

The outflow pathway ECM is relatively dynamic, undergoing constant turnover and remodeling. Regulated changes in enzymes responsible for ECM degradation and biosynthetic replacement are observed. IOP homeostasis, triggered by pressure changes or mechanical stretching of the TM, appears to involve ECM turnover. Several cytokines, growth factors and drugs, which affect the outflow resistance, change ECM component expression, mRNA alternative splicing, cellular cytoskeletal organization or all of these. Changes in ECM associated with open-angle glaucoma have been identified.

### Keywords

Aqueous humor outflow resistance; proteoglycans; extracellular matrix proteins; extracellular matrix turnover

## Introduction

The trabecular meshwork (TM) is thought to provide most of the flow resistance to aqueous humor outflow, although the alternative or uveoscleral outflow pathway does contribute to total aqueous humor drainage. (Bill and Maepea 1994; Schachtschabel et al. 2000; Weinreb 2000; Weinreb et al. 2002) Modulation of the trabecular outflow resistance is responsible for most of the regulation of intraocular pressure (IOP). (Brubaker 1970; Brubaker 1991) In open-angle glaucoma, sustained increases in the trabecular outflow resistance commonly result in

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elevated intraocular pressure (IOP). Elevated IOP is the primary risk factor for glaucomatous optic neuropathy, which is associated with progressive vision loss. Since glaucoma is responsible for around 25% of new blindness in developed nations, understanding the source, nature, and regulation of the normal and the glaucomatous trabecular outflow resistances is of considerable medical importance. (Quigley 1993,1996; Quigley and Vitale 1997)

The outer uveal and corneoscleral portions of the TM are highly fenestrated and composed of several irregular layers of ECM covered by TM beam cells (Fig. 1). The trabecular beams become more flattened and sheet-like in the deeper portions of this region. The beams and sheets are normally covered and maintained by continuous monolayers of TM cells, which reside on a typical basal lamina with a lamina rara and lamina densa. (Bhatt et al. 1995;Lütjen-Drecoll and Rohen 1996;Gong et al. 2002) The center or stroma of the beams exhibits typical collagen fibrils, elastic fibers and microfibril sheath-derived (SD) material. (Lütjen-Drecoll and Rohen 1996;Gong et al. 2002;Morrison and Acott 2003) Discontinuous tight junctional strands, which do not form continuous zonula occludens, and gap junctions are common between TM beam cells. (Grierson and Lee 1974;Raviola and Raviola 1981;Bhatt et al. 1995) Between the beams and sheets, the irregular intertrabecular spaces form torturous flow channels leading to the cribriform or juxtacanalicular (JCT) region, which lies adjacent to Schlemm's canal. The size of the intratrabecular spaces is sufficiently large that it is hard to imagine them contributing to the outflow resistance. The cells on the outer layers of the TM are actively phagocytic and are thought to act primarily as pre-filters, removing cellular debris from the aqueous humor prior to its passage through the less porous inner JCT and SC regions.

The JCT region is composed of an amorphous ECM with a discontinuous scattering of several layers of cribriform or JCT cells on the trabecular surface and embedded within the ECM (Fig. 1). (Tripathi 1971;Inomata et al. 1972;Tripathi 1977;Grierson et al. 1978;Rohen et al. 1981;Rohen 1983;Grierson and Calthorpe 1989;Epstein and Rohen 1991;Maepea and Bill 1992;Lutjen-Drecoll and Rohen 1994;Lütjen-Drecoll and Rohen 1996;Lutjen-Drecoll 1999;Parc et al. 2000) These JCT cells form occasional contacts with each other, often via extended processes using gap and adherens junctions.(Inomata et al. 1972;Grierson et al. 1978) They also form similar junctions with SC inner wall endothelial cells. (Inomata et al. 1972;Grierson et al. 1978) No continuous occludens junctions are apparent between JCT cells or between JCT and SC cells. SC inner wall cells form a continuous monolayer and exhibit occasional gap junctions and extensive continuous tight occludens junctions. (Inomata et al. 1972;Grierson et al. 1978;Raviola and Raviola 1981;Bhatt et al. 1995) The SC inner wall cells reside on a discontinuous basal lamina that may be affected by the movement of aqueous humor through these cells. (Hogan et al. 1971;Inomata et al. 1972;Grierson et al. 1978;Bhatt et al. 1995;Lutjen-Drecoll 1999;Gong et al. 2002;Fuchshofer et al. 2006) Whether JCT cells have basal lamina appears to be controversial. (Marshall et al. 1990;Tawara and Inomata 1994;Hernandez and Gong 1996;Lütjen-Drecoll and Rohen 1996;Fuchshofer et al. 2006) These cells have been considered to be fibroblast-like and thus without any basal lamina. However, basement membrane proteins, type IV collagen and laminin, and basement membrane proteoglycans have been identified in the JCT region and associated with the JCT cells. (Marshall et al. 1990;Tawara and Inomata 1994;Fuchshofer et al. 2006) One study found basement membrane-like material adjacent to all JCT cells. (Fuchshofer et al. 2006) This varied in amount and location with individual cells and was observed associated with at least half of the cell surface in some cases. It was generally not observed of the face of these cells where they abut the "open spaces" found scattered throughout the JCT region. (Fuchshofer et al. 2006)

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## Possible sites of the outflow resistance

Sequential serial dissection and microcapillary studies with perfused eyes suggest that the trabecular outflow resistance resides somewhere within the deepest 1/4 to 1/3 of the TM. (Grant 1958; Grant 1963; Ellingsen and Grant 1972; Rosenquist et al. 1989; Maepea and Bill 1992; Bill and Maepea 1994) When a microcapillary was inserted from Schlemm's canal toward the anterior chamber, the resistance was bypassed at 7–14 μm from the inner wall of Schlemm's canal. This is approximately the thickness of the juxtacanalicular region (Fig. 1). (Gong et al. 2002; Johnson 2006) This observation appears to eliminate the SC inner wall endothelium as a source of resistance, while positively implicating the remainder of the JCT. However, it has been argued that the microcapillary used in these studies was sufficiently large in bore that it may have deformed the inner wall a portion of this distance prior to breaching it. (Johnson 2006) Thus, the precise location of the resistance has not been definitively established. Most of the resistance does appear to reside within the JCT or SC inner wall endothelium, or both. (Lutjen-Drecoll 1999; Ethier 2002; Johnson 2006)

## Segmental flow

One caveat that may add complexity is the segmental flow pattern observed particularly in older human eyes. By all appearances, SC in humans and higher primates is completely open and unobstructed at normal IOP levels. Since there are approximately 25–30 collector channels distributed around a human eye, (Hogan et al. 1971) it seems that fluid within SC should exhibit relatively free circumferential flow. This would allow drainage via adjacent or even other more distant collector channels. However, a number of studies have documented segmental flow differences around the circumference of the eye. (Grant 1956; Grant 1958; Grant 1963; Buller and Johnson 1994; Parc et al. 2000; Ethier and Chan 2001; Gottanka et al. 2001; Hann et al. 2005; Hann and Johnson 2007) TM dissections studies and other observations suggest that fluid may not be free to move around the circumference of SC. (Hogan et al. 1971; Rosenquist et al. 1989; Lutjen-Drecoll 1999; Ethier 2002; Ethier et al. 2004; Johnstone 2004; Johnson 2006) Clinical observations that multiple site trabeculectomy or stent placement improves drainage, further highlight this phenomenon. Evaluation of F-actin cytoskeletal alignments within SC inner and outer wall endothelial cells and shear stress modeling of radial flow in SC, particularly relative to collector channels, suggests that this is more complicated than originally perceived. (Ethier et al. 2004)

## Possible sources of outflow resistance

Glycosaminoglycans (GAGs) within the TM have long been a favorite hypothetical source of the outflow resistance. (Bárány 1953; Bárány, E.H. and Scotchbrook, S. 1954; Pedler 1956; Francois 1975) These long repeat disaccharide chains with a high density of carboxyl and sulfate groups, which are negatively charged at physiologic pH, would be the ideal material to form the outflow resistance. (Francois 1975; Rohen and Lütjen-Drecoll 1981; Rohen and Lutjen-Drecoll 1982; Acott 1992, 1994a; Acott and Wirtz 1996; Hernandez and Gong 1996; Yue 1996) Initial perfusion studies with enzymes, which selectively degrade GAGs, were found to reduce the outflow resistance by approximately 50% in bovine eyes. (Bárány 1953; Bárány, E. H. and Scotchbrook, S. 1954; Bárány and Woodin 1954; Pedler 1956) Since then, variations of these experiments using eyes from different species perfused with several different GAG-degrading enzymes and conditions have given somewhat conflicting results. (Peterson and Jocson 1974; Knepper et al. 1984; Sawaguchi et al. 1992; Sawaguchi et al. 1993; Hubbard et al. 1997) In dog (Van Buskirk and Brett 1978) and rabbit (Knepper et al. 1984) eyes, in vivo, hyaluronidase treatment increased outflow facility. In bovine perfused organ culture, chondroitinase ABC increased outflow facility, while hyaluronidase and heparatinase did not have significant effects. (Sawaguchi et al. 1993) In porcine perfused

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anterior segment organ culture, chondroitinase ABC increased outflow facility significantly and a combination of chondroitinase ABC, heparatinase and hyaluronidase was even more effective. (Keller et al. 2007a) In primates, one study found that intracameral injection of chondroitinase ABC increased outflow (Sawaguchi et al. 1992), one study found that hyaluronidase increased outflow (Peterson and Jocson 1974), and one study found that neither chondroitinase ABC nor hyaluronidase had significant effects on outflow. (Hubbard et al. 1997) In human perfused anterior segment organ culture, heparatinase was shown to increase outflow (Johnson and Bahler 1999) and inhibition of GAG biosynthesis or GAG sulfation increased outflow. (Keller et al. 2007a) Thus, a contribution of GAGs to the outflow resistance seems highly likely, but a direct role in providing the resistance, particularly in humans and higher primates, has not been unequivocally established.

When combined with hydrodynamic modeling, initial morphometric analysis of transmission electron micrographs of the TM, suggested that the visible ECM within the JCT did not contain sufficient electron dense material to account for the outflow resistance. (McEwen 1958; Ethier et al. 1986) It was postulated that some “invisible” GAG-like substance filled the “empty spaces.” GAG chains would clearly not retain their native conformations through the harsh processing steps and are unlikely to be stained sufficiently to be visible in the micrographs. Recent quick-freeze deep-etch and electron microscopy studies show a much more extensive ECM in the JCT. (Gong et al. 2001; Gong et al. 2002) However, the actual visualized material is still insufficient to account for the outflow resistance. Although this method shows dramatically increased amounts of ECM, the highly-charged GAG side chains would still be expected to collapse completely in response to the processing necessary for even this approach. Thus, even this much-improved electron micrographic method highlights the presence of numerous micron-sized “open spaces” within the JCT that can easily account for more fluid conductivity than is observed experimentally. (Gong et al. 2001; Gong et al. 2002; Johnson 2006)

## Cells or ECM or both provide the outflow resistance

Studies comparing the outflow facility of *in vivo* to *in vitro* human eyes and evaluating the effects of temperature and of metabolic poisons on outflow facility all suggest that aqueous humor is not transported by an active cellular metabolic process. (Bárány 1953; Van Buskirk and Brant 1974; Epstein et al. 1981; Epstein and Anderson 1984; Suzuki and Anderson 1993) A number of agents that primarily act on cytoskeletal organization have been shown to modulate outflow facility. (Kaufman and Erickson 1982; Johnson 1997; Peterson et al. 1997; Tian et al. 1998; Epstein et al. 1999; Sabanay et al. 2000; Rao et al. 2001; Vittitow et al. 2002; Rao et al. 2005) These studies suggest that cells somewhere in the TM or the SC inner wall, or both, are directly involved in forming the outflow resistance. Perfusion of a fibronectin fragment that disrupts integrin-ECM interactions and could affect either ECM or cytoskeleton also increases outflow facility. (Santas et al. 2003) A recent study showing effects of cytoskeletal modifying agents on matrix metalloproteinases (MMPs) further adds to the complexity of assigning direct roles for the cells or ECM. (Sanka et al. 2007) The various GAG-modifying studies mentioned above suggest a direct ECM contribution to the outflow resistance. The initiation of ECM turnover in the TM, mediated by members of the MMP family, also increases outflow facility. (Bradley et al. 1998) Perhaps more importantly, the inhibition of endogenous trabecular MMP activity reduces outflow facility. Thus ongoing ECM turnover is required to maintain the appropriate outflow resistance. (Bradley et al. 1998) The microcapillary observations mentioned earlier also argue for the JCT ECM and against SC inner wall cells. (Maepea and Bill 1992)

However, due to the highly interdependent nature of the bidirectional interactions between cells and their ECMs, an approach which can clearly differentiate between the actual direct

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contribution of either the cells or the ECM to the aqueous outflow resistance has not yet been devised. Consequently, although both the cells and the ECM are clearly critical to maintaining the outflow resistance, their respective direct contributions remain unclear. Aspects of this uncertainty and a variety of other relevant studies have been discussed in detail. (Johnson and Erickson 2000; Ethier 2002; Fautsch and Johnson 2006; Johnson 2006)

A model reflecting a combined contribution of cells and ECM has considerable appeal, particularly in light of the inconclusive evidence discussed above. (Johnson et al. 1992; Ethier 2002; Overby et al. 2002; Johnson 2006) If flow through the inner wall cells of Schlemm's canal is constrained to a subpopulation of the inner wall "pores" that are not fixation artifacts, then the idea of "funneling" becomes tenable. With a limited number of real pores serving as the necks of funnels, fluid movement through the ECM of the JCT would be constrained to regions near and upstream from the funnel openings. These portions of the ECM and the SC inner wall cells would together provide the outflow resistance. Unfortunately, although this model helps to resolve the apparent conflicting observations, a clear method to test this combined resistance model has also yet to be devised.

## ECM components identified within the TM

A variety of ECM components have been identified within the TM. Most of these are similar to those found in other tissues, although a few apparently unique isoforms have been identified. Only limited information is available about the detailed molecular organization, exact composition and relative abundance of these components. Presumably, these nuances will be very important in defining the unique function(s) and properties of the outflow pathway. Studies with general microscopic stains and electron microscopy identified GAGs, fibrillar collagens, unevenly-spaced or curly collagen, elastic fibrils with associated sheath-derived plaque material, basement membrane and amorphous basement membrane-like materials and other non-specific ECM components. (Hogan et al. 1971; Francois 1975; Grierson and Lee 1975; Lütjen-Drecoll et al. 1981; Richardson 1982; Lütjen-Drecoll et al. 1986; Lütjen-Drecoll and Tamm 1987; Lütjen-Drecoll and Rohen 1996; Lutjen-Drecoll 1999; Gong et al. 2002)

## GAGs

The GAG composition in TM extracts was determined by sequential enzymatic degradation with various GAG-degrading enzymes. The sources included TM from a few hours postmortem human eyes, stationary human anterior segment organ cultures, perfused human anterior segment organ cultures and densely-confluent early-passage human TM cell cultures. (Grierson and Lee 1975; Schachtschabel et al. 1977; Knepper et al. 1981; Schachtschabel et al. 1982; Rohen et al. 1984; Acott et al. 1985; Yue and Elvart 1987; Acott et al. 1988; Tschumper et al. 1990; Acott 1994a; Knepper et al. 1996b) Relatively good agreement was found in the amount of each type of GAG in the TM using these models and all of the normal GAGs were identified. Of the total trabecular GAGS, approximately 20–25% is hyaluronic acid or hyaluronan (HA), 40–60% is chondroitin and dermatan sulfates (CS and DS), 5–10% is keratan sulfate (KS) and 15–20% is heparan sulfate (HS). (Francois 1975; Rohen and Lütjen-Drecoll 1981; Rohen and Lutjen-Drecoll 1982; Acott 1992, 1994a; Acott and Wirtz 1996; Yue 1996) These percentages are based on Alcian blue or similar GAG staining or $^{3}$H- or $^{35}$S-radiolabel incorporation and are not strictly calibrated molar amounts. When normalized to a relative ng amount based on GAG standards, HA was 15 %, CS/DS was 38%, KS was 6.5% and HS was 40% of the total. (Knepper et al. 1996a)

Localizations of the GAGs within the TM have been determined by similar enzymatic degradation methods using various GAG stains and light or transmission electron microscopy (TEM). Hyaluronan binding protein and GAG-specific antibodies have also been used to localize GAGs within the TM. Most studies found HA primarily in the JCT region with less

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in the remainder of the TM using HA binding protein as a probe (Keller et al, manuscript in review) (Lütjen-Drecoll et al. 1990; Hernandez and Gong 1996; Lerner et al. 1997) or quantitative hyaluronidase removal of Alcian blue staining. (Knepper et al. 1996b) The other GAGs are sulfated, facilitating alternative staining methods. When GAGs were stratified within the JCT of normal human eyes, fg/$\mu m^2$ values obtained were 7.78 for HA, 8.18 for CS, 0.29 for DS, and 2.48 for HS (where HS included a small contribution from undegraded material). (Knepper et al. 1996b) Using 4-layer stratification, HA and HS were found to be higher near SC and declined somewhat towards the trabecular side of the JCT, while CS and DS were found to increase modestly towards the trabecular side of the JCT. (Knepper et al. 1996b)

Using antibodies specific to the various sulfated GAG chains, we (Keller et al, manuscript in review) found for that all cell layers in the TM and JCT, CS, DS and HS were present at higher levels just beneath the cells. CS/DS GAGs were also found as small clumps in the center of TM beams, apparently associated with collagen fibrils and elastin microfibrils. The JCT showed relatively dense staining throughout for CS, DS and HS sulfated GAGs. Using Cuprolinic or Cupromeronic Blue to stain sulfated GAG chains in conjunction with GAGase treatments, sulfated proteoglycans have been localized at the ultrastructural level within the TM. (Tawara et al. 1989; Gong et al. 1992; Tawara and Inomata 1994; Hernandez and Gong 1996; Hubbard et al. 1997) Using Cuprolinic Blue, small thin structures were found to be closely associated with collagen fibrils. A large thick structure was associated more peripherally with and between collagen fibril bundles and associated with fine filaments. Both of these contained predominantly DS/CS GAGs. Basement membrane-associated structures contained HS GAGs. (Gong et al. 1992) Although they did not identify the proteoglycan core proteins in this study, the small thin structures were probably one or more small leucine-rich proteoglycans (SLRPs) such as decorin or biglycan; the large thick structure was probably versican; and the basement membrane-associated HS proteoglycan was probably perlecan. Using Cupromeronic Blue and a slightly different analysis, fairly similar distributions were obtained. (Tawara et al. 1989) In one study, these sulfated proteoglycans were found associated with JCT cell basement membrane-like material. (Tawara and Inomata 1994)

## Proteoglycans

With the exception of hyaluronan, (Itano and Kimata 2002) GAGs are synthesized directly as covalent side-chains on serine/threonine or asparagine groups of specific proteoglycan core proteins. (Funderburgh 2002; Silbert and Sugumaran 2002; Sugahara and Kitagawa 2002) Studies employing several methods of identification showed that the TM expresses a number of proteoglycans (PGs) (Table 1). This includes a number of SLRPs, CSPGs, HSPGs, and other types of PGs (Table 1). (Murphy et al. 1987; Tanihara et al. 1995; Wirtz et al. 1997; Knepper, P et al. 1998; Friedman et al. 2000; Friedman et al. 2002; Ishibashi et al. 2002; Knepper, P et al. 2002; Borras 2003; Lo et al. 2003; Filla et al. 2004; Vittitow and Borras 2004; Zhao et al. 2004; Chen et al. 2005; Vittal et al. 2005; Keller et al. 2007b)

Some specific core proteins have been localized to regions of the TM by immunohistochemistry using light or transmission electron microscopy. Perlecan was localized to the basement membranes of the TM beams and SC inner wall cells. (Murphy et al. 1987) At the light level, syndecan 1 immunostaining in the TM/SC was light or absent. Syndecan 2, 3 and 4 immunostaining was observed through the TM/SC region with syndecan 3 immunostaining being somewhat stronger in the JCT/SC region. (Filla et al. 2004) As would be expected for a transmembrane protein, syndecan immunostaining was strongest at the cell edges. At the ultrastructural level, decorin and versican immunostaining was found within the electron dense elastic fiber cores and the surrounding microfibril sheath material, both within the TM beams and the JCT region. (Ueda et al. 2002; Ueda and Yue 2003) Both proteoglycans were also localized to the long-spaced (100–120 nm periodicity) collagen and the normal collagen fibrils.

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At the light level, we see similar versican and fibromodulin distributions with a significant amount also associated with the TM beam, JCT and SC cells. (Keller et al. 2007a)

Many of these proteoglycans have complex structures with multiple binding domains and exhibit alternative splicing. (Zhao and Russell 2005; Keller et al. 2007b) For example, versican (Fig. 2) is a large lectican, which can bind to hyaluronan chains and interact with various other ECM proteins or cellular receptors and has four alternative mRNA splicing variants. (Wight 2002; Wu et al. 2005; Kenagy et al. 2006) TM cells express these different splice forms and the ratios are changed with mechanical stretch, TGFβ, TNFα and IL-1α treatments (Keller, et. al. manuscript in preparation). (Zhao and Russell 2005; Keller et al. 2007b) As indicated, these splice variants change the number of potential GAG attachment sites from over 20 to 0, thus dramatically affecting the structure and functional properties of this proteoglycan. Versican is also cleaved by specific proteinases at various sites, which results in fragments with very different activities such as stimulation of cell division, disruption of chemokine function and apoptosis. (Kenagy et al. 2006) In the TM, the versican fragment produced by ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) 1 or 4, which cleave between Glu441 and Ala442 in the alpha GAG domain, is found in moderate abundance and may be higher in areas of high segmental outflow (Bradley, et. al., manuscript in preparation).

## Other ECM components identified in the TM

Using molecular, biochemical and immunohistochemical approaches, a variety of other ECM proteins have been identified in the TM, or are expressed by TM cells. A select group is listed in Table 2. These ECM proteins and glycoproteins serve a variety of divergent functions in other tissues, although most have been studied in little detail in the TM.

## Basement Membrane

Several intrinsic basement membrane proteins, particularly laminin, type IV collagen and perlecan, are expressed in the outflow pathway and have been localized to basement membranes of TM beam cells, SC inner wall cells and the JCT cells. (Murphy et al. 1987; Marshall et al. 1990; Hernandez and Gong 1996; Yue 1996; Zhou et al. 1998a; Brilakis et al. 2001; Ueda et al. 2002; Ueda and Yue 2003; Fuchshofer et al. 2006) Interestingly, these components are not strictly limited to the basal lamina of the JCT but are also found dispersed throughout the JCT, perhaps indicating something novel about the origins of the JCT ECM. Laminins have a cruciform shape composed of a heterotrimer of different isoforms of α, β and γ subunits. (Scheele et al. 2007) The central shaft of the cross is a long coiled-coil formed by interaction of α-helix domains in the three subunits. The arms and head are formed by the individual subunits. The foot of the cross contains cell receptor binding sites and the various individual arms have binding sites for other cell-surface receptors, a number of ECM proteins, and several proteoglycans. Laminin can also form extended networks. The cellular laminin receptors include a number of integrins, the α-subunit of dystroglycan, specific HS GAG chains and sulfatides. (Scheele et al. 2007) Nidogen/entactin is a glycoprotein that binds to laminin and is thought to link it to type IV collagen as part of stabilizing basement membrane ECM networks. (Gersdorff et al. 2007) Nidogen 1 is expressed in TM. (Fuchshofer et al. 2006) Type IV collagen, a non-fibrillar collagen with one noncollagenous domain in the mature form, is also an integral component of basement membranes. It exists as a trimer of various α-subunits and can form larger three-dimensional networks. (Linsenmayer 1991) Several different α subunits have been identified in the TM. (Fuchshofer et al. 2006) Perlecan, the large basement membrane HSPG, is also an integral component of TM ECMs.

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## Fibronectin and Vitronectin

Fibronectin (Fig. 3) is present in basement membranes, but is also made by cells without basement membranes and found in other ECMs. It is found abundantly in TM beams and the JCT region, with slightly higher levels in the basement membranes of TM beam cells, JCT cells and SC inner wall cells (Keller et al, manuscript in review). (Floyd et al. 1985;Murphy et al. 1987;Zhou et al. 1998a;Bachmann et al. 2006;Keller et al. 2007a) In the JCT at the ultrastructural level, fibronectin is found in the amorphous material associated with the basement membranes and with the peripheral SD material of the elastic microfibrils. (Yue 1996;Brilakis et al. 2001;Ueda et al. 2002;Ueda and Yue 2003) In addition to forming a thin fibrous array that appears to encircle TM cells in culture, (Zhou et al. 1998b) fibronectin also forms thick fibrous strands. (Keller et al. 2007a) Fibronectin fibril formation has been studied in other tissues and involves interactions with cell surface integrins, proteoglycans and tenascin C. It produces a fibril with considerable elasticity. (Baneyx et al. 2002;Mao and Schwarzbauer 2005;Abu-Lail et al. 2006) This elasticity appears to come from unfolding domains of fibronectin and shifting to extended molecular conformations. (Baneyx et al. 2002;Abu-Lail et al. 2006;Vogel and Sheetz 2006) As discussed later, mechanical stretching of TM cells and their ECMs triggers homeostatic responses that reduce the outflow resistance. These fibronectin fibrils, which would have a number of cellular integrin attachments for each fibril, would provide an ideal sensing structure to communicate IOP-induced mechanical stretch to TM cells. Fibronectin has tandem repeats of three types of domains of around 50 amino acids each, which form protein-, GAG-, and integrin cell-binding domains. Three alternative splice regions are known, and TM cells express EIIIA, EIIIB and the variable regions V1, V2 and V3 of IIICS under different conditions. (Li et al. 2000;Vittal et al. 2005) Inclusion of the V1–V3 domains within the IIICS domain introduces a number of binding sites for heparin and several additional integrin α4β1 sites. (Vittal et al. 2005) Vitronectin has also been identified in the TM, associating with the cores and SD material of the elastic microfibrils. (Yue 1996;Ueda et al. 2002;Ueda and Yue 2003) Attachment of TM cells to vitronectin also has effects on cell properties. (Yue 1996)

## Collagens

In addition to the type IV basement membrane collagen mentioned earlier, the TM expresses a variety of other collagens. The classic interstitial fibrillar collagens, types I and III, form the standard collagen fibrils with a periodic 67-nm pattern, seen throughout the center of TM beams and JCT region. (Murphy et al. 1987; Marshall et al. 1991; Hernandez and Gong 1996; Yue 1996; Zhou et al. 1998a; Zhou et al. 1998b) They typically form evenly-spaced fibrils that serve primarily as a source of tensile strength for tissues. As mentioned above, type IV forms networks somewhat reminiscent of the hexagonal lattices in chicken wire, which coupled with other basement membrane proteins, provide a stable support for cell attachments.

Types V and VI collagens have been found in both the TM beams and JCT region. They have been observed associated with basement membranes in areas where collagen or elastic fibrils intersect or link to them; associated with the striated collagen fibrils forming a fine filamentous lattice around and between fibrils; associated with the SD microfibrils and to a lesser extent with the dense elastic cores; and associated with long-spaced collagen. (Lütjen-Drecoll et al. 1989; Marshall et al. 1991; Hernandez and Gong 1996; Lutjen-Drecoll 1999; Ueda et al. 2002; Ueda and Yue 2003) Some variations in their precise localizations were observed, probably reflecting differential epitope availability.

Type VI appears to be a key component of the connecting fibrils that have been observed between SC or JCT cells and the cribriform elastic-like plexus, which permeates the JCT region. (Lutjen-Drecoll et al. 1989 ; Tripathi, BJ et al. 1994; Lütjen-Drecoll and Rohen 1996; Lutjen-

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Drecoll 1999; Ueda et al. 2002; Ueda and Yue 2003; Fuchshofer et al. 2006) Type VI chains have a collagenous triple helix domain for assembly into a disulfide-bonded heterotrimeric type VI monomer, which then forms tetramers that assemble into beaded microfibrils (Fig. 4). Type VI collagen is a major component of 3–5 nm diameter microfibrils (Kielty et al. 1991) and fibrillin is a central component of the beaded 8–12 nm microfibrils discussed later. (Sakai et al. 1991) Several models for tetramer and higher level collagen VI microfibril have been proposed and one is shown in Fig. 4. (Bruns et al. 1986; Engvall et al. 1986; Ball et al. 2001; Reale et al. 2001; Baldock et al. 2003; Ball et al. 2003; Knupp et al. 2006) In the TM, type VI immunostaining of both heteromorphic aggregates and interband longitudinal sheets has been observed, supporting the model in Fig. 4. (Reale et al. 2001) This may relate to the 100 nm repeat structures in the JCT, although this has not been comprehensively studied in detail. (Hessle and Engvall 1984; Bruns et al. 1986; Engvall et al. 1986; Knupp et al. 2006) The type VI collagen N-terminal repeats bind hyaluronan and heparin, while the C-terminal domains are involved in self-assembly. (Kielty et al. 1992b; Specks et al. 1992; Ball et al. 2001; Ball et al. 2003) Decorin and biglycan associate with the N-terminal end of the central collagenous domain, affecting fibril formation. This domain also contains RGD sequences, which are responsible for cell-association. (Kielty et al. 1992a; Fitzgerald et al. 2001; Wiberg et al. 2001; Wiberg et al. 2002; Wiberg et al. 2003) It also interacts with a membrane-associated CSPG 4 and microfibril-associated glycoprotein (MAGP) 1. (Kreis and Vale 1999) Finally, type VI collagen exhibits a number of alternative mRNA splice variants, some of which are marked in Fig. 4. (Chu et al. 1989; Zanussi et al. 1992)

Non-fibrillar collagens type VII, VIII, XI, XV and XXII are produced by TM cells, as are fibril-associated collagens type XII and XIV (Chen et. al., manuscript in preparation). (Yue 1996;Zhao et al. 2004;Vittal et al. 2005;Fuchshofer et al. 2006;Keller et al. 2007b) Collagen VIII is a short-chain collagen also found in Descemet's membrane and in subendothelial ECMs that forms hexagonal lattice structures and may serve to provide an open, porous structure that can withstand compressive force. (Kreis and Vale 1999) Collagen type XV is among the multiplexins and is normally found in basement membranes forming short triple helices. (Kreis and Vale 1999) Additional specific details about the functions of these collagens in the outflow pathway have not been determined.

The FACIT (fibril associated collagens with interrupted triple helices), including type XII and XIV collagens, contain small collagenous domains. Their longer, non-collagenous (NC) domains are comprised of a number of typical ECM binding domains, including one for the DS chains of decorin (Fig. 5). (Kreis and Vale 1999) They are found in dense connective tissue associated with other collagen fibrils and appear to be important in fibril organization and interactions. They also often exhibit alternative mRNA splicing (Keller et al. 2007b) and type XII can have GAG chains. (Kreis and Vale 1999) Fig. 5 shows the domain structure, multimerization, and binding domains of type XII collagen, which is very similar to type XIV. (Kania et al. 1999) Alternative mRNA splice variants in the TM include short and long versions of NC3 and NC1. (Kania et al. 1999) Normally, the TM predominantly expresses the short form of both, but mechanical stretching increases expression of the long NC3 form and a novel alternative splice form missing exons 29 and 30 from the C-terminal end of NC3. (Keller et al. 2007b)

## Elastin and Microfibrils

The TM and JCT region has an extensive elastin-containing microfibril network that has been described as a cribriform elastic-like plexus. (Lütjen-Drecoll et al. 1981; Lütjen-Drecoll and Rohen 1996; Lutjen-Drecoll 1999) The elastin-containing microfibrils appear to attach to JCT cells and SC endothelial cells via the small connecting fibrils mentioned earlier that appear to contain type VI collagen. This elastic microfibril plexus also appears to attach to longitudinal

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processes of the scleral spur and eventually the ciliary muscle tendons. (Lütjen-Drecoll et al. 1981; Lütjen-Drecoll and Rohen 1996; Lutjen-Drecoll 1999; Fuchshofer et al. 2006) A contractile cell has also been identified in the scleral spur, which may have some function in modulating outflow via this elastic microfibril plexus. (Tamm et al. 1992, 1993; Lütjen-Drecoll and Rohen 1996; Lutjen-Drecoll 1999) TM beam and JCT cells appear to be highly similar or even identical, except that the latter expresses αB crystallin, both *in situ* and in primary cell culture. (Fuchshofer et al. 2006) Since TM cells do express αB crystallin in response to mechanical stretching (Gonzalez et al. 2000; Vittal et al. 2005), this expression difference *in situ* was interpreted as evidence that JCT cells experience chronic mechanical stress/stretching/tension via this elastic microfibril plexus. (Fuchshofer et al. 2006)

Elastin has been localized to the electron lucent central core of these microfibril structures in the TM beams and the JCT. (Gong et al. 1989; Umihira et al. 1994; Hernandez and Gong 1996; Ueda et al. 2002; Ueda and Yue 2003) The sheath-derived material shows a regular banding pattern and has been referred to as type III plaques. (Lütjen-Drecoll et al. 1981; Lütjen-Drecoll and Rohen 1996; Lutjen-Drecoll 1999) Fibulin 1, fibrillin 1 and 2, and microfibril-associated glycoproteins (MAGP) 1, 2 and 4 are apparent components of this microfibril sheath. (Ueda et al. 2002; Ueda and Yue 2003; Liton et al. 2006) A number of other ECM proteins have also been localized to this SD material, including fibronectin, versican and decorin. (Ueda et al. 2002; Ueda and Yue 2003) Binding interactions between various domains of all of these molecules, including several cell surface receptors, have been identified and a number of models developed to explain aspects of the structures. However, these models are complex and not completely in agreement and no details for the outflow pathway have been established. (Aspberg et al. 1999; Trask et al. 1999; Trask et al. 2000; Olin et al. 2001; Isogai et al. 2002; Kielty et al. 2002a; Kielty et al. 2002b; Segade et al. 2002; Timpl et al. 2003; Kielty et al. 2005) Several models could be proposed that would link this system to the ECM components that may be involved in the outflow resistance and to specific cellattachment components.

## Matricellular Proteins

Matricellular proteins are a group of modular ECM proteins that are often involved in reorganization of ECM and changes in ECM-cell interactions or relationships. (Bornstein and Sage 2002) Typically, they interact with both other ECM components and cell-surface receptors, often intervening in their interactions. They are often highly expressed during differentiation, wound healing, tissue remodeling and similar processes. In these processes, they are often anti-adhesive or interfere with binding interactions to allow changes in ECM organization. By contrast, they can also exhibit adhesive properties. Their functions and effects on cells and ECM are very cell type and context-sensitive, producing very different actions in different tissues under various conditions. With increased scrutiny, they continue to exhibit new and different functions and properties. The TM expresses a number of matricellular proteins.

SPARC/osteonectin (secreted protein, acidic and rich in cysteines) is expressed throughout the TM and JCT region and by TM cells in culture. (Wirtz et al. 1997; Rhee et al. 2003) Testicans 1 and 3, proteoglycan members of the SPARC family, are expressed in TM, as discussed earlier. (Chen et al. 2005) The domain structure of members of this family is shown in Figure 6.

Thrombospondins 1–4 are expressed in the TM. (Tripathi et al. 1991; Si et al. 2003; Flugel-Koch et al. 2004; Zhao et al. 2004; Chen et al. 2005; Fleenor et al. 2006; Liton et al. 2006; Fuchshofer et al. 2007) Figure 7 shows the domain structures and binding motifs of the thrombospondins. Thrombospondins 1 and 2 form homotrimeric structures and are considered to be matricellular proteins, while thrombospondins 3 and 4 are structurally somewhat different and are not considered to be matricellular proteins. (Bornstein and Sage 2002)

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Thrombospondin 1 is thought to be a major activator of TGFβ, most isoforms of which are present in latent forms that need activation for function. (Crawford et al. 1998) Since TGFβ is thought to be of significance in affecting the normal and glaucomatous ECMs of the outflow pathway and has been shown to modify outflow facility, this may be of considerable importance. (Tripathi, RC et al. 1994; Welge-Lussen et al. 1999; Li et al. 2000; Welge-Lussen et al. 2000; Lutjen-Drecoll and Rohen 2001; Gottanka et al. 2004) Immunostaining for thrombospondin 1 is observed throughout the TM, with the greatest density in the JCT region. (Flugel-Koch et al. 2004)

Tenascin C or hexabrachion is another matricellular protein expressed in the TM. (Chen et al. 2005; Vittal et al. 2005; Keller et al. 2007b) Tenascin C is composed of numerous repeat binding domains and forms a hexamer by disulfide bonding of the N-terminal tenascin assembly domain using heptad repeats (Fig. 8). (Ghert et al. 2001) It has both adhesive and anti-adhesive effects and is involved in cell migration, ECM remodeling, development, tumorogenesis and wound healing. (Chiquet-Ehrismann et al. 1988; Vainio et al. 1989; Ghert et al. 2001; Bornstein and Sage 2002; Chiquet-Ehrismann and Chiquet 2003; Chiquet-Ehrismann 2004) Some of these processes occur via tenascin C's association with fibronectin fibrils and effects on fibronectin-cell interactions. (Chung et al. 1995; Ghert et al. 2001) In addition to exhibiting changes in levels with various treatments that modify outflow facility (see later), tenascin C also undergoes a number of changes in mRNA splicing (Keller et al, manuscript in preparation). (Keller et al. 2007b) The inclusion of various partial or complete fibronectin type III domains between exons 5 and 6 of tenascin C introduces a number of heparin and other types of binding domains. Although the nuances of these changes on the properties of the outflow pathway have not been established, the potential seems significant.

Osteopontin is expressed by TM cells and has recently been classed as a matricellular protein. (Bornstein and Sage 2002; Chen et al. 2005) Unlike the other matricellular proteins, it also may serve some structural roles. (Denhardt and Guo 1993; O'Regan and Berman 2000; Bornstein and Sage 2002) It is a secreted, highly acidic and phosphorylated sialoprotein, which binds to cells via GRDGS interactions with αVβ3 integrin. (Denhardt and Guo 1993; Sodek et al. 2000) Thrombin cleavage releases two peptides with different functions. It interacts with fibronectin, type I collagen and osteocalcin (bone Gla protein). Additional details on its functional involvements are emerging. (Denhardt and Guo 1993; O'Regan and Berman 2000; Sodek et al. 2000; Bornstein and Sage 2002)

## Other ECM Proteins

Periostin or osteoblast specific factor 2, although not classed as a matricellular protein, appears to exhibit some characteristics and functions common to this group. Periostin is expressed by TM cells. (Vittitow and Borras 2004; Zhao et al. 2004; Chen et al. 2005; Vittal et al. 2005; Liton et al. 2006) Periostin contains several fasciclin-like binding domains, binds several different integrins, including αVβ3, αVβ5, and affects cell adhesion and migration. (Gillan et al. 2002; Litvin et al. 2004) It is expressed during development and active ECM remodeling, particularly where collagen is being modified. (Litvin et al. 2004; Kii et al. 2006) Initially involved in osteoblast function in mineralized tissues, it appears to have other functions in other tissues, such as the TM.

As may pertain to this latter pair of proteins, osteopontin and periostin (both thought initially to be bone-specific and limited to osteoblast mineralization functions) matrix GLA protein is also expressed by TM cells. (Vittitow and Borras 2004; Chen et al. 2005; Vittal et al. 2005) In this protein, a number of glutamate residues are converted post-translationally to γ-carboxylglutamic acid (GLA). Mediated by calcium-binding, this creates a domain, which binds bone morphogenic protein (BMP) 2. The exact mechanism of matrix GLA protein

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involvement in the outflow pathway remains unclear, but some type of calcium deposition event has been hypothesized. (Xue et al. 2006; Xue et al. 2007) Since it can inhibit BMP2-induced calcification, its presence and modulation by a number of modulators of outflow is intriguing. Matrix GLA protein is secreted and found in bone and cartilage, serving a regulator role in calcification in these tissues and in the vascular system, where it is involved in arterial wall stiffness. In the TM, it has been shown to affect levels of calcification markers. (Xue et al. 2006; Xue et al. 2007)

A number of other ECM proteins are expressed in the outflow pathway, but important functional roles have not been established for most of them.

## Cell-surface ECM receptors of the TM

Various typical ECM cell-surface receptors are expressed in the TM. Integrins α1, α2, α3, α4, α5, α6, αV, β1, β3, β4 and β5 subunits and α5β1, αVβ3 and α4β1 dimeric complexes have been specifically identified. (Yue 1996;Zhou et al. 1996;Zhou et al. 1998b;Zhou et al. 1999c;Vittal et al. 2005) Different combinations of α and β subunits create a 3-dimensional recognition site, providing a wide array of partially-overlapping specificities for different ECM macromolecules. Thus, it seems certain that a number of additional integrin pair combinations are also present on TM cells. The integrins are involved in fibronectin fibril formation, cell-ECM attachments and both inside-out and outside-in signaling processes. For many of these functions, they form large heterogeneous aggregates at the cell surface in which multiple integrin pairs, accessory and adaptor proteins, ECM macromolecules, signaling molecules and cytoskeletal attachments coalesce. Focal adhesions, focal contacts, podosomes and CLANs are among the more well-defined coalesced structures. (Geiger and Bershadsky 2001;Geiger, B et al. 2001;Geiger, B. et al. 2001) Attachment of TM cells to various integrins produces differential cellular responses. (Yue 1996;Zhou et al. 1996;Zhou et al. 1998b,1999a,b;Zhou et al. 1999c;Zhou et al. 2000;Filla et al. 2006) Additionally, both β1 and β3 integrins are involved in CLAN formation under some sets of conditions. (Filla et al. 2006) Integrins are thought to serve a key function in sensing the mechanical stretching of the JCT, which reflects changes in IOP and serves as a signal for homeostatic outflow resistance adjustments. (Acott 1994b;Acott and Wirtz 1996;Bradley et al. 2001;Borras et al. 2002;Bradley et al. 2003)

Anterior segment perfusion with the second heparin binding domain (Hep II) of fibronectin, composed of numbers 12–14 of the type III fibronectin repeat domains (Fig. 3), reversibly increases outflow facility. (Santas et al. 2003) The Hep II domain of fibronectin interacts with α4β1 integrin and the heparan sulfate chains of syndecans. After the Hep II domain was washed out of the system and the outflow facility had returned to normal, micrographs showed that Schlemm's inner wall lining cells were missing in some areas and their basement membranes in these areas were not intact. The remainder of the TM, including the JCT cells, seemed normal and relatively unaffected in both light and electron micrographs. A similar study using an RGD peptide (GRGDSP), which is from the 10th fibronectin type III domain and is the binding site for α5β1 integrin, showed similar disruptions of Schlemm's inner wall endothelium, but did not significantly change outflow facility. (Bahler et al. 2004) The remainder of the TM was not visibly affected.

Syndecans 1–4 are another family of cell-surface ECM receptors expressed in the TM (Table I). (Filla et al. 2004;Chen et al. 2005;Vittal et al. 2005;Filla et al. 2006) The syndecans are transmembrane HS proteoglycans that cooperate with specific integrins, bridge to specific cytoskeletal structures, and are involved in inside-out and outside-in signaling. (Couchman and Woods 1999;Couchman et al. 2001;Couchman 2003;Yoneda and Couchman 2003) As HS proteoglycans, they can modulate growth factor activity and availability. They are involved in focal adhesions (particularly syndecan-4), stress fiber formation (particularly syndecan-2),

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cytoskeletal organization and other cellular processes. (Couchman and Woods 1999;Couchman et al. 2001;Santas et al. 2002;Couchman 2003;Yoneda and Couchman 2003; FFilla et al. 2004;Peterson et al. 2005;Filla et al. 2006) Syndecan-4 also appears to localize with the verticies of CLANs and syndecan-2 localizes with podosomes in the TM (Aga, et. al. manuscript in preparation). (Filla et al. 2006) They clearly serve several important functions in the outflow pathway, but their exact roles here are only beginning to emerge. (Santas et al. 2002;Filla et al. 2006) The glypicans are another family of cellsurface HS proteoglycans. They are similar to the syndecans, except that they attach to the cell surface by glycosylphosphatidylinositol (GPI) lipid anchors instead of by a transmembrane tail. (Filmus and Selleck 2001;Song and Filmus 2002) Glypican 3, 4 and 5 are expressed in TM. (Zhao et al. 2004;Chen et al. 2005) The 6 glypicans share a conserved pattern of 14 Cys and 2–4 HS attachment sites near the C-terminal hydrophobic domain where the GPI anchor is attached. (De Cat and David 2001;Song and Filmus 2002) They are critical in development, serve a variety of roles in adult tissues, and are involved in Wnt, TGFβ and BMP signaling. (Hartwig et al. 2005;Song et al. 2005) Their GAGs share a pattern of iduronate and sulfate distributions that resemble the syndecans and facilitate comparable fibronectin Hep II domain interactions. (Tumova et al. 2000) Details of glypican involvement in the TM are unknown.

Another cell-surface ECM receptor, CD44, which is a hyaluronan receptor, has been studied in the TM in some detail and clearly plays an important role, directly or indirectly, in outflow and TM function. (Knepper, P et al. 1998; Knepper, P et al. 2002; Choi et al. 2005; Knepper et al. 2005) An important impact of the truncated soluble form of CD44 on TM cells has been reported. Alternative mRNA splicing of CD44 in the TM is also observed. (Jumper et al. 1998; Keller et al. 2007b)} The N-terminal portion of CD44 is coded by exons 1–5 and the transmembrane C-terminal portion is coded by exons 6–10. Between these two is an alternative splicing site where variable domains V1–V10 can be inserted. Some of these variable domains contain CS or HS GAG chains and can affect growth factor functions by binding and modulating their availability. CD44 is also directly involved in cellular signaling, where it can trigger several processes. It can also serve as a co-receptor and docking site for MMP-7, MMP-9 and MMP-14.

VCAM-1 is expressed by TM cells and PECAM-1 is expressed by SC cells. (Heimark et al. 2002; Chen et al. 2005; Vittal et al. 2005) A variety of other cell-surface receptors and cell-cell interaction proteins are expressed by TM/SC cells. These include sarcoglycan β and ε, dystroglycan and sarcospan, which are members of the dystroglycan glycoprotein complex that can serve as a laminin receptor in some tissues. (Kreis and Vale 1999) Fibronectin leucine-rich transmembrane proteins (FLRTs) 2 and 3 are also expressed by TM cells, although their functional significance has not been determined. (Chen et al. 2005; Liton et al. 2006)

## ECM turnover and outflow resistance

Several stimuli that affect outflow facility change the activity of a family of enzymes, the matrix metalloproteinases (MMPs), which are responsible for initiating most ECM turnover. (Parks and Mecham 1998) These stimuli include TNFα, IL-1α, TGFβ, dexamethasone, elevated pressure, and/or mechanical stretch. (Samples et al. 1993; Clark et al. 1995b; Alexander et al. 1998; Bradley et al. 1998; Bradley et al. 2000; Bradley et al. 2001; Gottanka et al. 2004; Chen et al. 2005; Clark et al. 2005; Vittal et al. 2005; Fleenor et al. 2006; Kelley et al. 2007) The process of ECM turnover is a concerted and highly coordinated program of degradation and biosynthetic replacement of specific ECM components. IOP homeostasis appears to be maintained, at lease in part by ECM turnover triggered in response to pressure changes or mechanical stretch or distortion, sensed by cells within the JCT. This triggers corrective steps by which these cells adjust the outflow resistance and restore the IOP to normal ranges.

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## Regulators of aqueous outflow and ECM component expression

A number of agents or modulators have been shown to affect aqueous humor outflow facility. Although the direct mode of action of each of these agents has been hypothesized, essentially all of them affect multiple aspects of TM cell behavior. Evaluating the TM cellular responses to several of these agents might provide insights into the mechanism(s) and actual components involved in the outflow resistance. Microarrays, proteomics or similar types of studies may thus provide some guidance for this approach.

## Elevated IOP, mechanical stretching and IOP homeostasis

We and others have developed evidence that TM cells can mount a homeostatic response to elevated IOP, adjusting the outflow resistance and restoring the IOP level. (Bradley et al. 1998; Bradley et al. 2001; Borras et al. 2002) When the flow rate in perfused anterior segment organ culture is doubled, the measured pressure immediately doubles. However, TM cells respond by increasing MMP enzyme activity and changing the expression of a number of genes. Over the next few days, the TM adjusts the outflow resistance and restores the initial IOP, although the flow rate is maintained at twice the initial value. (Bradley et al. 2001) TM cells sense mechanical stretching or distortion, probably of their ECMs via their cell-surface integrins and other cell-surface receptors, as an indication that the outflow resistance is too high. In addition to adjusting MMP activity, TM cells change expression levels of a number of ECM components, a few of which are shown below (Table 3). (Vittal et al. 2005) A number of changes in mRNA splicing patterns also occur. (Vittal et al. 2005; Keller et al. 2007b) Other cellular changes have also been observed. (Tumminia et al. 1998)

## IL-1 and TNF

Laser trabeculoplasty, a common glaucoma treatment, triggers IL-1 and TNF release within a few hours. (Bradley et al. 2000) These cytokines then stimulate sustained MMP-3 increases specifically within the juxtacanalicular region. (Parshley et al. 1995; Parshley et al. 1996) Adding either cytokine to perfused anterior segment organ cultures increases MMP-3 and outflow facility. (Bradley et al. 1998; Kelley et al. 2007) Thus, MMP-3 stimulation by these cytokines is probably a critical component of the effect of this treatment on outflow facility. Microarray analysis of TM cells treated with TNFα or IL-1α allows the identification of a number of ECM components that are changed and thus may be involved in the ECM remodeling and adjustment of the outflow resistance (Table 3) (Chen, et. al., Invest Ophthalmol Vis Sci 46: e-abstract #1349, 2005).

## TGFβ

Glaucomatous aqueous humor has been reported to contain elevated levels of TGFβ2. (Tripathi, RC et al. 1994) TGFβ2 perfusion reduces outflow facility and is generally thought to increase ECM production. (Gottanka et al. 2004; Fleenor et al. 2006) In TM cells, a number of ECM genes are modulated by TGFβ1 and/or TGFβ2 (Table 3). (Zhao et al. 2004; Fleenor et al. 2006) Earlier, it was shown that TGFβ1 β2, β3 and β5 all increased MMP-3 expression by the TM. (Alexander et al. 1998) In addition to these and many other changes observed, versican splicing was affected, as discussed later. (Zhao and Russell 2005)

**Glucocorticoids**. Dexamethasone has been shown to reduce outflow facility. (Clark et al. 1995b; Clark et al. 2005) Much of this effect may be due to dramatic cytoskeleton changes, i.e., the formation of cross-linked actin networks (CLANs) (Clark et al. 1994; Clark et al. 1995a), or to a number of other TM cell responses, e.g., increased expression of myocilin. (Polansky et al. 1997; Stone et al. 1997; Polansky and Nguyen 1998; Polansky et al. 2000; Lo et al. 2003) Myocilin has been shown to affect outflow facility, although the exact mechanism

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appears to be complex. (Borras et al. 2002; Goldwich et al. 2003; Zillig et al. 2005; Fautsch et al. 2006) Myocilin also binds several ECM components, including the hep II domain of fibronectin, (Filla et al. 2002) and is found in the JCT and SC region. (Ueda et al. 2002; Ueda and Yue 2003) An association with elastic microfibril SD plaques was also observed. Several ECM genes' expression was changed in response to dexamethasone treatment, including a reduction in versican and an increase in fibronectin levels. (Steely et al. 1992; Liu et al. 2003; Lo et al. 2003) Glucocorticoids also increase ECM deposition in the TM, producing a fingerprint-like arrangement somewhat resembling basement membrane material. (Johnson et al. 1997) This pattern differs from the SD plaques seen with glaucoma (see later). These studies may provide clues to the molecular mechanisms involved in steroid-induced glaucoma.

## Alternative splicing of ECM components

In addition to the changes in expression levels of a number of potentially relevant ECM components with these modulations of outflow facility, several components exhibit unusual mRNA splicing in the TM or change the relative amounts of different splice variants in response to outflow modulation. Some of these are indicated by the # symbol in Table 3. The presumed consequence is that the modified isoform will have an increase or decrease in some specific binding domain or possibly GAG attachment sites. In response to mechanical stretching, fibronectin mRNA splicing shifts to an increased proportion of transcripts containing the V1 and V3 regions. (Vittal et al. 2005) This introduces two additional α4β1 integrin binding sites and has been shown to affect fibronectin's Hep II domain interactions with cells. (Santas et al. 2002;Peterson et al. 2005) TNF and IL-1 have modest effects and TGFβ and dexamethasone have stronger effects of the EIIIA and EIIIB splice isoforms, changing availability of integrin α9 sites (Keller et. al. Manuscript in preparation). (Li et al. 2000)

Tenascin C, which is upregulated by mechanical stretch, TNFα and IL-1α treatments, also exhibits a wide variety of splice variants in the TM (Gregory, et. al., Invest Ophthalmol Vis Sci 46: e-abstract #1341, 2005). (Keller et al. 2007b) Some of these variants were not previously reported and several are changed by one or more of these treatments. Changes in TM cell splicing variations have also been identified in CD44, introducing a CS and a HS GAG attachment site, and in collagen type XII, introducing a heparin binding domain and a GAG attachment site. (Keller et al. 2007b)

Versican expression and alternative mRNA splicing is also modified with mechanical stretch, TNFα, IL-1α and TGFβ treatments (Chen, et. al., Invest Ophthalmol Vis Sci 46: e-abstract #1349, 2005). (Lo et al. 2003; Zhao et al. 2004; Vittal et al. 2005; Keller et al. 2007b) In addition, TGFβ has been shown to produce a shift in the proportion of TM versican mRNA splice variants increasing domains containing additional CS GAG attachment sites (Fig. 2). (Zhao and Russell 2005) Shifts in the relative amounts of these splice forms have also been detected with mechanical stretching (Keller et al. 2007b), TNFα and IL-1α treatment of TM cells (Keller, et. al. unpublished studies).

## Glaucoma and ECM

In glaucoma, an increase in plaque-like material with the ECM has been identified and studied in some detail. (Segawa 1979; Lütjen-Drecoll et al. 1981; Rohen et al. 1981; Rohen 1983; Lütjen-Drecoll et al. 1986; Lütjen-Drecoll and Tamm 1987; Lutjen-Drecoll and Rohen 2001) The exact molecular nature of this material is not clear and a direct causal link to the increased outflow resistance has been difficult to establish. An additional caveat to these glaucoma studies is that, with few exceptions, (Rohen et al. 1993) the tissue is from eyes that have been on various glaucoma medications for extended periods. None the less, valuable information can be gleaned from such studies. At least a portion of the plaque material appears to be from the elastic microfibril SD material. Three types of plaques have been identified by their

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appearance and properties. Increases with age are apparent, although less dramatic than with glaucoma. Morphometric and hydrodynamic analyses suggests that these plaques are not the direct source of the increased outflow resistance (Johnson 2006), but our inability to identify the normal or glaucomatous outflow resistance adds a caveat to this conclusion. Whether direct or indirect, this material seems likely to play some role in the elevated outflow resistance.

Flow segmentation appears to be higher in older eyes and particularly in glaucoma (Buller and Johnson 1994), although individual variability is high and a molecular cause has not yet been established. Long-lived, primarily ECM-related herniations of the JCT and SC inner wall at collector channels in glaucoma have also been identified, although their molecular nature is not established (Gong, et. al., Invest Ophthalmol Vis Sci 48:e-abstract #2079, 2007).

Gene expression analysis comparing glaucomatous and normal eyes and cells also shows a number of potentially important differences. (Wang et al. 2001; Diskin et al. 2006; Liton et al. 2006) The cell surface receptor ELAM-1 was found to be elevated in eyes with glaucoma, probably reflecting a chronic stress response that produces a sustained cellular phenotype change. (Wang et al. 2001) In one microarray study, the expression levels of mimecan, lumican, syndecan 4, ICAM-1 and several ECM glycolconjugate biosynthetic and degradative enzymes were changed. (Diskin et al. 2006) In another study, changes were observed in the expression of ECM proteins, including matrix Gla protein, chitinase 3-like 1 (glycoprotein 39), TIMP2, fibronectin, cartilage link protein, MAGP 2, collagens type I, III, V, VI, XI, XII and XIV, thrombospondin 3, periostin, MMP-1, -3 and -10, integrin α9, cochlin and myocilin. (Liton et al. 2006) The fibronectin change and perhaps other aspects of this study could be related to TGFβ2, BMP-4 and Gremlin studies recently reported. (Wordinger et al. 2007)

In other studies of glaucomatous outflow tissue, calcification markers relating to matrix Gla protein and BMP-2 action appear to be modified, although mechanisms remain to be established. (Xue et al. 2006; Xue et al. 2007) Increases in soluble CD44, particularly the phosphorylated form, have also been recently linked to glaucoma, although causal relationships remain to be established. (Knepper, PA et al. 1998; Knepper, P et al. 2002; Knepper, PA et al. 2002; Knepper et al. 2005) In an extension of the GAG stratification within the JCT region, discussed earlier, large changes in GAG distributions have also been observed in glaucoma. HA was decreased and CS was increased in all layers of the JCT of primary open-angle glaucoma eyes. (Knepper et al. 1996b)

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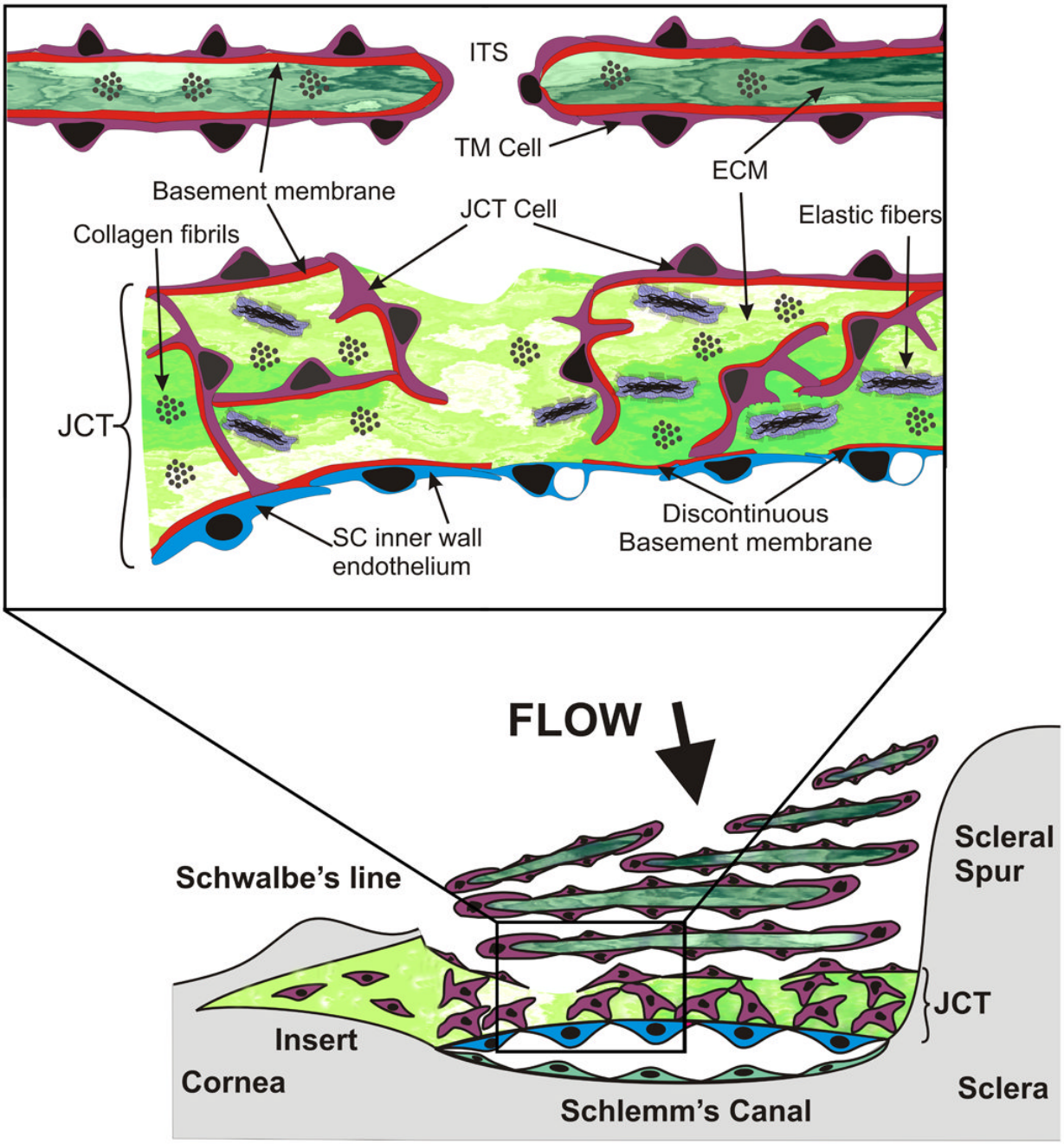


**Fig. 1.**

Diagram of the outflow pathway and juxtacanalicular or cribriform region. The lower portion of the figure shows a stylized view of the TM and the upper inset shows an expanded view of the JCT region. The figure is modified from several sources.(Tripathi 1971, 1977; Grierson et al. 1978; Rohen et al. 1981; Rohen 1983; Grierson and Calthorpe 1989; Epstein and Rohen 1991; Maepea and Bill 1992; Lutjen-Drecoll 1999; Parc et al. 2000)

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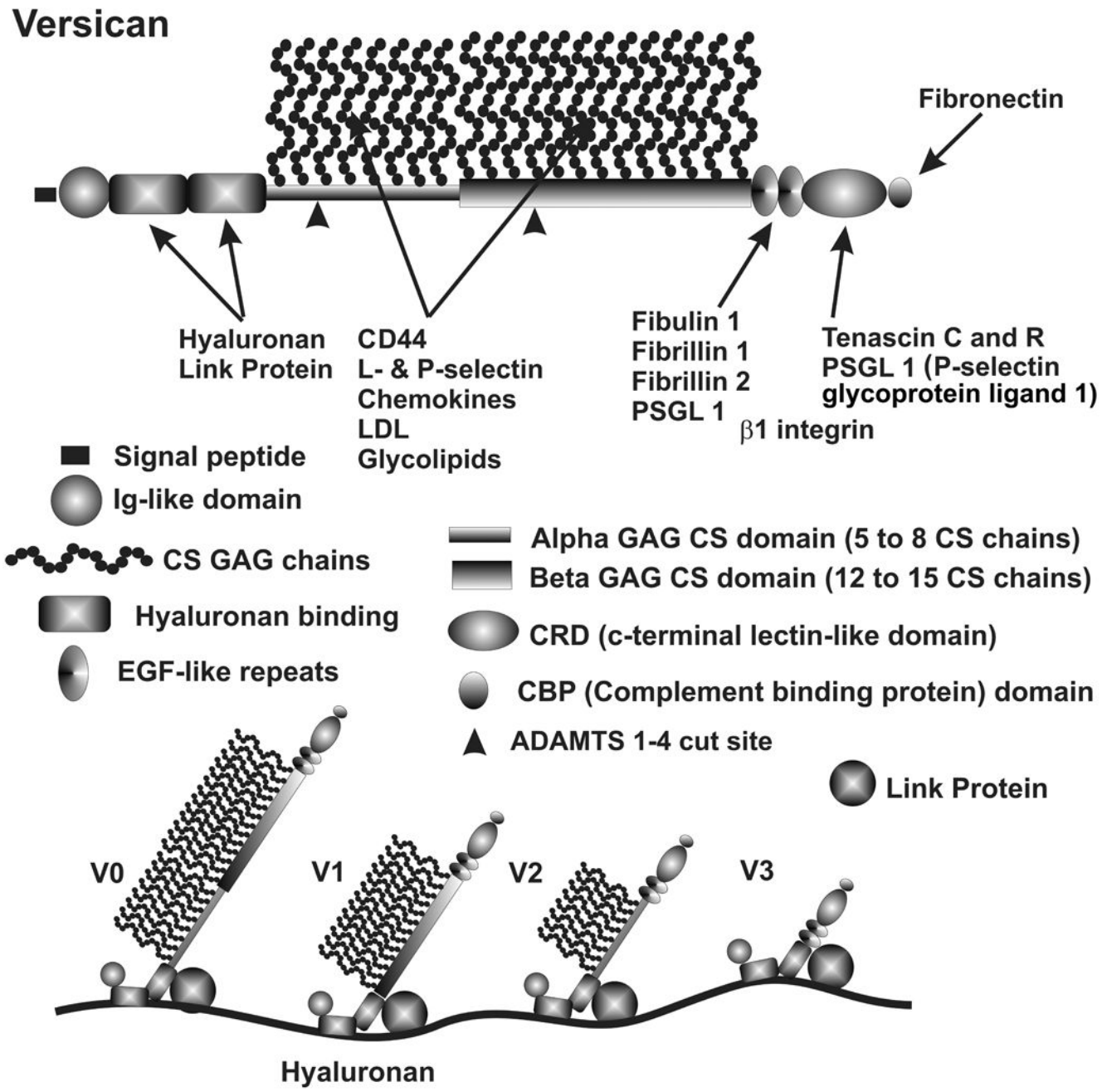


**Fig. 2.**

Versican structural domains, binding sites and alternative splicing. Key shows versican domains, positions of GAG chain attachments and position of ADAMTS 1–4 cut site. Various ECM molecules bind to these domains as indicated. The four alternative splice forms of versican are shown binding to hyaluronan with a stabilizing interaction with link protein. (Diagram modified from (Wight 2002; Wu et al. 2005)

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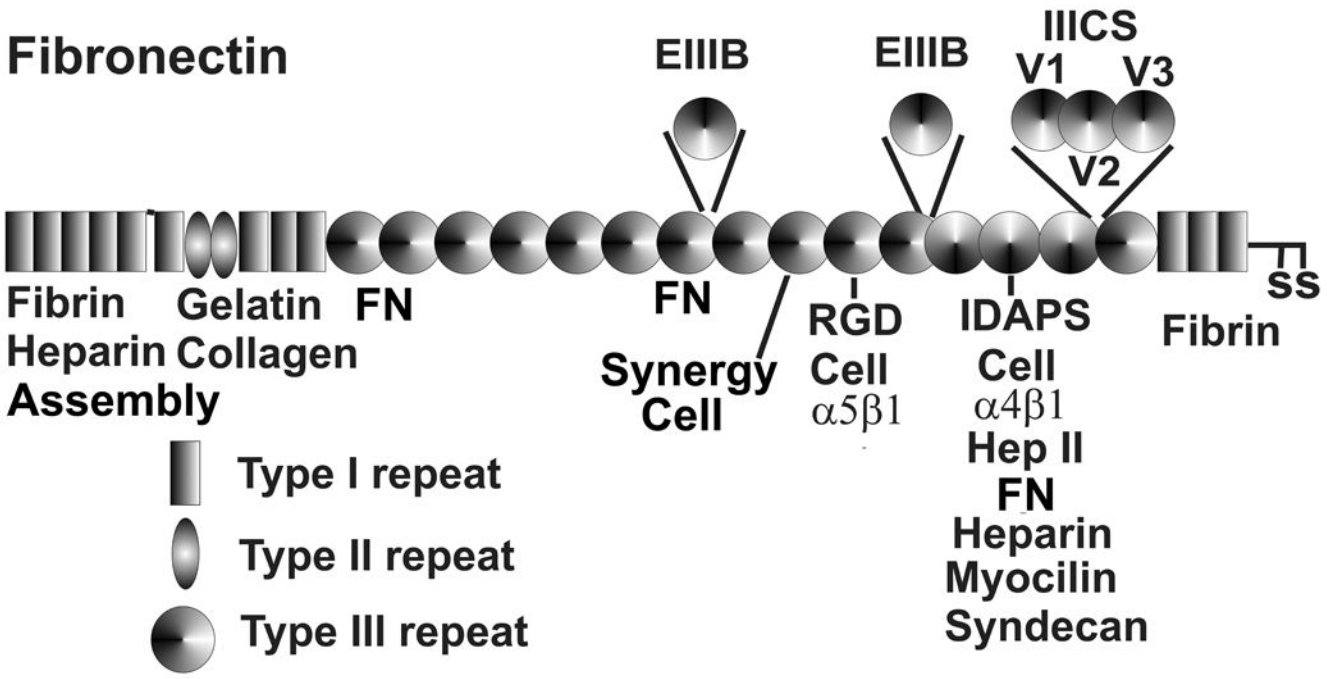


**Fig. 3.**

Fibronectin repeat type I, II and III domains, binding sites and alternative splicing. RGD, synergy and IDAPS sequences bind α5β1 and α4β1 integrins. Alternative splicing domains EIIIB, EIIIA and V1, V2 and V3 of IIICS are inserted as indicated. V1, V2 and V2 produce additional heparin, α4β1 integrin and a zinc binding site. FN and assembly represent fibril formation sites.

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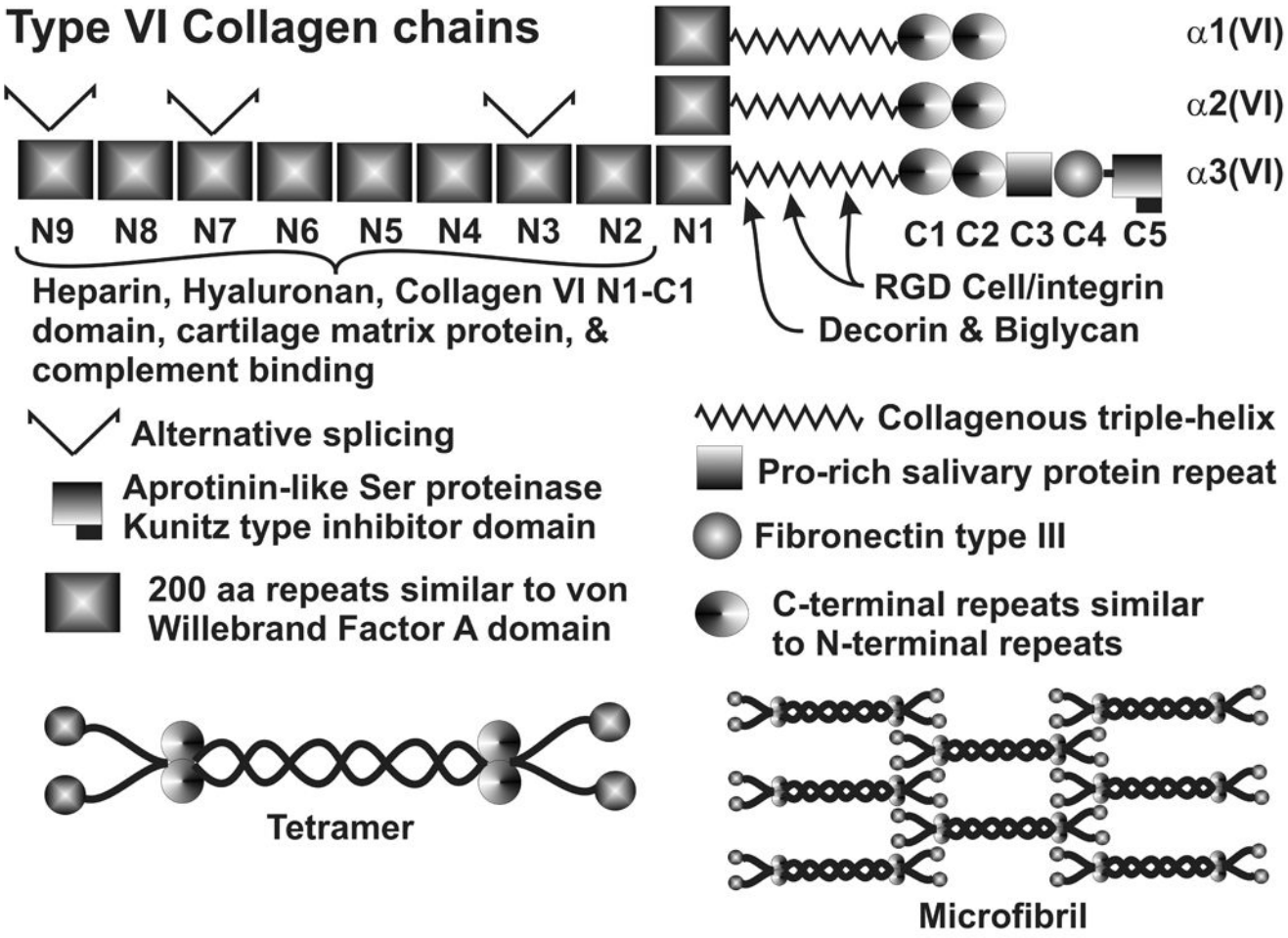


**Fig. 4.**
Type VI collagen chains, domain structure, binding domains and proposed microfibril organization.

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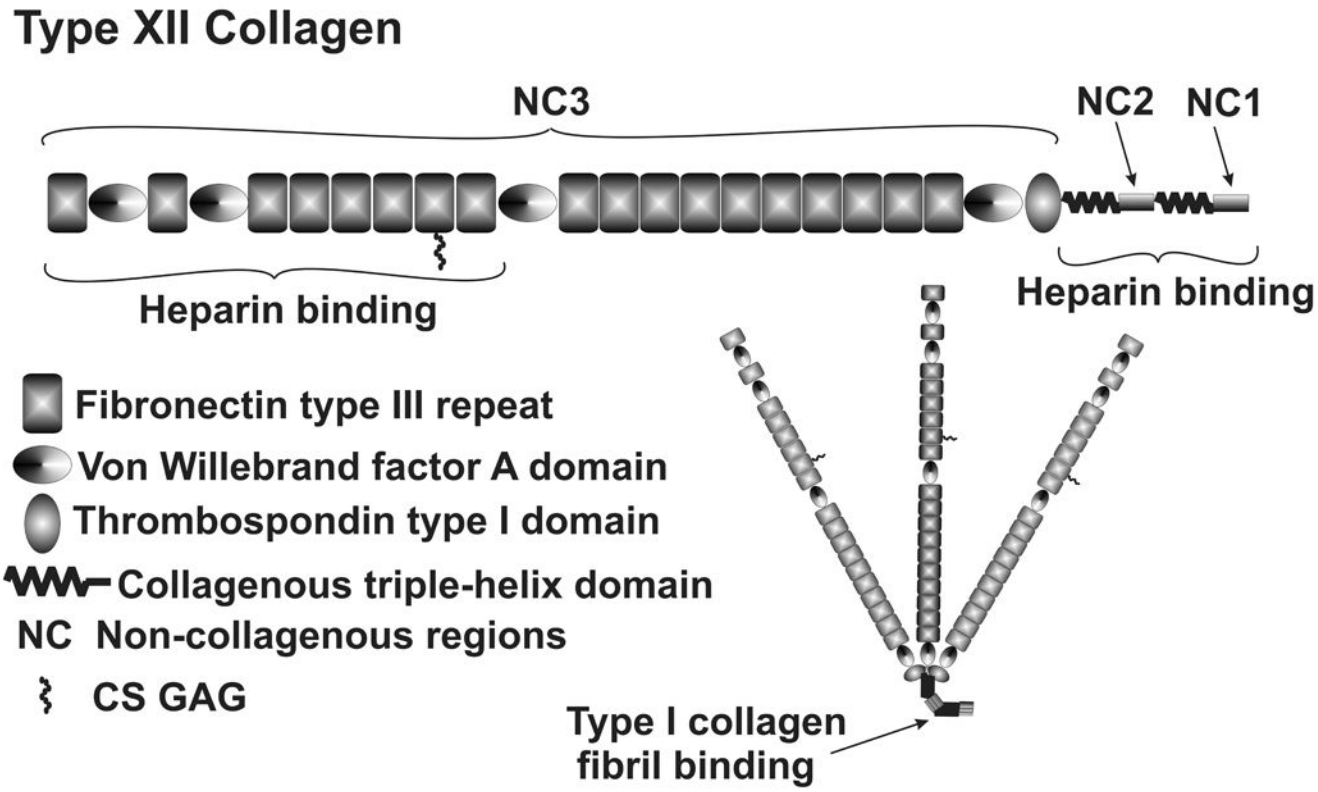


**Fig. 5.**
Type XII FACIT collagen domain structure, multimerization and binding domains.

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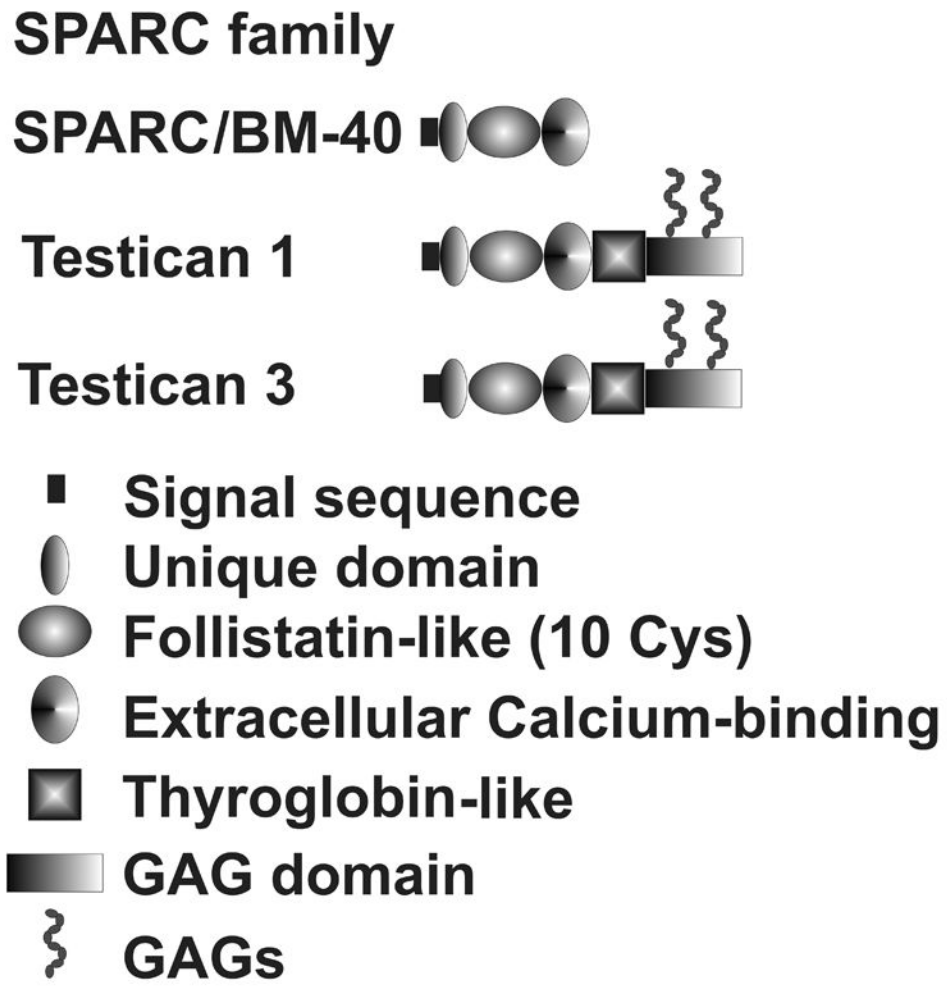


**Fig. 6.**
Structural domains of several SPARC family member expressed by outflow pathway cells.

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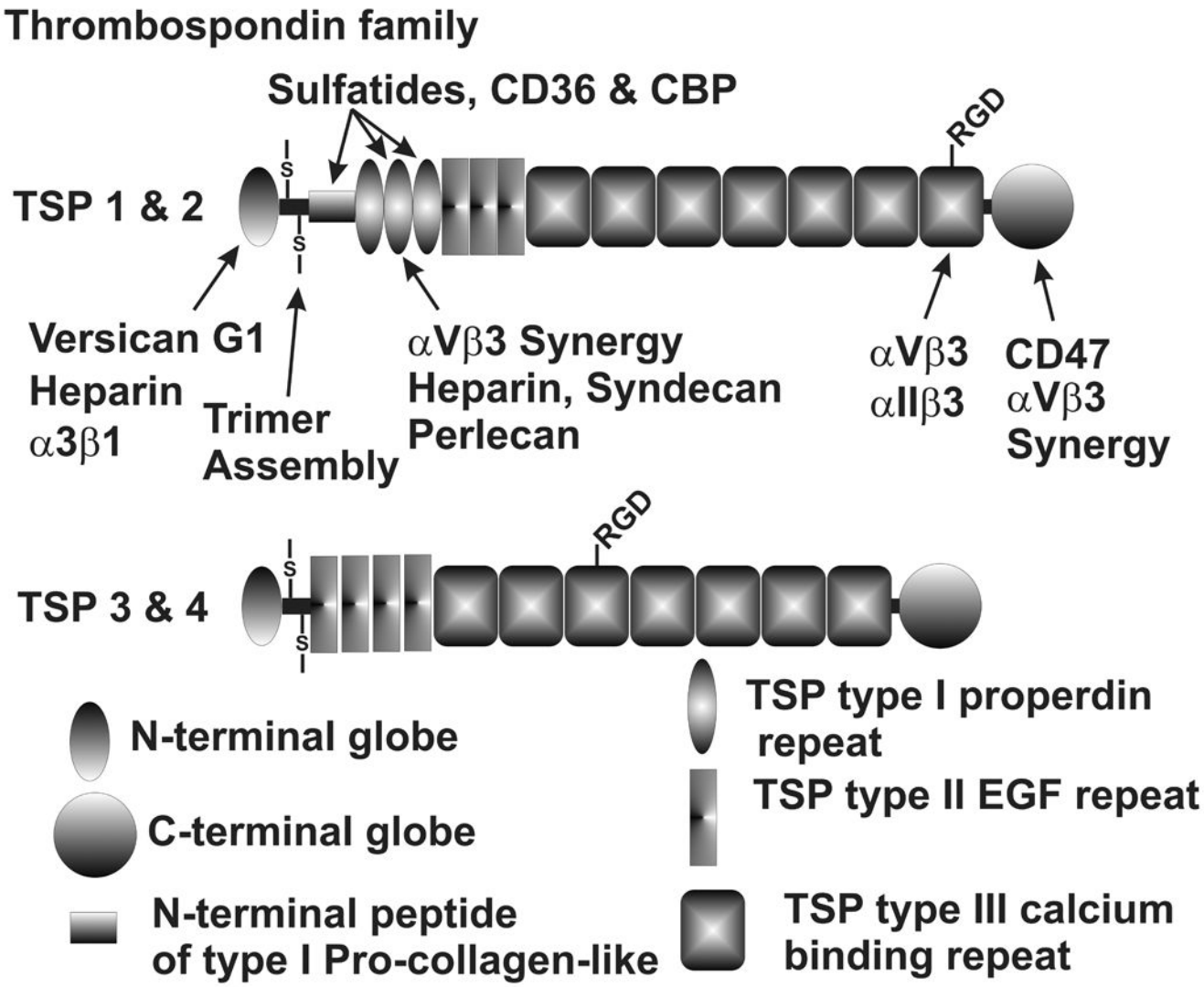


**Fig. 7.**

Thrombospondin family domain structures and binding sites. N- and C-terminal globe domains, a multimerization domain resembling the N-terminal region of type I procollagen and three types of thrombospondin (TSP) repeat domains are shown.

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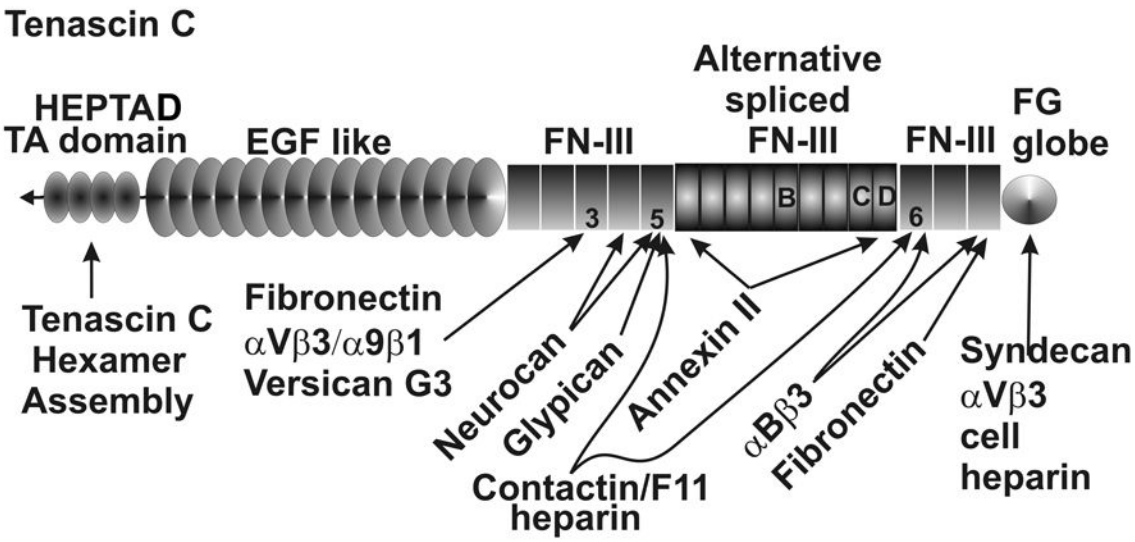


**Fig. 8.**
Tenascin C domain structure, binding sites and alternative splicing. Heptad repeat hexamer assembly domain, EGF-like domains, type III fibronectin and fibrin globular domains are as indicated.

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**Table 1**

Proteoglycans found in the outflow pathway.

| Proteoglycan | Family | Main GAG(s) |
|---|---|---|
| Biglycan | SLRP | DS/CS |
| Decorin | SLRP | DS/CS |
| Fibromodulin | SLRP | KS |
| Lumican | SLRP | KS |
| Mimecan | SLRP | KS |
| Versican, CSPG 2 | CSPG | CS |
| Neurocan, CSPG 3 | CSPG | CS |
| Melanoma-assoc., CSPG 4 | CSPG | CS |
| Bamacan, CSPG 6 | CSPG | CS |
| Perlecan, HSPG 1 | HSPG | HS |
| LDL receptor, HSPG 2 | HSPG | HS |
| Syndecan 1 | Transmembrane HSPG | HS |
| Syndecan 2 | Transmembrane HSPG | HS |
| Syndecan 3 | Transmembrane HSPG | HS |
| Syndecan 4 | Transmembrane HSPG | HS |
| Glypican 3 | GPI anchored HSPG | HS |
| Glypican 4 | GPI anchored HSPG | HS |
| Glypican 5 | GPI anchored HSPG | HS |
| Testican 1 | SPARC/osteonectin | CS |
| Testican 3 | SPARC/osteonectin | CS |
| Secretory granule PG 1 | Serglycin | CS/HS |
| Collagen XII | Non-fibrillar collagen | CS |
| Collagen XV, endostatin | Non-fibrillar collagen | CS |
| CD44 | Cell surface HA receptor | CS/HS |

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**Table 2**

Other ECM components found in the TM/SC

| ECM Component | Isoforms | Family |
|---|---|---|
| Fibronectin | Several splice variants | ECM organization, cell interaction, fibrils |
| Laminin | α2–4, β1–3, γ1–3 | Basement membrane |
| Nidogen | 1 | Basement membrane, laminin |
| Fibrillar collagen | I, III & V | ECM structure, tensile strength |
| Fibril network collagen IV | α1, α2, α4, α5 & α6 | Basement membrane |
| Filamentous collagen | VI | Microfibril structure |
| Non-fibrillar collagens | VII, VIII, XI, XV & XXII | ECM organization |
| Fibril-associated collagen | XII & XIV | FACIT, ECM organization |
| Elastin | | Elastic fibrils |
| Fibulin | 1, 5 & 6 | Elastic microfibril, ECM organizer |
| Fibrillin | 1 & 2 | Elastic microfibril protein |
| Microfibril-associated glycoprotein (MAGP) | 1,2 & 4 | Elastic microfibril protein |
| Vitronectin | | ECM organization, cell interaction |
| Tenascin | C | Matricellular, ECM reorganization |
| SPARC/osteonectin | | Matricellular, ECM reorganization |
| Thrombospondin | 1, 2 & 4 | Matricellular, ECM reorganization |
| NEL-like | 1 & 2 | ECM organization |
| Galectin | 1, 3, 6 & 8 | Lectin, galactose binding protein |
| Periostin | | Heparan binding cell adhesion |
| Osteopontin | 1 | Matricellular, Integrin binding |
| Oculoglycin, opticin | | SLRP |
| TNF-stimulated gene | TSG-6 | ECM & hyaluronan stabilization |
| Inter-α-trypsin inhibitor | H1, H2, H3, MEG3 | ECM & hyaluronan stabilization |
| Cartilage link protein | 1 | Versican & hyaluronan binding |

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**Table 3**

Changes in expression of a few select ECM genes in response to some treatments that modify aqueous outflow facility. Approximate fold changes are shown from microarray, quantitative RT-PCR, or proteomics analyses. NS indicates that changes were not statistically or biologically significant, N indicates information not available, and S indicates alternative mRNA splicing has been reported. Values are at the times of maximum change and range from 12 to 72 hrs for stretch, cytokines or TGFβ and up to 10 days for dex.

| ECM Component | Stretch | TNFα | IL-1α | TGFβ | Dex |
|---|---|---|---|---|---|
| Versican | 0.45, S | 0.47, S | 0.46, S | 4.8–9, S | 0.034 |
| Tenascin C | 2.6, S | 4.5, S | 3.7, S | N | N |
| Fibronectin | 1.5, S | NS, S | NS, S | 3–4, S | 2, S |
| CD44 | 1.8, S | 1.7, S | 1.36, NS, S | N | N |
| Syndecan 1 | 0.77, NS | 0.33 | 0.23 | N | N |
| Syndecan 2 | 1.5 | 0.32 | 0.46 | N | N |
| Synedcan 4 | NS | 2.1 | 1.87 | N | N |
| Glypican 3 | N | 1.98 | 2.78 | N | N |
| SPARC | 2.4 | 0.21 | 0.48 | N | N |
| Testican 1 | N | 0.37 | 0.48 | N | N |
| Testican 3 | N | 1.79 | NS | N | N |
| Thrombospondin 1 | NS | 0.32 | 0.49 | 2.4–5 | N |
| Thrombospondin 2 | 0.68, NS | 0.12 | 0.29 | N | N |
| TSG-6 | N | 10.24 | 24.5 | 3.3 | N |
| VCAM-1 | 1.69 | 47 | 28 | 0.31 | N |
| Collagen XIV | 3.25 | NS | NS | 0.38 | N |
| Periostin | 0.49–2.2 | 4.63 | 4.93 | 5.3 | N |
| Matrix Gla protein | 2.5–7 | 0.29 | 0.31 | N | N |
| Link protein | 1.3, NS | NS | 1.34, NS | 2.1 | N |
| MMP-2 | 1.4 | NS | NS | 4.9 | N |
| MMP-3 | NS | 49.5 | 54.6 | 8 | N |
| ADAMTS 4 | N | 25.7 | 36.6 | N | N |
| ADAMTS 5 | NS | 8.3 | 18.3 | 4.0 | N |