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The many faces of the trabecular meshwork cell

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Abstract

With the combined purpose of facilitating useful vision over a lifetime, a number of ocular cells have evolved specialized features not found elsewhere in the body. The trabecular meshwork (TM) cell at the irido-corneal angle, which is a key regulator of intraocular pressure, is no exception. Examination of cells in culture isolated from the human TM has shown that they are unique in many ways, displaying characteristic features of several different cell types. Thus, these neural crest derived cells display expression patterns and behaviors typical of endothelia, fibroblasts, smooth muscle and macrophages, owing to the multiple roles and two distinct environments where they operate to maintain intraocular pressure homeostasis. In most individuals, TM cells function normally over a lifetime in the face of persistent stressors, including phagocytic, oxidative, mechanical and metabolic stresses. Study of TM cells isolated from ocular hypertensive eyes has shown a compromised ability to perform their daily duties. This review highlights the many responsibilities of the TM cell and its challenges, progress in our understanding of TM biology over the past 30 years, as well as discusses unanswered questions about TM dysfunction that results in IOP dysregulation and glaucoma.

Keywords

trabecular meshwork; glaucoma; cell culture; cellular phenotype

I. Trabecular Meshwork Structure and Function

Trabecular meshwork (TM) cells are the primary cell type that occupy and form the proximal portion of the conventional outflow pathway, the primary egress route for aqueous humor from the eye. In this passageway resistance to unobstructed outflow is generated, regulated and responsible for homeostatic intraocular pressure (IOP) control. In coordination with the inner wall of Schlemm's canal (SC), IOP is rigorously maintained within a couple of millimeters of mercury by the TM and SC for about 90% of people over a lifetime (David

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et al., 1987, Klein et al., 1992). Unfortunately, cellular dysfunction in the conventional outflow pathway, including the TM, results in the generation of “extra” resistance that causes elevated IOP (ocular hypertension) characteristic of most types of primary open-angle glaucoma (POAG) (Grant, 1951, Grant, 1963). Thus, in POAG, the most common type of glaucoma, the irido-corneo angle is open and there are no gross abnormalities or clinically visible accumulation of material in the TM.

The TM is an avascular, architecturally complex connective tissue bridging Schwalbe’s line to the scleral spur/ciliary muscle; and spanning the entire length of SC. The TM can be anatomically divided into three regions (from inner to outermost): (i) the uveal meshwork, which is closest to the anterior chamber and consists of a network of collagen and elastin lamellae covered by TM cells with large “open spaces” in between individual lamellae; (ii) the corneoscleral meshwork is the “middle layer”, which is composed of a series of perforated collagen and elastin plates covered by TM cells; and (iii) the juxtacanalicular (JCT) TM is a loose connective tissue containing TM cells surrounded by extracellular matrix between the outermost corneoscleral plates and the inner wall of SC. The ciliary muscle is anatomically and functionally linked to the SC inner wall via a network of elastin fibers that extend from the tips of the longitudinal fibers through the corneoscleral and JCT TM, anchoring onto the inner wall (reviewed by (Tamm, 2009)). These three divisions are considered part of the “filtering” TM since they lay directly over SC. A fourth division called the “insert” region of the TM is considered “non-filtering” since it resides just below Schwalbe’s line, adjacent to, but not in front of SC. Data suggest that this region contains a population of TM stem cells (reviewed by (Kelley et al., 2009)).

The TM tissue has two primary responsibilities to assure effective outflow resistance regulation over nearly a century of life. As aqueous humor leaves the eye, it first encounters the uveal and corneoscleral TM, which function as a self-cleaning biological filter; intercepting cellular debris and reactive oxygen species before reaching the resistance generating region of the JCT. The JCT region of the TM is populated and maintained by JCT-TM and inner wall of SC cells. The precise manner by which JCT-TM cells and SC cells function to generate and regulate outflow resistance (and thus IOP) is not completely known. However, it appears that the two cell types work together physically and functionally to generate outflow resistance, to restrict flow of aqueous humor into the lumen of SC and onto the systemic venous system (Reviewed by (Overby et al., 2009)).

While TM cells that populate the conventional outflow tissues have two different morphologies, both have a common embryological origin, the neural crest (Tripathi and Tripathi, 1989). Uniquely, these mesenchymal cells display characteristics of four different cell types, supporting the two primary responsibilities of the TM (Table 1). Thus, requisite for an occupation as a biological filter, cells of the inner TM have macrophage-like activity. Acting as professional phagocytes, TM cells clear cellular debris derived from shed pigmented epithelia propelled forward toward the irido-corneo angle by the flow of aqueous humor (Samuelson et al., 1984, Grierson and Lee, 1973, Rohen and van der Zypen, 1968). Importantly, debris is rapidly cleared by the inner TM before reaching deep into the TM where it might accumulate and interfere with resistance generation and regulation. Cultured TM cells as well as TM cells in vivo are actively phagocytic (Johnson et al., 1989, Epstein et

al., 1986, Tripathi and Tripathi, 1982), which appears to be an essential part of keeping the "outflow filter" clean. In addition to engulfing pigment granules and debris, TM cells also experimentally phagocytize latex beads and other labeled particles (Johnson et al., 1989, Yue et al., 1987, Grierson et al., 1986). Similar to macrophages, TM cells express scavenger receptors, thought to be used for uptake of foreign and waste materials. In fact, early identification of TM cells was partially dependent upon their unique expression of receptors that mediate acetylated low density lipoprotein uptake (Chang et al., 1991, Stamer et al., 1995b). Interestingly, factors other than defects in phagocytosis are thought responsible for secondary forms of glaucoma that display accumulation of pigment or exfoliation material (Matsumoto and Johnson, 1997, Epstein et al., 1986).

To maintain a clear outflow pathway, the cells of the inner TM completely cover the elaborate collagen-elastin lamellae and plates as thin continuous monolayers. Functioning as endothelia, TM cells produce large quantities of antithrombogenic substances, like heparin sulfate and tissue-plasminogen activator (tPA). Incredibly, TM cells produce one hundred times more tPA than vascular endothelia, emphasizing the importance of passageway patency (Snyder et al., 1993, Shuman et al., 1988). Also like endothelia, cells from the inner TM participate in antigen presentation and inflammation mediation, producing major histocompatibility proteins and a wide array of inflammatory cytokines, respectively (Shifera et al., 2010, Tripathi et al., 1990a, Latina et al., 1988, Lynch et al., 1987). In fact, laser trabeculoplasty takes advantage of the TM's role in local inflammatory mediation and resolution to decrease outflow resistance. Hence, delivery of laser energy to the TM results in the rapid secretion of IL-1α and TNFα in response to the injury, stimulating extracellular matrix turnover and debris phagocytosis (Bradley et al., 2000, Latina et al., 1998).

In line with their second responsibility, resistance generation, TM cells in the JCT region have both fibroblastic and smooth muscle-like qualities. For example, TM cells secrete a number of extracellular matrix proteins and their cognate degradation enzymes to support the continual remodeling of extracellular matrix (reviewed by (Keller et al., 2009). Indeed, the activity of TM cells resembles fibroblasts at a wound site, turning over matrix proteins as rapidly as every 48 hours (Acott et al., 1988). In keeping with the interplay between extracellular matrix turnover and cytoskeletal tension, TM cells in the JCT and corneoscleral region are contractile, expressing smooth muscle actin and myosin (reviewed by (Tian et al., 2009)). Cellular contractile force generation by the TM cells counter tension that is imposed by the ciliary muscle, which extends elastic tendons into the TM, connecting with its elastic network that extends to the inner wall of SC (Overby et al., 2014, Rohen et al., 1981). Thus, contraction of the ciliary muscle pulls on the TM elastic network, opening spaces between lamellae and cribiform plates plus preventing collapse of the SC lumen (Li et al., 2014, Lutjen-Drecoll, 1973). Together, the relationship between the ciliary muscle, TM and SC modifies/maintains flow pathways to increase outflow facility. Paradoxically, treatment of TM tissues with drugs that decrease contractility, such as actin depolymerizing agents or rho kinase inhibitors, also increases outflow facility (Rao et al., 2001, Kaufman and Erickson, 1982). While the precise mechanism is unknown, rho kinase inhibitors increase the separation distance between the outermost cribiform plate and the SC inner wall (Yang et al., 2013), emphasizing the importance of the relationship between JCT TM and SC inner wall cells in outflow resistance generation and dampening of IOP spikes.

II. TM cells reside in a biologically demanding environment

Owing to their location, pressure-sensitivity, lack of a blood supply and continual exposure to byproducts of UV radiation and cellular metabolism, the cells of the TM weather a combination of stresses not experienced by most other cells in the body. For instance, TM cells on a daily basis experience mechanical stress due to routine daily activities such as eye rubbing, squinting, blinking, ocular pulse and saccades that range from a 20–105% increase of their original dimensions (Coleman and Trokel, 1969, Johnstone and Grant, 1973). In fact, recent studies by Downs show that about 15% of all energy in the eye is due to pressure spikes (on top of steady state IOP)(Downs, 2015). To survive such insults, TM cells possess adaptations such as a prominent cytoskeleton, complex cell-cell and cell-matrix attachments and expression of water channels to facilitate rapid changes in cell volume following stretch (Baetz et al., 2009, Grierson and Lee, 1975, Bhatt et al., 1995, Tumminia et al., 1998).

TM cells, particularly in the inner meshwork, continually endure oxidative stress in the form of byproducts from UV energy interacting with aqueous humor, cornea and lens epithelium. Cells of the TM express a number of oxidative reducing agents, including super oxide dismutase, glutathione reductase and peroxidase to neutralize reactive species (Russell and Johnson, 1996, De La Paz and Epstein, 1996, Nguyen et al., 1985). An example of the extraordinary oxidative capacity of TM cells was demonstrated when 1 mM hydrogen peroxide was injected intracamerally into calf eyes and hydrogen peroxide was not detectable in effluent exiting the aqueous veins (Nguyen et al., 1988).

Because the conventional outflow tract is the final destination for aqueous humor before it leaves the eye, TM cells experience constant metabolic and phagocytic stress. Metabolic activity of other avascular ocular cells upstream of the TM, such as crystalline lens and cornea deplete the aqueous humor of nutrients and deposit waste products. Aqueous humor also carries cell debris from epithelial turnover and apoptosis events upstream plus pigment granules from the iris rubbing on the crystalline lens that increases with age. The inner TM cells intercept these materials, protecting the functionality of the outer TM for proper resistance generation. Thus, filtering of aqueous humor of cell debris, in addition to the normal remodeling of the tissue, impose a continual phagocytic stress on the TM. Like a macrophage, TM cells that have phagocytosed large cellular debris detach from their matrix and leave the TM (Sherwood and Richardson, 1988). It is unclear in healthy eyes whether detached TM cells are replaced by TM insert (stem) cells; however, trabecular beams in healthy eyes remain cellularized, suggesting that lost cells are replaced.

III. Original isolation and characterization of cultured TM cells

Significant advances in our understanding of the normal function of the TM and TM cells have come from pioneering studies on cultured of TM cells from several species. The first report of TM cell culture was published by Rohen and colleagues, who cultured TM cells from dissected primate TM explants (Rohen et al., 1975). The cultured TM cells appeared metabolically active with high amounts of endoplasmic reticulum, ribosomes, and mitochondria. These cells were also capable of phagocytosis of pigment granules. This same group subsequently showed that these primate TM cells synthesized the glycosaminoglycans

hyaluronic acid, dermatan sulfate, and chondroitin-4-sulfate and suggested that these GAGs might function as an outflow filter for the aqueous humor (Schachtschabel et al., 1982). Soon after, Jon Polansky, Jorge Alvarado and colleagues cultured and characterized human TM cells from explants dissected from postmortem eyes (Alvarado et al., 1982, Polansky et al., 1979). They morphologically and ultrastructurally compared these cells to human corneal keratinocytes, scleral fibroblasts, and corneal endothelial cells, which could be potential contaminating cells upon dissection of the TM explants. TM cells formed confluent monolayers with long overlapping processes, whereas the keratinocytes and scleral fibroblasts grew much faster, in multiple layers, and had the characteristic elongated fibroblast morphology and cellular foci. They reported that the cultured human TM cells had many features of TM cells in vivo, including the expression of fibronectin, gap junctions, apical villous processes (including cilia), and numerous coated vesicles at the basal surface. About the same time, Ramesh and Brenda Tripathi cultured human TM cells and morphologically and ultrastructurally compared these cells to corneal endothelial cells, keratocytes, and scleral fibroblasts, with similar findings (Tripathi and Tripathi, 1982). They also reported that these human TM cells had greater phagocytic activity compared to the other cell types, which could be blocked with the microfilament disrupting agent cytochalasin B. Ian Grierson and colleagues cultured bovine TM cells and found that confluent, contact inhibited monolayers better reflected the behavior and activities of TM cells in vivo (Grierson et al., 1985). They also showed that the cultured bovine TM cells were phagocytically active (Grierson et al., 1986). Therefore, early studies of cultured TM cells from 3 different species displayed a number of common morphological and functional characteristics of TM cells in vivo.

Cell isolates from the TM have one of two general morphologies: Cells obtained from the inner TM that cover collagen/elastic beams/plates of the uveal and corneoscleral TM have a round to oval shape, a large cell body with some overlapping processes. These TM cells appear “endothelial-like” upon reaching confluence in culture, with cobblestone morphology (Figure 1). In contrast, cells isolated from the deeper JCT region of the TM have a more elongated, spindle-shaped morphology with many overlapping processes. Upon reaching confluence in culture, JCT cells appear to have a smooth muscle-like or fibroblastic appearing morphology (Figure 1). Whether TM and JCT cells are the same cell “type” in different biological environments or two different cell types has been the subject of debate for decades (Flugel et al., 1991, Coroneo et al., 1991) (Ge et al., 2016). Regardless of their phenotype, TM cells are contact inhibited with primary isolates and low passage cells having a doubling time in culture of less than two days (Polansky et al., 1979). The proportion of endothelial-like cells versus fibroblastic/smooth muscle-like cells obtained during isolations depends upon two factors: the depth of dissection into the TM and the age of the donor eyes. For example, cells obtained from eyes of younger donors with well-populated lamellae tend to give preferentially endothelial-like cultures, whereas eyes from older donors with less populated lamellae generally give cultures dominated by fibroblastic/smooth muscle-like TM cells. Of course, the fresher the donor tissue, the more cells are obtained in either case. Not surprising, TM cells isolated from eyes of younger donors are smaller, more abundant, have faster doubling times and can be passaged more times before reaching senescence.

IV. Types of TM Cell Cultures

TM cells have a very limited replicative potential in situ (Kimpel and Johnson, 1992, Dueker et al., 1990). However, unlike their corneal endothelial neighbors, isolated primary TM cells proliferate until they form a contact inhibited monolayer in culture. As mentioned above, primary TM cell cultures are often started from TM tissue explants (Steely et al., 1992, Grierson et al., 1985, Tripathi and Tripathi, 1982, Polansky et al., 1979, Rohen et al., 1975). However, some use proteases to partially digest the dissected TM tissue prior to culture (Stamer et al., 1995b). Although primary TM cell cultures are normally passaged by trypsin, the cells become more senescent with each passage. Therefore some laboratories routinely passage cells using gelatin-coated Cytodex beads (Steely et al., 1992), where the TM cells are not routinely exposed to trypsin and appear to maintain their initial phenotypes for longer periods in culture. Using these methods, primary TM cell cultures have been derived from multiple species including human (Polansky et al., 1979, Snyder et al., 1993, Steely et al., 1992, Tripathi and Tripathi, 1982), nonhuman primates (Rohen et al., 1975)], cows (Mao et al., 2012a, Grierson et al., 1985), pigs (Alexander et al., 1998, O'Brien et al., 1996, Tripathi et al., 1991), and mice (Mao et al., 2013). All these TM cells have consistent phenotypes; however, TM cell cultures from cows and pigs tend to be dominated by "endothelial-like" cells due to their anatomy; having prominent inner TM and angular aqueous plexi instead of SC.

TM cells are routinely grown on plastic culture plates or flasks in defined culture medium containing fetal bovine serum (FBS). Most investigators use high amounts of serum (10–20%) to promote cell division while expanding cultures. Some, use a differentiation step once cells are confluent whereby supplementary growth factors are withdrawn (such as FGF) and/or fetal bovine serum levels are reduced (to 1–10%) (Wax et al., 1989, Ge et al., 2016). Freddo and colleagues have reported in vivo leakage of serum proteins at the iris root adjacent to TM tissue, so it is likely that the TM in vivo is also exposed to serum components (Freddo, 1993). Estimates place serum concentrations in the TM at about 1% v/v of aqueous humor. Alternatively, Fautsch and colleagues have cultured human TM cells in medium mixed with aqueous humor rather than FBS (Resch et al., 2010) because TM cells in situ are bathed in AH. For example, myocilin expression is significantly diminished in cultured TM cells, but expression is greatly increased in the presence of AH. While TM cells effectively and rapidly lay down ECM on plastic, there was concern that plastic is not ideal for studying TM cell biology. Paul Russell has measured the normal elasticity (Young's modulus) of human TM tissue (Last et al., 2011) and has cultured human TM cells on substrates of varying stiffness, demonstrating that TM cell behavior and phenotypes are quite different compared to cells grown on plastic (Wood et al., 2011, McKee et al., 2011). Others have cultured TM cells on porous filters and exposed the cells in attempt to more closely mimic aqueous flow through the TM (Ramos et al., 2009, Perkins et al., 1988). Confluent TM cells make their own ECM substrate, and depending on the culture conditions, the ECM content and stiffness can change. For example, NTM cells treated with the glucocorticoid, dexamethasone, deposit a stiffer substrate compared to control TM cells (Raghunathan et al., 2015).

Because some primary TM cells grow rather slowly in culture, particularly from older donor eyes, and have a finite passage lifetime, several labs have generated transformed or spontaneously immortalized TM cells. Transformed human TM cell lines, including GTM3 and NTM5 cells (Pang et al., 1994), grow faster and proliferate indefinitely, and these cell lines are widely used (Figure 2). However, these cell lines have a very different phenotype compared to primary TM cells: (1) they are not contact inhibited and continue to grow past the monolayer stage, (2) the cells are smaller and have less cytoplasm, (3) the cells do not form an extensive cytoskeleton, and (4) they do not make as much ECM as primary cultures. One major advantage of these transformed and immortalized cells is that they are much easier to transfect compared to primary TM cell cultures. Note that many eye research journals (including *Experimental Eye Research*, *IOVS*, and *Molecular Vision*) require that any work done with transformed cell lines be replicated in primary cell cultures, ex vivo or in vivo models. Unlike the other transformed human TM cell lines, the spontaneous immortalized bovine BTM28T cell line is diploid with 60 pairs of chromosomes and is contact inhibited growing as a monolayer (Mao et al., 2012b). This cell line has the same phenotype as primary bovine TM cells except that it has been propagated greater than 50 passages.

In attempt to provide a more natural environment for cultured TM cells, perfusion “organ” cultured anterior segments have been developed for human (Johnson and Tschumper, 1987), nonhuman primate (Hu et al., 2006), bovine (Mao et al., 2011, Erickson-Lamy et al., 1988), and porcine (Keller et al., 2008) eyes. The lens, iris, and ciliary body are removed from these anterior segments prior to being mounted on specially designed Plexiglass dishes, sealed with an O-ring, and perfusion cultured with culture medium (generally lacking FBS). The perfusion medium enters the bottom of the dish and is perfused through the trabecular outflow pathway and into the episcleral veins. These ex vivo cultures can be run under conditions of constant flow (using a perfusion pump) and variable pressure (continuously recording pressure with a pressure transducer) or as constant pressure (medium reservoir set at specific height) and variable flow (flow measured by loss of fluid from medium reservoir). In many labs, these ex vivo models can be cultured for weeks with very healthy TM tissue. These ex vivo models have been extremely useful to study and validate glaucoma related pathways (discussed below), drug effects on the trabecular outflow system (Pang et al., 2001, Pang et al., 2000, Gottanka et al., 2004, Wan et al., 2007), and circumferential differences in outflow resistance and biochemistry (Keller et al., 2011).

V. TM cell markers

The search for marker proteins that specifically identify TM cells in culture began over 30 years ago, when TM cells were first isolated. Unfortunately, decades later a specific marker is still not available. In addition to positive identification of TM cells in culture, specific marker proteins will enable selective genetic targeting and modification of TM cells in organ culture and transgenic mouse models. Moreover, the future of gene therapy and genome editing in the TM, a tissue ideally suited for such modifications, is likely dependent upon tissue-specific promoters. There are two main reasons that have made TM marker protein discovery difficult. Firstly, TM cell responsibilities overlap with neighboring cells and secondly, TM cells reside in two distinct environments within the conventional pathway,

demanding different aptitudes. Thus, when TM cells are isolated, cultures are generally mixed, comprised of both cell “phenotypes” (Figure 1). In the future, it will be useful to expression profile cells from each region, isolated from the same donor eye. Alternatively, profiling of TM versus neighboring cells using RNAseq in inbred mice will speed up the identification of unique promotors/proteins in the TM.

Since the detection of a single TM marker has been elusive, researchers rely on a panel of proteins (positive selection markers) and behaviors that typify TM cells in culture (Table 2). Studies have revealed a few proteins preferentially expressed by TM cells including aquaporin-1, alpha 2A adrenergic receptors, collagen IV, alpha-B crystallin and myocilin (Stamer et al., 1996, Stamer et al., 1998, Stamer et al., 1995a, Nguyen et al., 1998a, Polansky et al., 2000, Siegner et al., 1996). These proteins are differentially expressed or induced by TM cells compared to their cell neighbors that include ciliary muscle cells, scleral spur cells, scleral fibroblasts, Schlemm’s canal endothelia and corneal endothelia (Welge-Lussen et al., 1999, Hernandez et al., 1987). For example, myocilin is a protein whose expression is inordinately high in the TM compared to cell neighbors, but decreases with time in culture (Fautsch et al., 2005). Interestingly, myocilin is dramatically upregulated when TM cells are treated with glucocoticoids, a feature that is not shared by neighboring cells (Figures 1 and 3) (Polansky et al., 2000). Thus, glucocorticoid induction of myocilin is the most common and reliable way used today to identify TM cells. It is important to monitor changes in myocilin expression, both by immunocytochemistry and western blot. Immunocytochemistry shows the percentage of isolated cells responsive to glucocorticoid (70–80% is typical) and whether myocilin immunoreactivity displays a peri-Golgi and vesicular phenotype (O’Brien et al., 1999, Stamer et al., 1998). Western blot will validate specificity of anti-myocilin antibody being used, appearing as a 55–57 kDa doublet band. Some anti-myocilin antibodies have reported a larger 65 kDa band that appears to be non-specific staining, likely cross-reacting with albumin (molecular mass of 66.5 kDa). On a cautionary note, cells that are purchased commercially (Sciencell, Innoprot, etc) must be characterized before use, since methods used by companies to characterize commercial cells often are not standard practice for the field. For example, Figure 3 shows that while a commercial “TM” cell strain appears similar morphologically to human TM cells, this particular strain did not upregulate myocilin in response to glucocorticoid treatment and thus is likely not a TM cell.

Other examples of TM behavior are TGFβ induction of PAI-I, MMP2 and myocilin (Tamm et al., 1999). Moreover, TM cells consistently upregulate MMP3 and MMP9 when treated with IL-1α, TNFα or phorbol ester (Kelley et al., 2007a, Kelley et al., 2007b, Hosseini et al., 2006, Fleenor et al., 2003, Alexander and Acott, 2003, Shearer and Crosson, 2001) (Table 2). As mentioned above, TM cells readily take up acetylated low density lipoprotein (Chang et al., 1991).

Array and proteomic expression studies have also revealed a number of proteins that are preferentially expressed by TM cells, including, matrix GLA protein and chitinase-3 like-1 (Gonzalez et al., 2004, Liton et al., 2005). Unfortunately, these proteins are not expressed by all cells in the TM, but only subpopulations. Similarly, only a portion of cells of the TM express smooth muscle actin and myosin (de Kater et al., 1992, de Kater et al., 1990). There

are a few marker proteins that TM cells do not express that are used as negative selection markers. For example the endothelial proteins PECAM-1, VE-cadherin, integrin α6, VEGFR3, Prox-1 and fibulin-2 are expressed by SC cells, but not by TM (Heimark et al., 2002, Kizhatil et al., 2014, Perkumas and Stamer, 2012, VanderWyst et al., 2011). Likewise, the epithelial proteins keratin and desmin are not expressed by TM cells (Welge-Lussen et al., 1998, Weinreb and Ryder, 1990, Tripathi and Tripathi, 1982).

VI. TM Cell responses to stress hormones and cytokines

Cultured TM cells respond to a wide variety of glaucoma-like insults. Glucocorticoid (GC) treatment of TM cells in culture led to the identification of the first POAG gene *Myocilin (MYOC)* (Nguyen et al., 1998b, Stone et al., 1997, Polansky et al., 1997). Since then, GC induction of myocilin has been described in bovine (Mao et al., 2012a) and mouse TM cells (Mao et al., 2013), making this a very reproducible phenotype to characterize and validate TM cells as mentioned above. GCs also induce a major actin cytoskeletal rearrangement to form cross-linked actin networks (CLANs) in confluent cultured human TM cells (Clark et al., 1994, Wilson et al., 1993), which has also been shown in cultured bovine (Wade et al., 2009) and mouse (Mao et al., 2013) TM cells. Significantly, these CLANs are observed in the TM tissues of perfusion cultured human anterior segments treated with dexamethasone (Clark et al., 2005). Moreover, CLANs are more prevalent in cultured glaucomatous TM cells (Clark and Wordinger, 2009, Clark et al., 1995) and in situ in human glaucoma eyes (Hoare et al., 2009). GCs have also been shown to induce extracellular matrix production in cultured TM cells and tissues, including fibronectin (Steely et al., 1992), laminin (Dickerson et al., 1998), collagens (Zhou et al., 1998, Hernandez et al., 1985), and glycosaminoglycans (Engelbrecht-Schnur et al., 1997, Johnson et al., 1990). GCs inhibit TM cell proliferation and migration (Clark et al., 1994) as well as inhibit TM cell phagocytosis (Zhang et al., 2007). Therefore, GC treatment of TM cells has been used extensively to better understand GC-induced ocular hypertension. Importantly, GC-induced changes in TM cells are similar in many ways to glaucomatous changes in the TM (Wordinger and Clark, 1999).

The anti-inflammatory profibrotic cytokine, TGFβ2, has been implicated in the pathogenesis of POAG because: (1) TGFβ2 levels are elevated in the aqueous humor (Lutjen-Drecoll, 2005, Tripathi et al., 1994, Agarwal et al., 2015, Trivedi et al., 2011, Min et al., 2006, Yamamoto et al., 2005, Ozcan et al., 2004, Ochiai and Ochiai, 2002, Picht et al., 2001, Inatani et al., 2001) and TM (Tovar-Vidales et al., 2008) of POAG patients, and (2) activated TGFβ2 elevates IOP in perfusion cultured human (Fleenor et al., 2006, Gottanka et al., 2004) and porcine (Bachmann et al., 2006) anterior segments as well as in mouse eyes in vivo where the TM was transduced with an Ad5.TGFβ2 expression vector (Shepard et al., 2010). The time course of IOP induction occurred over days, consistent with observed effects on ECM accumulation in the TM. TGFβ serves as a profibrotic signal for cultured TM cells due to induced expression of α-smooth muscle actin (Tamm et al., 1996), a variety of extracellular matrix proteins, including fibronectin (Medina-Ortiz et al., 2013, Fleenor et al., 2006), collagens (Fuchshofer et al., 2007), plasminogen activator inhibitor-1 (PAI-1) (Fuchshofer et al., 2003), and extracellular matrix crosslinking enzymes transglutaminase-2 (TGM2) (Tovar-Vidales et al., 2011, Welge-Lussen et al., 2000), lysyl oxidase (LOX), and LOXL1–4 (Sethi et al., 2011b). TGFβ2 also inhibits TM cell proliferation (Wordinger et al.,

1998), which may at least partially be responsible for the decreased cell density in the inner TM tissues of POAG eyes (Alvarado et al., 1984).

Expression of connective tissue growth factor (CTGF) is induced by TGFβ2 and has been implicated in a number of fibrotic diseases (Moussad and Brigstock, 2000, Franklin, 1997). CTGF also is induced in human TM cells by TGFβ1, mechanical stretch, and increased IOP (Chudgar et al., 2006). CTGF directly increases the expression of a wide variety of ECM proteins, including fibronectin, collagens I, III, IV, and VI as well as self-induction of CTGF, thereby generating feed-forward signaling. Blocking CTGF expression by RNA interference inhibited the TGFβ2 induction of CTGF and fibronectin (Junglas et al., 2009). Anti-CTGF antibody treatment also decreased ECM production in cultured TM cells (Wallace et al., 2013). In addition, a recent study showed that nanoparticles coated with cyclic RGD inhibited CTGF signaling in TM cells (Hennig et al., 2016). These studies suggest that at least part of the profibrotic effects of TGFβ2 are mediated by CTGF. The question of whether CTGF plays a role in regulating IOP was nicely answered in mice with Ad5.CTGF transduction of TM tissue and a transgenic mouse model where CTGF expression was driven by a βB1-crystallin promoter (Junglas et al., 2012). Junglas and colleagues showed increased expression of CTGF in the TM in both the inducible and transgenic models, which led to significantly elevated IOP and optic nerve damage. This was associated increased actin stress fiber formation and increased expression of α-smooth muscle actin.

As implicated with TGFβ2/CTGF treatments, aqueous humor outflow resistance is mediated in part by the TM ECM. Inhibition of ECM degrading matrix metalloproteinases (MMPs) leads to increased outflow resistance in perfusion cultured human anterior segments (Bradley et al., 1998), and TM cells express a number of MMPs, including MMP1–3, MMP9, and MMP14 (Conley et al., 2004, Fuchshofer et al., 2003, Pang et al., 2003, Alexander and Acott, 2001). The therapeutic success of argon laser trabeculectomy is mediated by activation and release of proinflammatory cytokines, especially TNFα and IL-1α from TM cells (Bradley et al., 2000), which “remodel” the TM tissue via activation of MMP3 (Fleenor et al., 2003, Alexander and Acott, 2003, Parshley et al., 1995). Urokinase and tissue plasminogen activators, which are also expressed in TM cells (Tripathi et al., 1990b, Park et al., 1987), help proteolytically convert the zymogen proMMPs to MMPs.

VII. Glaucomatous TM Cells

There are a number of pathological changes that have been reported in the glaucomatous TM. There is a progressive decrease of TM cellularity with age that is exacerbated in POAG eyes (Grierson and Howes, 1987, Alvarado et al., 1984). There also is the accumulation of plaque-like material, extracellular material in the JCT (Lutjen-Drecoll et al., 1986, Rohen, 1983, Lutjen-Drecoll et al., 1981), and increased deposition of fibronectin (Medina-Ortiz et al., 2013, Babizhayev and Brodskaya, 1989) in the TM tissues of POAG eyes. There is increased endoplasmic reticulum and protein misfolding stress in glaucomatous TM tissue and cells, as seen by significantly increased expression of GRP78, GRP94 and CHOP, suggesting that the GTM cells undergo chronic protein stress in glaucoma (Peters et al., 2015). Using atomic force microscopy, Last and colleagues showed that TM tissue at the level of the JCT is stiffer (decreased elasticity) and regionally more variable in POAG eyes

compared to aged-matched normal eyes (Last et al., 2011). However, the circumferential elasticity of POAG TM (including uveal, corneoscleral and JCT) tissue appears to be greater (Camras et al., 2014) and may contribute to enhanced SC collapse and irreversible herniations into collector channels found in glaucoma (personal communication, Haiyan Gong).

Knepper and colleagues have shown the aqueous humor of POAG eyes has higher concentrations of soluble CD44 (sCD44) compared to control eyes (Knepper et al., 2002) and that this sCD44 isoform was hypophosphorylated (Knepper et al., 2005). Moreover, aqueous humor concentrations of sCD44 correlate with progressive visual field loss in POAG patients (Nolan et al., 2007). Consistent with this observation, transduction of the TM in mouse eyes with either Ad5.sCD44 or Ad5.CD44 significantly elevates IOP (Giovingo et al., 2013). These data suggest that sCD44, a multifunctional glycoprotein protein involved in cell-cell interactions and adhesion, may play a pathogenic role in POAG.

Normal TM cells have been cultured with a wide variety of glaucoma-like insults and display some features of glaucomatous TM cells. For example, treatment of normal TM cells with exogenous TGFβ2 enhances the production of some ECM molecules (including the profibrotic fibronectin isoform EDA) and increases PAI1 expression, thereby inhibiting plasminogen activator activation of MMPs. TGFβ2 also increases the expression of ECM cross-linking enzymes such as TGM2 and LOXs as well as induces CLAN cytoskeletal rearrangement (O'Reilly et al., 2011) in cultured TM cells. There is increased expression of the EDA fibronectin isoform (Medina-Ortiz et al., 2013), TGM2 (Tovar-Vidales et al., 2008), and CLANs (Hoare et al., 2009) in POAG TM tissues.

Some laboratories have been able to culture TM cells from OAG donor eyes that display detectible differences compared to cells isolated from donor eyes without a history of OAG (Table 3). For example, primary cultures of GTM cells demonstrate decreased migratory ability from explants and defects in autophagy (Porter et al., 2015, Stamer et al., 2000). Moreover, the majority of POAG patients are considered GC responders (Clark and Wordinger, 2009, Wordinger and Clark, 1999), and cultured GTM cells are more responsive to GCs compared to NTM cells. For example, these GTM cells have a greater basal level of CLANs and are more responsive to GC-induced CLAN formation (Uchida et al., 2008, Clark et al., 1995). GTM cells have a greater induction of a GRE-luciferase reporter gene compared to NTM cells (Zhang et al., 2005). An alternatively spliced form of the glucocorticoid receptor (GRβ) acts as a dominant regulator of GC activity (Schaaf and Cidlowski, 2002), and GRβ expression is lower in GTM cells, making these more sensitive to GCs (Zhang et al., 2005), possibly due to altered expression of splicesome protein SFRS5.1, which increases splicing of GRβ in cultured TM cells (Jain et al., 2012).

Gene and protein expression comparing normal TM cells and tissues to GTM cells and tissues have lead to the discovery of a number of new pathogenic pathways. Cultured GTM cells express higher levels of TGFβ2 (Tovar-Vidales et al., 2008), possibly due to epigenetic regulation of histone acetylation in TM cells [Bermudez et al., submitted for publication]. Treatment with a potent HDAC inhibitor elevates TGFβ2 expression in TM cells and tissues, with a corresponding increase in IOP in perfusion cultured anterior segments. TM cells

express bone morphogentic proteins (BMPs) and BMP receptors (Wordinger et al., 2002), which normally antagonize TGFβ2 signaling (Wordinger et al., 2007, Fuchshofer et al., 2007). However, expression of the BMP antagonist gremlin is elevated in GTM cells, and over-expression of gremlin elevates IOP in perfusion cultured human anterior segments (Wordinger et al., 2007) and in mouse eyes (McDowell et al., 2015). TGFβ2 increases gremlin expression and gremlin increases TGFβ2 expression in cultured TM cells (Sethi et al., 2011a), creating a profibrotic environment. In addition to TGFβ2/BMP signaling, TM cells also have a functional canonical β-catenin Wnt pathway that regulates aqueous outflow and IOP (Mao et al., 2012b, Wang et al., 2008b). GTM cells and tissues have higher mRNA and protein expression of SFRP1, an inhibitor of Wnt signaling (Wang et al., 2008b), and overexpression of SFRP1 increases aqueous humor outflow resistance and elevates IOP in perfusion cultured human anterior segments and in mouse eyes (Mao et al., 2012a, Wang et al., 2008b). mRNA and protein expression of the acute phase gene SAA2 is elevated in cultured GTM cells, and treatment of perfusion cultured human anterior segments with recombinant SAA2 elevated IOP (Wang et al., 2008a). SAA2 treated TM cells increased IL-8 secretion and did not cause obvious amyloid deposits in the TM. A proteomic analysis of trabeculectomy tissue from POAG patients compared to TM tissue dissected from normal donor eyes identified increased expression of cochlin, and expression of this protein was also elevated in the TM region of the glaucomatous DBA/2J mouse (Bhattacharya et al., 2005a, Bhattacharya et al., 2005b). Perfusion culture of monkey anterior segments with recombinant cochlin increased aqueous outflow resistance (Lee et al., 2010). Expression of ELAM1 in POAG TM tissue has also been reported to be a distinct marker of POAG, and ELAM1 expression activated by IL-1 in an autocrine feedback loop in GTM cells (Wang et al., 2001). Global changes in gene expression of normal and POAG TM tissues have recently been reported (Liu et al., 2013), which may further increase our understanding of glaucomatous damage to the TM.

VIII. Unresolved questions and future directions

Given the handful of laboratories working in the area of TM cell biology, progress has been exemplary over the past 30 years. For example, despite only 20–25 laboratories/year funded over the past decade by NIH to study TM biology (NIH Reporter, using trabecular meshwork as searchable term in abstracts) these research teams have been instrumental in helping bring 5 drugs to late stage clinical trials that target the trabecular meshwork (Kopczynski and Epstein, 2014).

Drugs in trials are thought to function by relaxing the TM, improving access of aqueous humor to SC and opening new flow pathways plus enhancing turnover of ECM. For example, basic research on TM cell biology have resulted in the development of drugs that target rho kinase (Rao et al., 2001), adenosine A1 receptors (Shearer and Crosson, 2002) and cyclic GMP (Wiederholt et al., 1994). In addition to these, there will likely be multiple other drug classes that target the conventional outflow pathway to restore functionality. Consequently, there is a clinical need for (i) drugs that transiently “clean out” accumulated debris, particularly after some cataract removals and in exfoliation glaucoma; (ii) drugs that assist in phagocytic processing of materials; (iii) drugs that stimulate resident stem cell division (Bradley et al., 2000), (v) specific disease modifying therapies based on our

understanding of molecular pathogenesis, and (vi) gene therapeutics. Several drugs that target the diseased conventional outflow tract will be needed because “glaucoma” is a group of diseases, most of which have in common elevated IOP and defects in conventional outflow. Depending upon the type of glaucoma (e.g.: myocilin versus psuedoexfoliation versus steroid-induced), the diseased pathway in the conventional tract will likely be different.

Even with such progress, there remain many questions about this complicated tissue that when answered will uncover new drug targets and specific treatments for TM pathologies.

- **A.** Which TM cell functions and/or interactions with the inner wall of SC altered in glaucoma are responsible for increased outflow resistance and elevated IOP?
- **B.** What strategies are needed to identify specific TM cell marker(s)?
- **C.** Are JCT cells really TM cells in a different environment or are they a separate cell type or epigenetically modified?
- **D.** What is the relationship between JCT cells and SC inner wall cells and how do they determine outflow resistance in normal and glaucoma eyes?
- **E.** Does TM cell and tissue stiffness affect outflow resistance?
- **F.** Are CLANs involved in outflow resistance, and why are they increased in POAG?
- **G.** Does enhanced TM ECM crosslinking affect outflow resistance?
- **H.** Which of the plethora of GC and TGFβ1/2 effects on the TM are responsible for IOP elevation?
- **I.** Is there cross-talk between the pathogenic signaling pathways identified to date?
- **J.** What awakens stem cell population in TM insert region- are these cells unresponsive in some types of glaucoma?
- **K.** What happens to TM cells in psuedoexfoliation/pigment dispersion syndrome vs. glaucoma that makes them unresponsive to shed cellular materials?
- **L.** How can mouse models help us better understand the TM?

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Highlights

- Trabecular meshwork cells are responsible for regulating aqueous humor outflow resistance
- Defective trabecular meshwork function can cause elevated IOP and the development of glaucoma
- Trabecular meshwork cells are a hybrid cells, containing properties of endothelia, myofibroblasts, and monocytes
- The study of TM cells in culture has led to the development of new glaucoma therapies

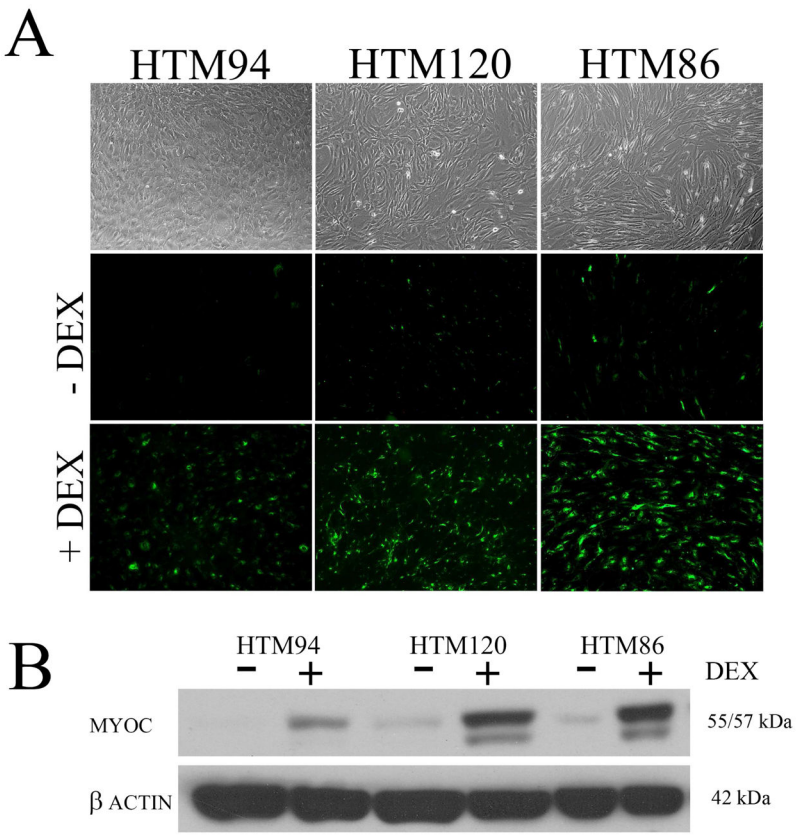

Figure 1.

Morphology and response to corticosteroid of human trabecular meshwork cell monolayers in culture. Panel A shows phase contrast and immunofluorescence microscopic images of three human trabecular meshwork cell monolayers, each isolated from different human donor eyes and displaying different morphologies. HTM 94 shows predominant “endothelial-like” morphology, while HTM120 and HTM86 are mixed cultures with primarily “myofibroblast” morphologies. Cell monolayers were probed with antibodies raised against myocilin in the presence or absence of 100 nM dexamethasone (DEX) treatment for 5 days. To assess magnitude of myocilin induction, corresponding western blots of treated and untreated TM cell monolayers are shown in panel B.

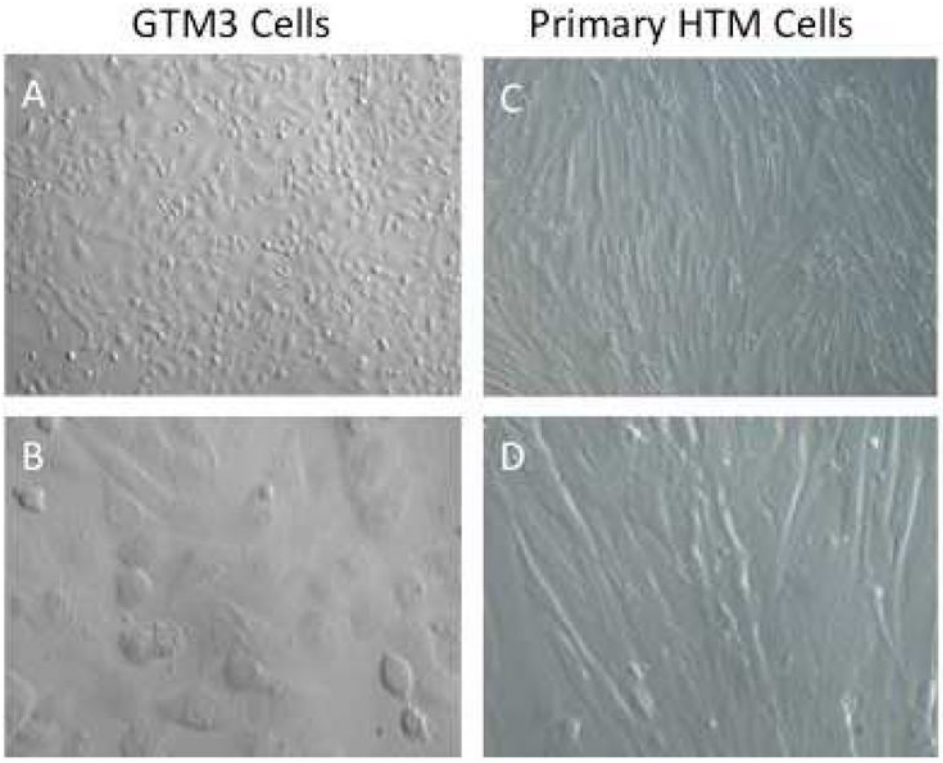

Figure 2.

Morphological comparison between transformed GTM3 cells and primary cultures of human TM cells. GTM3 cells (A-10X and B-40X) are smaller cells with less cytoplasm and continue cell division (rounded cells) despite being confluent. Primary human TM cells (NTM176-04)(C-10X and D-40X) are larger and more elongated with overlapping processes. These cells are contact inhibited and stop dividing when they become a confluent monolayer.

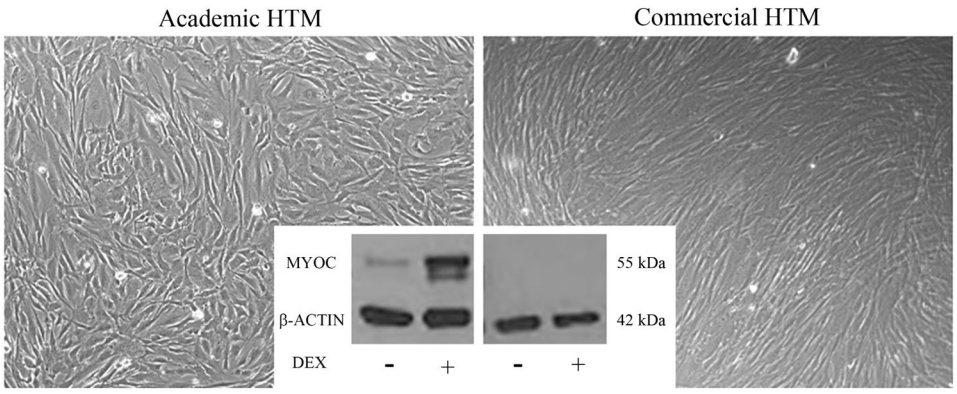

Figure 3.

Morphology and corticosteroid response of a commercial human “trabecular meshwork” cell (HTM) culture compared to a human trabecular meshwork cell strain isolated in an academic setting. The phase contrast images show subtle phenotypic differences; however, western blot analysis of myocilin show clear differences in both basal expression and induction by dexamethasone (DEX).

Table 1

TM phenotypes, tissue location, cell behavior and associated responsibilities

Phenotype	Location	Cell Behavior	Responsibilities
Endothelial	Uveal/corneoscleral	Endothelia	Maintain passageway patency
			Neutralize reactive oxygen species
		Macrophage	Biological filter/phagocytosis
			Immune mediation
Fibroblastic	Juxtacanalicular tissue	Fibroblast	ECM turnover/tissue repair
		Smooth muscle	Contractile tone
			Mechanotrasduction

Table 2

Identification of TM cells in culture; differentially expressed proteins and responses to stimuli compared to potential contaminating cells

Positive Markers	Negative Markers	Cellular responses
Myocilin	VE-cadherin	Acetylated LDL uptake
Matrix GLA protein	Fibulin-2	αvb5/β3-mediated phagocytosis
Chitinase-3 like-1	Integrin α6	CS induction of myocilin, FN, CLANs
Aquaporin-1	Keratin	CS down regulation of tPA, phagocytosis
Smooth muscle actin	Desmin	PMA, TNFα and IL-1α stimulation of MMP-3/-9
Smooth muscle myosin	VEGFR3	TGFβ induction of alpha-B crystallin, PAI-I, MMP2, CLANs
Alpha-B crystallin	Prox-1	Stretch induction of MMP-2/-3

Abbreviations: VE=vascular endothelial; LDL=low density lipoprotein; CS= corticosteroid; FN=fibronectin; CLANs=cross-linked actin networks; tPA= tissue-type plasminogen activator; PMA=phorbol ester; TNF= tumor necrosis factor; IL= interleukin; MMP= matrix metalloproteinase; PAI= plasminogen activator inhibitor; VEGFR3: vascular endothelial growth factor receptor type 3.

Table 3

Summary of Differences Between NTM and GTM Cells

	Feature	**Difference in GTM**
TM Tissue (in vivo)	TM cellularity	Decrease
	Deposition of ECM in outflow pathway	Increase
	Fibronectin expression	Increase total Increase EDA isoform
	Elasticity	Decrease at JCT Increase circumferentially
	TGFβ2 expression	Increase
	TGM2 expression	Increase
	sCD44 expression	Increase
	ELAM1 expression	Increase
	Cochlin expression	Increase
	ER/protein misfolding stress	Increase
TM Cells (in vitro)	Migratory ability	Decrease
	Autophagy	Decrease
	GC responsiveness	Increase
	GRβ expression	Decrease
	CLANs	Increase
	TGFβ2 expression	Increase
	Gremlin expression	Increase
	SFRP1 expression	Increase
	Cochlin expression	Increase
	SAA2 expression	Increase

[See Sections VI and VII for details and references]