ABSTRACT

Primary open angle glaucoma (POAG) is the most common form of glaucoma. Ocular hypertension is a major risk factor for POAG and is caused by increased aqueous humor (AH) outflow resistance in the trabecular meshwork (TM). Increased extracellular matrix (ECM) deposition within the TM is correlated with ocular hypertension. Transforming Growth Factor beta 2 (TGFβ2) levels are elevated in the AH and TM of POAG patients, and TGFβ2 increases ECM protein expression, aqueous outflow resistance, and intraocular pressure (IOP). Recently, TGFβ2 was found to induce bone morphogenetic protein 1 (BMP1) expression in TM cells suggesting that BMP1 activity might be involved in glaucoma pathogenesis. Procollagen C proteinase enhancers (PCOLCE 1 and PCOLCE 2) regulate BMP1 activity. Therefore, PCOLCE1 and PCOLCE2 may play an important role(s) in regulating ECM structural changes in the TM, and contribute to AH outflow resistance and elevated IOP in glaucoma. The purpose of this study was to determine if human TM cells and tissues express PCOLCE1 and PCOLCE2 and whether TGFβ2 induces their expression. This is the first documentation that PCOLCE1 and PCOLCE2 are expressed in TM cells and tissues and that TGFβ2 does induce expression of PCOLCE1.

EXPRESSION OF PROCOLLAGEN C PROTEINASE ENHANCER PROTEINS IN HUMAN TRABECULAR MESHWORK CELLS AND TISSUES

THESIS

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Monal Naik, B.S.

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CHAPTER 1: INTRODUCTION

Glaucoma

Glaucoma is defined as a group of heterogeneous optic neuropathies that progressively damage the optic nerve head leading to visual dysfunction and blindness. It has been reported to affect over 70 million people worldwide[1]. The most common form of glaucoma is primary open-angle glaucoma (POAG). POAG is a chronic disease that is characterized by open anterior chamber angles of normal appearance with no other apparent cause for increased IOP. It is also characterized by changes in the optic nerve, retinal nerve fiber layer and visual field [2]. Elevated intraocular pressure (IOP) is a major risk factor in the development and progression of POAG [3,4]. Elevated IOP is primarily due to an increase in aqueous humor (AH) outflow resistance within the trabecular meshwork (TM) [1]. The extracellular matrix (ECM) in the TM is important in regulating IOP in the eye [5].

Trabecular Meshwork

The trabecular meshwork (TM) is a specialized connective tissue at the irido-corneal angle that regulates the drainage of aqueous humor in the eye so that normal IOP can be maintained[6]. The TM can be subdivided into three regions: uveal, corneoscleral, and juxtacanalicular or cribriform meshworks [5]. The layer closest to the anterior chamber is known as the uveal meshwork, and is located near the anterior chamber from the iris root and ciliary body to the peripheral cornea. It is composed of irregular layers of ECM surrounded by one to three layers of trabeculae or beams covered by endothelial-like cells and has large pores (e.g. 25–75 μm) in the proximal regions

of the uveal meshwork [7,8]. Limited resistance to AH outflow exists because of the presence of these large sized pores [8].

The middle layer is the corneoscleral meshwork, which extends from the scleral spur (a wedge-shaped circular ridge that protrudes from the inner sclera) to the anterior wall of the scleral sulcus (indentation). This layer consists of flattened sheet-like trabeculae (3-5 layers) covered by monolayers of TM cells. The presence of narrower intercellular spaces sized 2–15 μm in the deeper layers of the corneoscleral meshwork[5,9] contribute to the increase resistance of the AH outflow in the TM, but is not the major site of outflow resistance[8]. In the uveal and corneoscleral meshwork core beams lie densely packed elastin fibers region, collagen I and III fibrils, and microfibril sheath derived material [10,11]. The cells covering the beams bind to the basal lamina that consists of collagen IV and laminin [12,13]

The outermost layer, the juxtacanalicular (JCT) or cribriform meshwork, is adjacent to the inner wall of endothelial cells of Schlemm's canal. It is the thinnest part of the TM with a thickness of 2-20 μm[8,14]. This region does not consist of trabecular lamellae but instead has loose connective tissue containing 2-5 layers of JCT cells embedded in a loosely arranged ECM[8]. The ECM of the beams and the JCT are composed of proteoglycans, fibrillar and non-fibrillar collagens, elastin, fibronectin, and matricellular protein[15]. Direct pressure measurements [16-18] and the presence of glycosaminoglycans and proteoglycans in the open spaces in the JCT region [19,20] are evidence that support the idea that the JCT region of the TM is where the majority of the outflow resistance resides.

Once ECM components are synthesized and secreted (i.e. collagens, elastin, fibronectin), they are cross-linked to become stabilized and resistant to degradation. Transglutaminase (TGM2) is

responsible for cross-linking collagen and fibronectin[21], while lysyl oxidase (LOX) is responsible for cross-linking collagen and elastin[22].

Aqueous Humor Outflow System

Aqueous humor is a clear fluid that helps fill and form the anterior and posterior segments of the eye and also provides nutrition to the avascular anterior segment part of the eye. It is secreted by the ciliary body where it then enters the posterior chamber and flows around the lens and through the pupil into the anterior chamber [23]. Aqueous humor then exits the eye via two distinct pathways. In the conventional outflow pathway, AH passes through the TM into Schlemm's canal and then drains into the aqueous veins [5,24]. The second pathway is known as the non-conventional/uveoscleral pathway where AH leaves the anterior chamber by diffusion through the iris root and intercellular spaces among the ciliary muscle fibers [25]. In POAG, there is an increase in outflow resistance in the juxtacanalicular region of the TM, Schlemm's canal inner wall endothelium, or both leading to elevated IOP. There is a debate currently as to which region contributes most to increased outflow resistance. [19,26,27].

ECM Turnover

Within the TM there is an important balance between deposition and degradation of ECM proteins. An imbalance between ECM protein deposition and degradation may lead to the accumulation of ECM components as seen in POAG. Normal homeostatic adjustment of outflow resistance is triggered by pressure changes or mechanical stretching of the JCT region of the TM, which subsequently leads to alteration of ECM components (e.g. ECM turnover) [15]. Alteration of ECM components after pressure change/mechanical triggering is due to proteolytic degradation by members of a family of 23 related matrix metalloproteinases (MMPs) [28]. MMPs are produced as inactive proenzymes, but are then proteolytically cleaved by other MMPs or furin proprotein

convertase to yield a biologically active proteinase [29]. Several MMPs (e.g. MMP2, MMP14, MMP15 and MMP16) are constitutively expressed in the TM, while MMP1, MMP3, MMP9 are expressed at lower levels (to see an extensive list of MMPs found in the TM, see [30]). MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs). There are four TIMPs that are expressed in human TM cells and are involved in inhibiting MMP activity [30]. MMPs have been associated with increase in outflow facility when anterior segments in perfusion culture were treated with MMPs. In contrast, when anterior segments in perfusion culture were treated with TIMP or specific inhibitors of MMP, MMP activity decreased outflow facility [31].

Morphological Changes in the TM of eyes with POAG

In POAG, plaque-like material has been found in greater amounts compared to in normal eyes and is thought to be derive from sheaths of elastic fiber networks [32] that also contain type VI collagen[33]. Other changes include an abundance of long spacing collagen, thickened elastic sheath or basement membrane material in the trabeculae, loss of TM cells and thickening of basement membrane tendons extending from ciliary muscle in glaucomatous samples[37,] . Moreover, ECM components such as fibronectin, vitronectin, laminin, tenascin, decorin, versican collagen I, III, IV, V, microfibril-associated glycoprotein (MAGP) 1, fibrillin and myocilin were found to be components of these sheaths [34,35]. Large changes in GAG distribution are also observed. For example, GAGs such as hyaluronic acid (HA) are decreased while chondroitin sulfate (CS) was increased in the JCT in POAG eyes [26,36]. Thus, these findings suggest that sheaths containing increased ECM components, a decrease in HA and an increase in CS might contribute to increased outflow resistance in POAG TM.

Transforming Growth Factor Beta 2 (TGFβ2) and Glaucoma

The molecular mechanism that leads to AH outflow resistance is not clearly understood. One major player thought to be involved in the pathogenesis of POAG is transforming growth factor beta-2 (TGFβ2). TGFβ2 is member of the transforming growth factor beta superfamily; a group of structurally related multifunctional regulatory proteins that include (a) TGFβ1-3, (b) bone morphogenetic proteins, (c) activins, (d) inhibins, and (e) growth and differentiation factors[37]. Members of the TGFβ superfamily are involved in inflammation, wound healing, ECM accumulation, bone formation, tissue development, cell differentiation and tumor progression [38-41].

Various groups have reported significantly increased levels of TGFβ2 in glaucomatous cells/tissues and from AH from human POAG patients [42-46]. Higher than normal levels of TGFβ2 in the AH of POAG patients are likely to induce ECM deposition since TGFβ2 increases synthesis and secretion of ECM molecules. For example, in vitro studies have shown that cultured TM cells treated with exogenous TGFβ2 had a substantial increase in the expression of various ECM molecules including type I, III, IV and VI collagen, laminin, elastin, cochlin, fibronectin, fibulin, versican, thrombospondin-1, myocilin, plasminogen activator inhibitor 1 (PAI-1), tissue inhibitor of metalloproteinase 1, and ECM crosslinking enzymes transglutaminase and lysyl oxidase [47-52]. Additionally, elevated TGFβ2 levels also increase AH outflow resistance and elevate IOP in ex-vivo perfusion-cultured human and porcine eye models, and in vivo in rodent eyes [53-56]. These results are consistent with the hypothesis that TGFβ2 is involved in the pathogenesis of glaucoma.

Bone Morphogenetic Protein 1 (BMP1)

Recently, Bone Morphogenetic Protein 1 (BMP1) was reported to be involved in regulating the formation of the ECM. Bone morphogenetic proteins (BMPs) were first identified as factors derived from bone extracts that induce de novo endochondral bone formation [57-59]. Unlike other BMPs that belong to the TGFβ superfamily, BMP1 is known to be a metalloproteinase[60,61].

BMP1 consists of an amino terminal prodomain, followed by a conserved protease domain, which is characteristic of the astacin family of metalloproteinases. Next to the prodomain is the carboxyl-terminal CUB (complement-uegf-BMP1) and EGF (epidermal growth factor)-like domains [62-64]. The prodomains are proteolytically cleaved by subtilisin-like proprotein convertases (SPCs) in some cells (e.g. fibrosarcoma cells) [65] while in other cells (e.g. fibrogenic cells such as human osteosarcoma cells, murine osteoblastic cells, human AH1F neonatal foreskin fibroblasts and primary keratinocytes), BMP1 is secreted with the prodomain intact [66,67]. BMP1 is known to play a role in ECM deposition since it processes protein precursors into functional proteins. These precursors include collagens, small leucine-rich proteoglycans (SLRPs), small integrin-binding ligand N-linked glycoproteins (SIBLINGs), the cross-linking enzyme lysyl oxidase (LOX), and basement membrane components such as perlecan and laminin-5 [60,61,68-70].

More importantly, BMP1 is well known to have procollagen C proteinase activity. A comparison was made of procollagen C proteinase activity between a purified mouse procollagen C proteinase and recombinant BMP1 protein produced from a Baculovirus system [71]. Procollagen C proteinase is involved in the cleavage of carboxyl propeptides of collagen types I-III to form mature monomers that then have the capability to assemble into fibrils [71]. BMP1 is known to cleave the C terminal end of these propeptides intracellularly or extracellularly in a tissue and

developmental stage-specific manner [60,72,73]. BMP1 also cleaves the C terminal of other procollagens (V, VII, and XI) [74-76]. In vitro and in vivo studies have also demonstrated that BMP1 activates TGFβ1.

TGFβ1 is produced with a large latent complex (LLC) that inhibits TGFβ-1 signaling. The LLC is bound to the TGFβ1 cleaved prodomain (known as latency-associated peptide [LAP]) and via LAP, the LLC is bound to latent TGF beta-binding proteins (LTBPs). This allows LLC to bind the ECM[77]. BMP1 cleaves LTBP1[78] freeing the LLC from the ECM thus resulting in activation of TGFb-1 via cleavage of LAP by non-BMP1-like proteinases (e.g. plasmin and/or matrix metalloproteinases) [79-84].

Recently, Tovar-Vidales et al (2013) found that BMP1 is expressed in human TM cells and tissues and that exogenous TGFβ2 induces BMP1 protein levels in cultured human TM cells. Since TGFβ2 levels are elevated in POAG and BMP1 is induced by this growth factor, this suggests that BMP1 may play a role in glaucoma pathogenesis. Compared with other extracellular metalloproteinases such as the matrix metalloproteinases whose activity is regulated by TIMPS, BMP1 activity depends on proteins that enhance its activity. Such proteins known to be involved with enhancing BMP1 activity include twisted gastrulation, olfactomedin-noelin-tarin factor 1, periostin[85-88], and procollagen C proteinase enhancer proteins (PCOLCE1 and PCOLCE2). The procollagen C proteinase enhancer proteins are the more extensively studied ones and are of importance because they have been shown to be associated with myocardial fibrosis and liver fibrotic diseases in vitro and in vivo[89-92].

Procollagen C Proteinase Enhancer Proteins

Procollagen C proteinase enhancers (PCOLCE1 and PCOLCE2) regulate BMP1 activity[88,93-95]. PCOLCE1 (formerly known as PCOLCE) is a secreted glycoprotein. In mouse fibroblasts, full

expression of BMP1 was dependent upon 55 kDa PCOLCE1 or its 36 and 34 kDa proteolytically processed forms of the same protein [71]. None of the three-enhancer proteins exhibited intrinsic procollagen processing activity, but all were capable of enhancing the activity of the C-proteinase by a 1 order of magnitude [76].

PCOLCE1 and PCOLCE2 consist of two CUB domains (which account for procollagen C proteinase enhancing activity) followed by a netrin-like domain. The function of this domain is controversial as one study suggests it to have inhibitory protease activity [96] (e.g. the domain is homologous to netrin domain in TIMP) while another study showed the opposite effect[97]. PCOLCE1 is highly expressed in adult and fetal human tissues including heart, uterus, digestive tract, adrenal gland, but barely is detectable in brain and liver [88,93,94]. More recently, a group used linkage and expression sequence tag analysis to identify a potential glaucoma candidate gene (PCOLCE2) in the 3q21-q24-chromosome locus [95]. However, no glaucoma-associated changes were detected in PCOLCE2 [95]. PCOLCE2 is a protein C proteinase enhancer with activity levels similar to PCOLCE1 [94]. PCOLCE2 also was found to enhance the procollagen I cleavage activity of BMP1[94]. Both PCOLCE1 and PCOLCE2 have been reported to increase BMP1 activity greater than ten fold [94,98]. Other reports have shown that the PCOLCE1 enhancement of BMP1 activity is substrate specific, being limited to the C terminal end processing of fibrillar procollagens I-III [75,88,93,94]. PCOLCE2 is expressed in heart, pituitary gland, bladder, mammary gland, and trachea, non-ossified cartilage in developing bone, and in the TM [94,95].

Finally, progress has been made on the mechanism by which PCOLCE1/PCOLCE2 enhances BMP1 activity. Studies have shown that PCOLCE1 binds C propeptide [88,93] or the telopeptide region [93,99] (a short non-triple region of ~25-residues that remains intact at the C terminus of the collagen molecule following C-propeptide cleavage) of the procollagen molecule. This induces a

conformational change in the procollagen molecule that allows for more efficient binding of BMP1 to the procollagen molecule. The net result is an increase in BMP1 catalytic activity and cleavage of fibrillar procollagens [93,99]. PCOLCE1 also binds the 300 nm long triple helical region of the procollagen molecule and then displaces BMP1 bound to the collagen protein [94].

Hypothesis and Significance

No previous studies have reported the presence of both PCOLCE 1 and PCOLCE2 in human TM cells and tissues. Therefore, we hypothesize that (a) PCOLCE1 and PCOLCE2 are expressed in human TM cells and tissues and that (b) TGFβ2 regulates PCOLCE1 and PCOLCE2 secretion by cultured human TM cells.

This study is significant because it would demonstrate for the first time that PCOLCE 1 and PCOLCE2 are expressed at both the mRNA and protein levels in the human TM cells and would localize POCOLCE1 and PCOLCE2 proteins in human TM tissues. It is important to know the expression of these proteins so that further studies can investigate the role of PCOLCE1 and PCOLCE2 in the human TM. An understanding of PCOLCE1 and PCOLCE2 in the TM will foster further studies of the molecular mechanisms that contribute to ECM changes (e.g. excess collagen deposition and cross-linking) in the TM, AH outflow resistance, and elevated IOP in glaucoma (see Figure 1). This will expand our current knowledge of the pathogenesis mechanisms in the glaucomatous in the TM and allow the identification and development of new therapeutic targets.

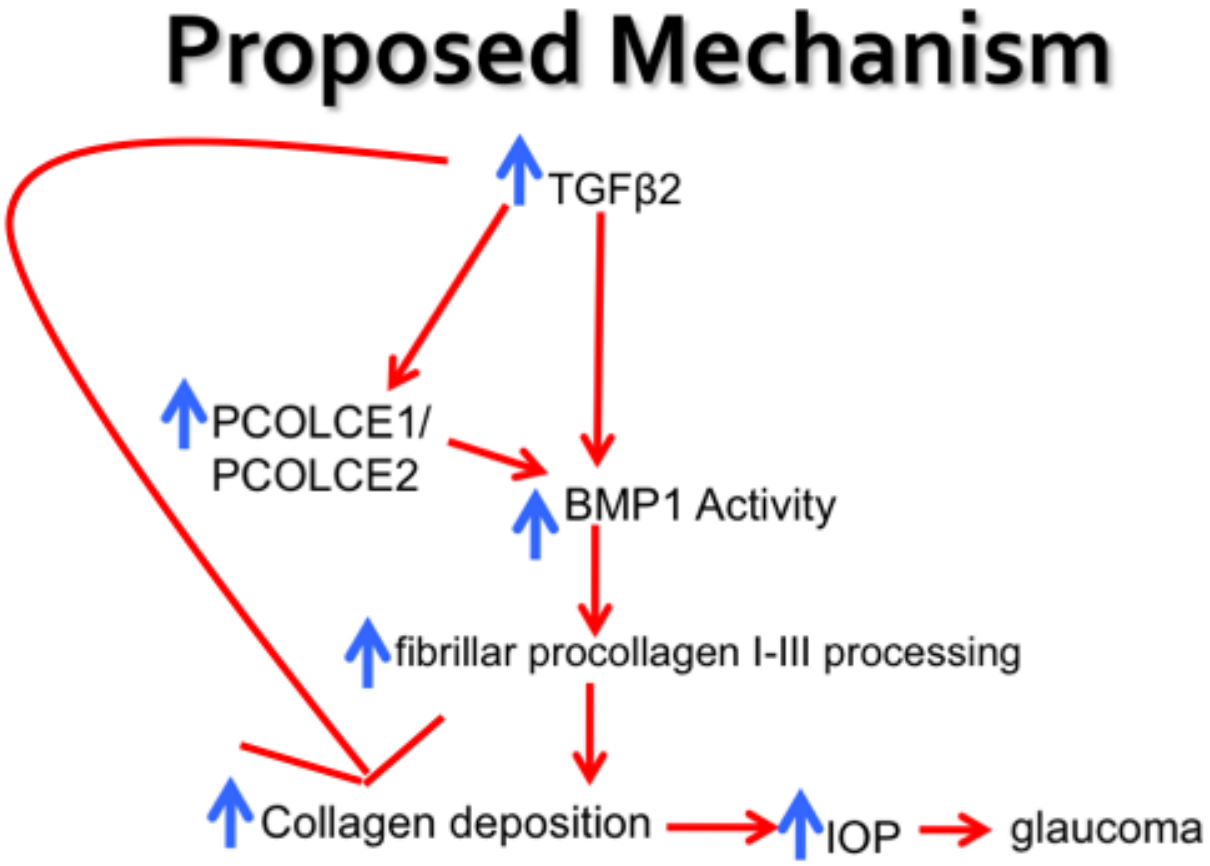

Figure 1.1: A proposed mechanism for PCOLCE 1 and 2 in the processing of procollagen to collagen accumulation in the glaucoma TM.

References

1. Quigley HA. Glaucoma. *Lancet*. 2011;377(9774):1367-1377. doi: 10.1016/S0140-6736(10)61423-7; 10.1016/S0140-6736(10)61423-7.

2. Weinreb RN, Khaw PT. Primary open-angle glaucoma. *Lancet*. 2004;363(9422):1711-1720. doi: 10.1016/S0140-6736(04)16257-0.

3. Kass MA, Heuer DK, Higginbotham EJ, et al. The ocular hypertension treatment study: A randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch Ophthalmol*. 2002;120(6):701-13; discussion 829-30.

4. The Advanced glaucoma intervention study (AGIS): 7. the relationship between control of intraocular pressure and visual field deterioration.the AGIS investigators. *Am J Ophthalmol*. 2000;130(4):429-440.

5. Llobet A, Gasull X, Gual A. Understanding trabecular meshwork physiology: A key to the control of intraocular pressure? *News Physiol Sci*. 2003;18:205-209.

6. Bill A. Editorial: The drainage of aqueous humor. *Invest Ophthalmol*. 1975;14(1):1-3.

7. Flocks M. The anatomy of the trabecular meshwork as seen in tangential section. *AMA Arch Ophthalmol*. 1956;56(5):708-718.

8. Tamm ER. The trabecular meshwork outflow pathways: Structural and functional aspects. *Exp Eye Res*. 2009;88(4):648-655. doi: 10.1016/j.exer.2009.02.007; 10.1016/j.exer.2009.02.007.

9. Tripathi R. Tracing the bulk outflow route of cerebrospinal fluid by transmission and scanning electron microscopy. *Brain Res*. 1974;80(3):503-506.

10. Marshall GE, Konstas AG, Lee WR. Immunogold ultrastructural localization of collagens in the aged human outflow system. *Ophthalmology*. 1991;98(5):692-700.

11. Gong H, Ruberti J, Overby D, Johnson M, Freddo TF. A new view of the human trabecular meshwork using quick-freeze, deep-etch electron microscopy. *Exp Eye Res*. 2002;75(3):347-358.

12. Murphy CG, Yun AJ, Newsome DA, Alvarado JA. Localization of extracellular proteins of the human trabecular meshwork by indirect immunofluorescence. *Am J Ophthalmol*. 1987;104(1):33-43.

13. Marshall GE, Konstas AG, Lee WR. Immunogold localization of type IV collagen and laminin in the aging human outflow system. *Exp Eye Res*. 1990;51(6):691-699.

14. McEWEN WK. Application of poiseuille's law to aqueous outflow. *AMA Arch Ophthalmol*. 1958;60(2):290-294.

15. Acott TS, Kelley MJ. Extracellular matrix in the trabecular meshwork. *Exp Eye Res*. 2008;86(4):543-561. doi: 10.1016/j.exer.2008.01.013; 10.1016/j.exer.2008.01.013.

16. Bill A, Svedbergh B. Scanning electron microscopic studies of the trabecular meshwork and the canal of schlemm--an attempt to localize the main resistance to outflow of aqueous humor in man. *Acta Ophthalmol (Copenh)*. 1972;50(3):295-320.

17. Maepea O, Bill A. Pressures in the juxtacanalicular tissue and schlemm's canal in monkeys. *Exp Eye Res*. 1992;54(6):879-883.

18. Maepea O, Bill A. The pressures in the episcleral veins, schlemm's canal and the trabecular meshwork in monkeys: Effects of changes in intraocular pressure. *Exp Eye Res*. 1989;49(4):645-663.

19. Ethier CR. The inner wall of schlemm's canal. *Exp Eye Res*. 2002;74(2):161-172. doi: 10.1006/exer.2002.1144.

20. Seiler T, Wollensak J. The resistance of the trabecular meshwork to aqueous humor outflow. *Graefes Arch Clin Exp Ophthalmol*. 1985;223(2):88-91.

21. Tovar-Vidales T, Clark AF, Wordinger RJ. Focus on molecules: Transglutaminase 2. *Exp Eye Res*. 2011;93(1):2-3. doi: 10.1016/j.exer.2010.06.006; 10.1016/j.exer.2010.06.006.

22. Sethi A, Wordinger RJ, Clark AF. Focus on molecules: Lysyl oxidase. Exp Eye Res. 2012. 104;97-8.

23. Goel M, Picciani RG, Lee RK, Bhattacharya SK. Aqueous humor dynamics: A review. *Open Ophthalmol J*. 2010;4:52-59. doi: 10.2174/1874364101004010052; 10.2174/1874364101004010052.

24. Lütjen-Drecoll E, Rohen J. Glaucomatous I. In: Ritch R, Shields M, Krupin T, eds. *Morphology of aqueous outflow pathways in normal and glaucomatous eyes.* . St Louis: Mosby-Year Book, Inc; Morphology of aqueous outflow pathways in normal and glaucomatous eyes.:89-124.

25. Bill A. Blood circulation and fluid dynamics in the eye. *Physiol Rev*. 1975;55(3):383-417.

26. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog Retin Eye Res*. 1999;18(1):91-119.

27. Johnson M. 'What controls aqueous humour outflow resistance?'. *Exp Eye Res*. 2006;82(4):545-557. doi: 10.1016/j.exer.2005.10.011.

28. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol*. 2007;8(3):221-233. doi: 10.1038/nrm2125.

29. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: An overview. *Mol Cell Biochem*. 2003;253(1-2):269-285.

30. Keller KE, Aga M, Bradley JM, Kelley MJ, Acott TS. Extracellular matrix turnover and outflow resistance. *Exp Eye Res*. 2009;88(4):676-682. doi: 10.1016/j.exer.2008.11.023; 10.1016/j.exer.2008.11.023.

31. Bradley JM, Vranka J, Colvis CM, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. *Invest Ophthalmol Vis Sci*. 1998;39(13):2649-2658.

32. Gottanka J et al. Severity of optic nerve damage in eyes with POAG. J glaucoma. 1997 ;6(2):123-32.

33. Lutjen-Drecoll E, Futa R, Rohen JW. Ultrahistochemical studies on tangential sections of the trabecular meshwork in normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 1981;21(4):563-573.

34. Ueda J, Wentz-Hunter K, Yue BY. Distribution of myocilin and extracellular matrix components in the juxtacanalicular tissue of human eyes. *Invest Ophthalmol Vis Sci*. 2002;43(4):1068-1076.

35. Lutjen-Drecoll E, May CA, Polansky JR, Johnson DH, Bloemendal H, Nguyen TD. Localization of the stress proteins alpha B-crystallin and trabecular meshwork inducible glucocorticoid response protein in normal and glaucomatous trabecular meshwork. *Invest Ophthalmol Vis Sci*. 1998;39(3):517-525.

36. Rohen JW, Witmer R. Electrn microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1972;183(4):251-266.

37. De Caestecker M. The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev*. 2004;15(1):1-11.

38. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta1 to the bone. *Endocr Rev*. 2005;26(6):743-774. doi: 10.1210/er.2004-0001.

39. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov*. 2004;3(12):1011-1022. doi: 10.1038/nrd1580.

40. Sporn MB, Roberts AB. Transforming growth factor-beta: Recent progress and new challenges. *J Cell Biol*. 1992;119(5):1017-1021.

41. Barnard JA, Lyons RM, Moses HL. The cell biology of transforming growth factor beta. *Biochim Biophys Acta*. 1990;1032(1):79-87.

42. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol*. 2001;239(2):109-113.

43. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol*. 2002;46(3):249-253.

44. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol*. 2004;25(1):19-22.

45. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol*. 2001;239(3):199-207.

46. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res*. 1994;59(6):723-727.

47. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (LOX) genes in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2011;52(8):5240-5250. doi: 10.1167/iovs.11-7287; 10.1167/iovs.11-7287.

48. Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGFbeta/smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci*. 2011;52(8):5251-5259. doi: 10.1167/iovs.11-7587; 10.1167/iovs.11-7587.

49. Zhao X, Ramsey KE, Stephan DA, Russell P. Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 2004;45(11):4023-4034. doi: 10.1167/iovs.04-0535.

50. Fuchshofer R, Tamm ER. The role of TGF-beta in the pathogenesis of primary open-angle glaucoma. *Cell Tissue Res*. 2012;347(1):279-290. doi: 10.1007/s00441-011-1274-7; 10.1007/s00441-011-1274-7.

51. Fuchshofer R, Welge-Lussen U, Lutjen-Drecoll E. The effect of TGF-beta2 on human trabecular meshwork extracellular proteolytic system. *Exp Eye Res*. 2003;77(6):757-765.

52. Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res*. 2011;93(4):442-451. doi: 10.1016/j.exer.2011.06.011; 10.1016/j.exer.2011.06.011.

53. Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor-{beta}2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51(4):2067-2076. doi: 10.1167/iovs.09-4567; 10.1167/iovs.09-4567.

54. Bachmann B, Birke M, Kook D, Eichhorn M, Lutjen-Drecoll E. Ultrastructural and biochemical evaluation of the porcine anterior chamber perfusion model. *Invest Ophthalmol Vis Sci*. 2006;47(5):2011-2020. doi: 10.1167/iovs.05-1393.

55. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: Implications for intraocular pressure. *Invest Ophthalmol Vis Sci*. 2006;47(1):226-234. doi: 10.1167/iovs.05-1060.

56. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004;45(1):153-158.

57. Wozney JM, Rosen V, Celeste AJ, et al. Novel regulators of bone formation: Molecular clones and activities. *Science*. 1988;242(4885):1528-1534.

58. Urist MR. Bone: Formation by autoinduction. *Science*. 1965;150(3698):893-899.

59. Van de Putte KA, Urist MR. Osteogenesis in the interior of intramuscular implants of decalcified bone matrix. *Clin Orthop Relat Res*. 1965;43:257-270.

60. Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS. Bone morphogenetic protein-1: The type I procollagen C-proteinase. *Science*. 1996;271(5247):360-362.

61. Li SW, Sieron AL, Fertala A, Hojima Y, Arnold WV, Prockop DJ. The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci U S A*. 1996;93(10):5127-5130.

62. Gomis-Ruth FX. Catalytic domain architecture of metzincin metalloproteases. *J Biol Chem*. 2009;284(23):15353-15357. doi: 10.1074/jbc.R800069200; 10.1074/jbc.R800069200.

63. Bond JS, Beynon RJ. The astacin family of metalloendopeptidases. *Protein Sci*. 1995;4(7):1247-1261. doi: 10.1002/pro.5560040701.

64. Bork P, Beckmann G. The CUB domain. A widespread module in developmentally regulated proteins. *J Mol Biol*. 1993;231(2):539-545. doi: 10.1006/jmbi.1993.1305.

65. Leighton M, Kadler KE. Paired basic/furin-like proprotein convertase cleavage of pro-BMP-1 in the trans-golgi network. *J Biol Chem*. 2003;278(20):18478-18484. doi: 10.1074/jbc.M213021200.

66. Lee S, Solow-Cordero DE, Kessler E, Takahara K, Greenspan DS. Transforming growth factor-beta regulation of bone morphogenetic protein-1/procollagen C-proteinase and related proteins in fibrogenic cells and keratinocytes. *J Biol Chem*. 1997;272(30):19059-19066.

67. Jasuja R, Ge G, Voss NG, et al. Bone morphogenetic protein 1 prodomain specifically binds and regulates signaling by bone morphogenetic proteins 2 and 4. *J Biol Chem*. 2007;282(12):9053-9062. doi: 10.1074/jbc.M610929200.

68. Borel A, Eichenberger D, Farjanel J, et al. Lysyl oxidase-like protein from bovine aorta. isolation and maturation to an active form by bone morphogenetic protein-1. *J Biol Chem*. 2001;276(52):48944-48949. doi: 10.1074/jbc.M109499200.

69. Uzel MI, Scott IC, Babakhanlou-Chase H, et al. Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control

lysyl oxidase activation in mouse embryo fibroblast cultures. *J Biol Chem*. 2001;276(25):22537-22543. doi: 10.1074/jbc.M102352200.

70. Unsold C, Pappano WN, Imamura Y, Steiglitz BM, Greenspan DS. Biosynthetic processing of the pro-alpha 1(V)2pro-alpha 2(V) collagen heterotrimer by bone morphogenetic protein-1 and furin-like proprotein convertases. *J Biol Chem*. 2002;277(7):5596-5602. doi: 10.1074/jbc.M110003200.

71. Kessler E, Adar R. Type I procollagen C-proteinase from mouse fibroblasts. purification and demonstration of a 55-kDa enhancer glycoprotein. *Eur J Biochem*. 1989;186(1-2):115-121.

72. Canty EG, Lu Y, Meadows RS, Shaw MK, Holmes DF, Kadler KE. Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. *J Cell Biol*. 2004;165(4):553-563. doi: 10.1083/jcb.200312071.

73. Prockop DJ, Kivirikko KI. Collagens: Molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem*. 1995;64:403-434. doi: 10.1146/annurev.bi.64.070195.002155.

74. Ge G, Zhang Y, Steiglitz BM, Greenspan DS. Mammalian tolloid-like 1 binds procollagen C-proteinase enhancer protein 1 and differs from bone morphogenetic protein 1 in the functional roles of homologous protein domains. *J Biol Chem*. 2006;281(16):10786-10798. doi: 10.1074/jbc.M511111200.

75. Petropoulou V, Garrigue-Antar L, Kadler KE. Identification of the minimal domain structure of bone morphogenetic protein-1 (BMP-1) for chordinase activity: Chordinase activity is not enhanced by procollagen C-proteinase enhancer-1 (PCPE-1). *J Biol Chem*. 2005;280(24):22616-22623. doi: 10.1074/jbc.M413468200.

76. Takahara K, Kessler E, Biniaminov L, et al. Type I procollagen COOH-terminal proteinase enhancer protein: Identification, primary structure, and chromosomal localization of the cognate human gene (PCOLCE). *J Biol Chem*. 1994;269(42):26280-26285.

77. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci*. 2003;116(Pt 2):217-224.

78. Ge G, Greenspan DS. BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J Cell Biol*. 2006;175(1):111-120. doi: 10.1083/jcb.200606058.

79. Mu D, Cambier S, Fjellbirkeland L, et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J Cell Biol*. 2002;157(3):493-507. doi: 10.1083/jcb.200109100.

80. Maeda S, Dean DD, Gay I, Schwartz Z, Boyan BD. Activation of latent transforming growth factor beta1 by stromelysin 1 in extracts of growth plate chondrocyte-derived matrix vesicles. *J Bone Miner Res*. 2001;16(7):1281-1290. doi: 10.1359/jbmr.2001.16.7.1281.

81. D'Angelo M, Billings PC, Pacifici M, Leboy PS, Kirsch T. Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. *J Biol Chem*. 2001;276(14):11347-11353. doi: 10.1074/jbc.M009725200.

82. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev*. 2000;14(2):163-176.

83. Sato Y, Rifkin DB. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol*. 1989;109(1):309-315.

84. Lyons RM, Keski-Oja J, Moses HL. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J Cell Biol*. 1988;106(5):1659-1665.

85. Maruhashi T, Kii I, Saito M, Kudo A. Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase. *J Biol Chem*. 2010;285(17):13294-13303. doi: 10.1074/jbc.M109.088864; 10.1074/jbc.M109.088864.

86. Inomata H, Haraguchi T, Sasai Y. Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. *Cell*. 2008;134(5):854-865. doi: 10.1016/j.cell.2008.07.008; 10.1016/j.cell.2008.07.008.

87. Scott IC, Blitz IL, Pappano WN, Maas SA, Cho KW, Greenspan DS. Homologues of twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. *Nature*. 2001;410(6827):475-478. doi: 10.1038/35068572.

88. Vadon-Le Goff S, Kronenberg D, Bourhis JM, et al. Procollagen C-proteinase enhancer stimulates procollagen processing by binding to the C-propeptide region only. *J Biol Chem*. 2011;286(45):38932-38938. doi: 10.1074/jbc.M111.274944; 10.1074/jbc.M111.274944.

89. Goldsmith EC, Bradshaw AD, Spinale FG. Contributory pathways leading to myocardial fibrosis - moving beyond collagen expression. *Am J Physiol Cell Physiol*. 2012. doi: 10.1152/ajpcell.00347.2012.

90. Baicu CF, Zhang Y, Van Laer AO, Renaud L, Zile MR, Bradshaw AD. Effects of the absence of procollagen C-endopeptidase enhancer-2 on myocardial collagen accumulation in chronic pressure overload. *Am J Physiol Heart Circ Physiol*. 2012;303(2):H234-40. doi: 10.1152/ajpheart.00227.2012; 10.1152/ajpheart.00227.2012.

91. Kessler-Icekson G, Schlesinger H, Freimann S, Kessler E. Expression of procollagen C-proteinase enhancer-1 in the remodeling rat heart is stimulated by aldosterone. *Int J Biochem Cell Biol*. 2006;38(3):358-365. doi: 10.1016/j.biocel.2005.10.007.

92. Ogata I, Auster AS, Matsui A, et al. Up-regulation of type I procollagen C-proteinase enhancer protein messenger RNA in rats with CCl4-induced liver fibrosis. *Hepatology*. 1997;26(3):611-617. doi: 10.1002/hep.510260312.

93. Moali C, Font B, Ruggiero F, et al. Substrate-specific modulation of a multisubstrate proteinase. C-terminal processing of fibrillar procollagens is the only BMP-1-dependent activity to be enhanced by PCPE-1. *J Biol Chem*. 2005;280(25):24188-24194. doi: 10.1074/jbc.M501486200.

94. Steiglitz BM, Keene DR, Greenspan DS. PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1. *J Biol Chem*. 2002;277(51):49820-49830. doi: 10.1074/jbc.M209891200.

95. Xu H, Acott TS, Wirtz MK. Identification and expression of a novel type I procollagen C-proteinase enhancer protein gene from the glaucoma candidate region on 3q21-q24. *Genomics*. 2000;66(3):264-273. doi: 10.1006/geno.2000.6229.

96. Williamson RA, Panagiotidou P, Mott JD, Howard MJ. Dynamic characterisation of the netrin-like domain of human type 1 procollagen C-proteinase enhancer and comparison to the N-terminal domain of tissue inhibitor of metalloproteinases (TIMP). *Mol Biosyst*. 2008;4(5):417-425. doi: 10.1039/b717901d; 10.1039/b717901d.

97. Bekhouche M, Kronenberg D, Vadon-Le Goff S, et al. Role of the netrin-like domain of procollagen C-proteinase enhancer-1 in the control of metalloproteinase activity. *J Biol Chem*. 2010;285(21):15950-15959. doi: 10.1074/jbc.M109.086447; 10.1074/jbc.M109.086447.

98. Adar R, Kessler E, Goldberg B. Evidence for a protein that enhances the activity of type I procollagen C-proteinase. *Coll Relat Res*. 1986;6(3):267-277.

99. Ricard-Blum S, Bernocco S, Font B, et al. Interaction properties of the procollagen C-proteinase enhancer protein shed light on the mechanism of stimulation of BMP-1. *J Biol Chem*. 2002;277(37):33864-33869. doi: 10.1074/jbc.M205018200.

CHAPTER II

Expression of Procollagen C Proteinase Enhancer Proteins in Human Trabecular Meshwork Cells and Tissues

Monal Naik[1], Tara Tovar-Vidales [1,2], Ashley F. Fitzgerald[1,2], Abbot F. Clark[1,2] and Robert J. Wordinger[1,2],

[1] Department of Cell Biology and Anatomy, and [2]North Texas Eye Research Institute, University of North Texas Health Science Center, Fort Worth, Texas 76107

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Corresponding author: Robert J. Wordinger, NTERI CBH-457, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107; Robert.Wordinger@unthsc.edu

Abstract

Purpose: To determine if: a) Procollagen C proteinase enhancer proteins 1 and 2 (PCOLCE1 and PCOLCE2) are expressed in human TM cells and tissues and (b) TGFβ2 regulates PCOLCE1 and PCOLCE2 secretion in cultured human TM cells.

Methods: RT-PCR and Western immunoblot analyses are used to determine mRNA expression and protein levels of PCOLCE1 and PCOLCE2 in transformed and primary human TM cells. Immunohistochemistry was used to localize PCOLCE1 and PCOLCE2 in the TM of human donor eyes. Western immunoblot analysis of conditioned medium was used to determine if TGFβ2 increased secretion of PCOLCE1 and PCOLCE2 in primary human TM cells.

Results: PCOLCE1 and PCOLCE2 mRNA were expressed in cultured transformed and primary TM cell cultures. TM cells also secreted both PCOLCE1 and PCOLCE2. Both proteins were localized in human TM tissues as demonstrated by immunohistochemistry. Under the conditions tested, TGFβ2 did appear to regulate PCOLCE1 but not PCOLCE2 protein secretion.

Conclusion: PCOLCE1 and PCOLCE2 proteins are present in human TM tissues and is secreted by cultured TM cells. TGFβ2 appears to regulate PCOLCE1 but not PCOLCE2 secretion.

Introduction:

Primary open-angle glaucoma (POAG) is a leading cause of irreversible visual impairment and blindness in the world [1]. Elevated intraocular pressure (IOP) is a major risk factor in the development and progression of glaucoma [2,3]. Elevated IOP is primarily due to an increase in aqueous humor (AH) outflow resistance in the TM [1]. Increased AH outflow resistance is associated with deposition of extracellular matrix (ECM) proteins within the TM [2-4]. However, the exact molecular mechanism that leads to AH outflow resistance and increased IOP in glaucoma is not clearly understood.

Transforming growth factor beta-2 (TGFβ2) has been suggested to be involved in the molecular pathogenesis of glaucoma. TGFβ2 levels are significantly increased in AH of POAG patients [5-9]. Higher than normal levels of TGFβ2 in AH, may induce TM ECM deposition. In vitro studies have shown that cultured TM cells treated with TGF-β2 resulted in a substantial increase in the expression of various ECM molecules including type I, III, IV and VI collagens, laminin, elastin, cochlin, fibronectin, fibulin, versican, thrombospondin-1, myocilin, plasminogen activator inhibitor 1 (PAI-1), tissue inhibitor metalloproteinase 1, and ECM crosslinking enzymes transglutaminase and lysyl oxidase [10-15]. Elevated TGFβ2 levels also increase AH outflow resistance and elevate IOP in perfusion-cultured human and porcine eyes as well as *in vivo* in rodent eyes [16-19]. These results are consistent with the hypothesis that TGFβ-2 is involved in the pathogenesis of glaucoma.

To further understand potential pathogenesis changes in the ECM of the glaucomatous TM, other cellular mechanisms need to be examined. Recently bone morphogenetic protein 1(BMP1) was shown to be involved in regulating the formation of the ECM. Bone morphogenetic proteins (BMPs) were first identified as bone derived factors that induce *de novo* endochondral bone

formation [20-22]. However, unlike other BMPs that belong to the TGFβ superfamily, BMP1 is a matrix metalloproteinase [23,24]. BMP1 cleaves ECM components such as small leucine rich proteoglycans, small integrin binding N-linked glycoproteins, lysyl oxidase and laminin into functional proteins. BMP1 possesses procollagen C proteinase activity [25].

Procollagen C proteinase is involved in the cleavage of carboxyl propeptides of collagen types I-III to form mature monomers that then have the capability to associate into fibrils [25]. BMP1 is known to cleave the C terminal end of these propeptides intracellularly or extracellularly in a tissue and developmental stage-specific manner [23,26,27]. BMP1 also cleaves the C terminal of other procollagens (V, VII, and XI) [28-30]. Recently, Tovar- Vidales et al (2013) reported that BMP1 is present at higher levels in glaucomatous human TM tissues and that TGFβ2 induces BMP1 expression in cultured human TM cells. This suggests that BMP1 may play a role in glaucoma pathogenesis. Compared with extracellular metalloproteinases such as the matrix metalloproteinases whose activity is regulated by TIMPS, BMP1 activity depends on proteins that enhance its activity. Proteins involved with enhancing BMP1 activity include twisted gastrulation, olfactomedin-noelin-tarin factor 1, periostin [31-34], and procollagen C proteinase enhancer proteins (PCOLCE1 and PCOLCE2). The procollagen C proteinase enhancer proteins have been more extensively studied and are of importance since they are associated with myocardial fibrosis and liver fibrotic diseases [35-38].

PCOLCE1 and PCOLCE2 are secreted glycoproteins that regulate BMP1 activity [34,39-41]. In mouse fibroblasts, full expression of BMP1 was dependent upon 55 kDa PCOLCE1 or 36 and 34 kDa proteolytically processed forms of the same protein [25]. None of these proteins exhibited intrinsic procollagen processing activity, but all were capable of enhancing the activity of the C-proteinase by an order of magnitude [30]. PCOLCE1 and PCOLCE2 each consist of two CUB

domains (which account for procollagen C proteinase enhancing activity [30]) followed by a netrin-like domain. The function of this domain is controversial as one study suggests it inhibits protease activity [42] (e.g. the domain is homologous to netrin domain in TIMP) while another study showed the opposite effect [43].

PCOLCE1 is highly expressed in diverse adult and fetal human tissues including heart, uterus, digestive tract, adrenal gland, but is barely detectable in brain and liver [34,39,40]. More recently, a group used linkage and expression sequence tag analysis to identify a potential glaucoma candidate gene (PCOLCE2) in the 3q21-q24-chromosome locus [41]. However, no coding sequence mutations were detected in PCOLCE2 [41]. PCOLCE2 is a protein C proteinase enhancer that has activity levels similar to PCOLCE1 [40]. PCOLCE2 also enhances the procollagen I cleavage activity of BMP1 [40]. Both PCOLCE1 and PCOLCE2 have been reported to increase BMP1 activity greater than ten fold [40,44]. Other reports have shown that the PCOLCE1 enhancement of BMP1 activity is substrate specific, being limited to the processing of C terminal ends of fibrillar procollagens I-III [29,34,39,40]. PCOLCE2 is expressed in heart, pituitary gland, bladder, mammary gland, trachea, non-ossified cartilage in developing bone, and in the TM [40,41].

Progress has been made on the mechanism by which PCOLCE1/PCOLCE2 enhances BMP1 activity. PCOLCE1 binds C propeptide [34,39] or the telopeptide region [39,45] (a short non-triple region of ~25-residues that remains intact at the C terminus of the collagen molecule following C-propeptide cleavage) of the procollagen molecule, inducing a conformational change in the procollagen molecule that allows for more efficient binding of BMP1. The net result is an increase in BMP1 catalytic activity and cleavage of fibrillar procollagens [39,45]. Another study has shown that PCOLCE1 binds the 300 nm long triple helical region of the procollagen molecule and then displaces BMP1 bound to the collagen protein [40].

No previous studies have reported the presence of both PCOLCE 1 and PCOLCE2 in human TM cells and tissues. Therefore, we hypothesize: (a) PCOLCE1 and PCOLCE2 are expressed in human TM cells and tissues and (b) TGFβ2 regulates PCOLCE1 and PCOLCE2 secretion by cultured human TM cells.

Methods

TM Cell Culture:

Well-characterized, transformed TM cell lines and primary human TM cell strains were obtained from Novartis (Fort Worth, TX). Human TM cells were grown in Dulbecco's modified Eagle's medium (DMEM) (low glucose; Gibco, Grand Island, NY) that was supplemented with 10% fetal bovine serum (HyClone Labs, Logan, UT), L glutamine (0.292mg/ml), penicillin (100 units/ml), streptomycin (0.1mg/ml) and amphotericin B (4 mg/ml). Antibiotics were purchased from Gibco BRL, Grand Island, NY. Cells were cultured at 37°C in 5% CO_2, 95% air, and fresh medium was exchanged every three days. No visual evidence of cellular senescence was observed. Cells were grown to near 100% confluency followed by washing and then culturing in serum-free for 24 hours. Cells were then treated with or without recombinant TGFβ2 (5ng/ml) protein (#302-B2, R&D Systems, Minneapolis, MN) for 24 and 48 hours for whole cell lysate (WCL) and conditioned medium (CM) studies.

RNA Isolation and PCR

Total RNA was isolated with TRIzol (5 Prime Inc, Gaithersburg, MD) and reverse transcribed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Life Science, Hercules, CA). PCR primers were designed using the National Center for Biotechnology Information (NCBI) primer blast tool program: PCOLCE1 Forward Primer (5'→3') CCCCAACTACACCAGACCCGT, PCOLCE1 Reverse Primer (5'→3')

TCCAGAGCATCGTAGCGGCA; PCOLCE2 Reverse Primer CTCCAGAGTCCCCGTCCGTC; β-actin Forward Primer (5'→3') CTGGAACGGTGAAGGTGACA, β-actin Reverse Primer (5'→3') AAGGGACTTCCTGTAACAATGCA. 1μg-2μg of cDNA was used in a reaction consisting of Go Taq DNA polymerase (M300B; Promega, Madison, WI), 5x PCR buffer (M791A; Promega), 25mM $MgCl_2$ (A351H; Promega), 10mM dNTP mix (U1515; Promega), dimethyl sulfoxide (BP231-100; Fisher Scientific, Pittsburg, PA) dH_2O, betaine, and 100 nM PCR primers. RT-PCR reactions were run in a PTC-100 thermal cycler (MJ Research, Inc., Ramsey, MN) with cycling parameters of initial denaturation at 95°C; 30 cycles of 95°C for 1min; 58°C for 38 seconds; and 72°C for 45 seconds. PCR amplified products were run on 2% agarose gels, stained with SYBR green and analyzed with the Fluorchem 8900 UVP system (Alpha Innotech, Logan, UT). β-actin was used as a loading control.

Western Blot Analysis of Whole Cell Lysate (WCL) and Conditioned Media (CM) Following TGF-β2 Treatment

WCL and CM were obtained from three normal primary TM cell strains and used for Western blot analysis with antibodies against PCOLCE1 and PCOLCE2 (Table 1). β-actin, was used to normalize protein loading. Whole cell lysate of whole human heart (Alpha Diagnostic HAL 1301. San Antonio, TX) was used as a positive control for both PCOLCE1 and PCOLCE2 protein expression. Induction of fibronectin (FN) was used as a positive control for TGFβ2 activity.

TM cells were treated with and without TGFβ2 (5ng/ml) for 24 and 48 hours. Total cellular protein was isolated from cultured TM cells using M-PER extraction buffer (#78501; Pierce Biotech, Rockford, IL) and Protease Inhibitor Cocktail (#78415; Pierce Biotech), and

StrataClean resin (400714; Agilent Technologies, Santa Clara, CA) was used to concentrate conditioned medium. Protein concentrations were determined using the Bio-Rad D_c protein assay for cell lysate. Denaturing SDS–polyacrylamide gel electrophoresis, separated protein samples. Proteins were transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated in 10% milk in Tris Buffered Saline Tween for 90 minutes to block nonspecific binding. Blots were processed using primary antibodies and appropriate secondary antibodies (Table 1). The Super Signal West Femto Maximus sensitivity substrate (#34095; Pierce Biotech) was used for protein detection using a FluroChem 8900 Imager (Alpha Innotech, San Leandro, CA). β-actin was used as a loading control for WCL samples. Fibronectin was used as a positive control for TGFβ2 induction and WCL of whole heart was used as a positive control for PCOLCE1 (50μg) and PCOLCE2 (20-30μg) expression.

Immunohistochemical Localization of PCOLCE1 and PCOLCE2 in TM Tissues

Six pairs of formalin fixed normal human eyes (74, 79, and 85 years of age for PCOLCE1; 79, 82, 92 years of age for PCOLCE2) were used to localize PCOLCE1 and PCOLCE2 in human TM tissues. Paraffin sections were deparaffinized, rehydrated and placed in antigen retrieval citrate buffer (pH 6.0) for 30 minutes followed by three PBS washes for 5 minutes each and then placed in 0.05M glycine for 15 minutes. Sections were incubated in 5% goat serum for 1 hour to block non-specific staining. Subsequently, sections were incubated at 4°C overnight with primary antibodies for PCOLCE1 or PCOLCE2 (Table 1). Secondary antibody staining was performed for 1 hour at room temperature with goat anti-rabbit Alexa 488 conjugated secondary antibody (Table 1). To visualize cell nuclei, tissue sections were stained with DAPI for 10 minutes. IgG and omission of no primary antibodies served as negative controls. Images were captured using the Zeiss LSM-410 confocal microscope and Eclipse Ti-U microscope (Nikon,

Melville, NY) containing the Nuance FX imaging system (CRI, Burlington, MA). Liver and prostate cancer tissue sections (Biomax Inc. Rockville, MD) were used as positive controls for PCOLCE1 and PCOLCE2 immunostaining respectively.

Results

PCOLCE1 and PCOLCE2 mRNA Expression in Human TM Cells

Transformed (n=2) and non-transformed primary (n=4) TM human TM cell strains were examined for PCOLCE1 and PCOLCE2 expression by RT-PCR. PCOLCE1 (207bp PCR amplimer) and PCOLCE2 (223bp PCR amplimer) were expressed in both transformed TM cell lines (Fig.1A). Additionally, PCOLCE1 was expressed in all four of the non-transformed human TM cell strains examined with faint expression of PCOLCE1 in one of the non-transformed cell lines (Fig. 1B lane 2). In contrast to PCOLCE1, only two out of the four non-transformed human TM cell strains expressed PCOLCE2 (Fig. 1B lanes 3 and 4).

Western Blot Analysis of PCOLCE1 and PCOLCE2 in TM Conditioned Media (CM)

Since PCOLCE1 and PCOLCE2 are secreted glycoproteins, we first wanted to determine whether these proteins were in CM from human TM cells. CM was collected from four normal primary TM cell strains cultured for 48 hours. An expected protein band at 48kDa for PCOLCE2 was seen in the CM of all four non-transformed human TM cell strains (Fig. 2A). Two additional non-specific bands appeared at 75kDa (Fig. 2A). An expected 48kDa band for PCOLCE2 was also observed in two out of the four non-transformed human TM cell strains. Additional bands at 34kDa and ~20kDa were also observed for PCOLCE2 (Fig 2B). Whole cell lysate (WCL) from human heart was used as a positive control for both PCOLCE1 and PCOLCE2. (Fig 2B).

Western Blot Analysis of PCOLCE1 and PCOLCE2 in TM WCL and CM following TGFβ2 Treatment

Since TGFβ2 is elevated in the AH of glaucoma patients and exogenous TGFβ2 induces BMP1 in human TM cells, we wanted to see if exogenous TGFβ2 also induces PCOLCE1 and PCOLCE2 protein expression. Three non-transformed human TM cell strains were treated with or without 5 ng/ml of TGFβ2 for 24 or 48 hours, and whole WCL as well as CM were collected. Inconsistent protein bands were seen in WCL for both PCOLCE1 and PCOLCE2 (data not shown). An expected protein band at 48kDa for PCOLCE1 was seen in the CM of all 3 primary human TM cell strains (Fig. 2.3A) Two additional non-specific bands appeared at 75kDa (Fig. 3A). WCL from human normal heart was used as a positive control for PCOLCE1 (Fig. 3A). Treatment of human TM cells with TGFβ2 (5 ng/ml) for 48 hours did increase PCOLCE1 protein levels in two of the three cell strains. Western blot analysis of CM for PCOLCE2 demonstrated protein bands of ~25kDa, 34kDa, and 48-50kDa in all 3 primary human TM cell strains (Fig. 3B). WCL from human heart was used as a positive control for PCOLCE2 and similar protein bands were also observed (Fig. 3B). Treatment of human TM cells with TGFβ2 (5 ng/ml) for 24 or 48 hours did not increase PCOLCE2 protein levels (Fig 3A and 3B). Fibronectin was used as a positive control to demonstrate that TGFβ2 was biologically active. TM cells were cultured with 5 ng/ml of TGFβ-2 for 24 or 48 hours. All three non-transformed human TM cells responded to TGFβ-2 with increased fibronectin protein levels at both time points (Fig. 3A and Fig. 3B).

Immunohistochemical Localization of PCOLCE1 and PCOLCE2 Proteins in Human TM Tissues

Representative images for PCOLCE1 and PCOLCE2 protein expression in human TM tissues were obtained from three normal human donor eyes. Liver cancer and prostate cancer tissue sections were used as positive controls for PCOLCE1 and PCOLCE2 immunostaining respectively. All sections were stained with DAPI to visualize cell nuclei. PCOLCE1 expression was associated with trabecular beams in two out of the three human TM tissues (Fig. 4B and Fig. 4D). No primary antibody and IgG were used as negative controls (Fig..4A and Fig. 4C). PCOLCE1 immunolocalization was observed in liver cancer tissue sections, which served as a positive control (Fig. 4E). There was no staining for PCOLCE1 in the negative control (Fig. 4F).

In comparison to PCOLCE1, PCOLCE2 protein expression was low in TM tissue sections. Staining for PCOLCE2 was diffused across the TM in one out of three normal donor eyes (Fig. 5B). There were no apparent differences in PCOLCE2 localization in the various regions of the human TM (Fig. 2.5D). No primary antibody and IgG were used as negative controls (Fig. 5A and Fig. 5C). PCOLCE2 immunolocalization was observed in prostate cancer tissue sections that served as the positive control (Fig. 5E). There was no staining for PCOLCE2 in the negative control (Fig. 5F).

Discussion

This study has demonstrated for the first time expression of PCOLCE1 and PCOLCE2 in human TM cells and tissues. Both PCOLCE1 and PCOLCE2 mRNA and proteins are expressed in human TM cells. Immunostaining analysis showed that both proteins are present in TM tissues from normal donor eyes.

Additionally, protein bands less than 37kDa that appeared in our western blot studies could represent processed forms of PCOLCE2 (e.g. 36kDa or 34kDa) as previously reported in the literature [25,46]. The appearance of these bands could result from (a) non-identical subunits of a dimeric protein, (b) distinct forms of a single chain protein that differs in chain length and glycosylation, (c) proteolytic fragments of the 48kDa form, or (d) a degraded product of the 36kDa protein [25,46]. The additional protein band observed at ~20/25kDa for PCOLCE2 could represent a splice variant of PCOLCE2 [47] with unknown protein function or be degradation product of PCOLCE2. Differences in protein bands for PCOLCE1 and PCOLCE2 in Western blot studies may be an indication of differential glycosylation as reported in a previous study [40].

Immunohistochemical results demonstrated that PCOLCE1 is present along the TM beams. This observation agrees with what was seen in previous studies that identified PCOLCE1 associated with fibrillar-collagen in interstitial connective tissues [40,48]. The presence of PCOLCE1 along or within trabecular meshwork beams suggests that PCOLCE1 can play an important role in the excess collagen deposition in TM of glaucoma patients. Further studies would have to be conducted to support this role in excess collagen deposition and pathogenesis of glaucoma. In contrast to PCOLCE1, PCOLCE2 was diffuse within the TM agreeing with a previous study that showed that PCOLCE2 has a higher affinity for heparan sulfate than PCOLCE1 suggesting that it is it closely associated with the cell surface [40]. In a previous study, PCOLCE2 was also found to be a collagen-associated glycoprotein, [40] thus suggesting that it may also play an important role in pathogenesis of glaucoma. Additional studies would have to confirm its role in this disease.

Exogenous treatment with TGFβ2 at 5 ng/ml for 48 hours induced PCOLCE1 but not PCOLCE2 protein expression in CM of TM cells. Low expression of PCOLCE2 in TM tissue and no

increased expression of PCOLCE2 by TGFβ2 may imply that PCOLCE2 plays a less prominent role than PCOLCE1. Increased expression of PCOLCE1 by TGFβ2 may lead to increase facilitation of procollagen processing via enhancement of BMP1 activity, resulting in excess extracellular collagen deposition thereby leading to increase outflow resistance and elevated IOP (Fig. 2.6). Taken together, our results demonstrate PCOLCE1 and PCOLCE2 mRNA and protein expression in human TM cells and tissues. Further studies are needed to evaluate their role in pathogenesis of glaucoma.

Figure Legends

Figure 1: mRNA expression of PCOLCE1 and PCOLCE2 in human TM cells (A) SYBR green stained gels of RT-PCR products for biological triplicates of PCOLCE1, PCOLCE2, and β-actin from normal (HTM5) and glaucomatous (GTM3) transformed TM cell lines (B) SYBR green stained gels of RT-PCR products for PCOLCE1, PCOLCE2, and β-actin from normal primary human TM cell strains (n=4).

Figure 2: Protein expression of PCOLCE1 and PCOLCE2 in primary human TM cells conditioned media. (A) PCOLCE1 expression (48kDa) in normal TM cell strains (n=4). (B) PCOLCE2 expression (48 kDa) in normal TM cell strains (n=4) . (+) Human normal heart cell lysate served as a positive control for both PCOLCE1 and PCOLCE2.

Figure 3: Protein expression of PCOLCE1 and PCOLCE2 at 24 and 48 hours post TGFβ2 treatment in conditioned medium from primary human TM cells. (A) Representative Western immunoblot image of PCOLCE1 expression in CM of primary TM cells treated with 5ng/ml human recombinant TGFβ2 for 24 and 48 hours (n=3). (B) Representative western immunoblot

image of PCOLCE2 expression in CM of primary TM cells treated with 5ng/ml human recombinant TGFβ2 for 24 and 48 hours (n=3). (+) Human normal heart lysate served as a positive control (+) for PCOLCE1 and PCOLCE2. FN= Fibronectin positive control for TGFβ2 treatment (5ng/ml) at 24 and 48 hours.

Figure 4: Representative Image of PCOLCE1 localization in normal human TM tissue (A) No Primary antibody served as a negative control for TM. (B) Merged PCOLCE1 and DAPI nuclei staining in normal TM tissue. TM (Trabecular meshwork). SC (Schlemm's canal). (C) Enlarged area of A. (D) Enlarged area of B showing PCOLCE1 localization in normal TM tissue. (E) No primary antibody served as a negative control of human liver cancer tissue (F) Human liver cancer tissue served as a positive control for PCOLCE1 immunolocalization. Scale bar (100μm).

Figure 5: Representative Image of PCOLCE2 localization in normal human TM tissue. (A) No Primary antibody served as a negative control for TM. (B) Merged PCOLCE2 and DAPI nuclei staining in normal TM tissue. TM (Trabecular meshwork). SC (Schlemm's canal). (C) Enlarged area A. (D) Enlarged area of B showing PCOLCE2 localization in normal TM tissue. (E) No primary antibody served as a negative control of human prostate cancer tissue (F) Human prostate cancer served as a positive control for PCOLCE2 immunolocalization. Scale bar (100μm).

Figure 6: A proposed mechanism for PCOLCE 1 and 2 in processing of procollagen and collagen accumulation in the glaucomatous TM.

Table 1: List of Antibodies used

Antibody	Catalog number	Antibody dilution for Western Blot	Antibody dilution for IHC	Source
Anti-PCOLCE 1	ab39204	1 to 3000	…….	Abcam
Anti-PCOLCE 2	ab28345	1 to 1000	…….	Abcam
β-actin mouse monoclonal	MAB1501	1 to 1000	…….	Millipore
Rb X Human Fibronectin	AB1945	1 to 1000	…….	Millipore
anti-rabbit IgG HRP	70745	1 to 10 000	…….	Cell Signaling
Stabilized peroxidase conjugated goat anti-mouse	32430	1 to 10 000	…….	Thermo Scientific
PCOLCE 1	14993-1-AP	…….	1 to 100	Protein tech Inc
PCOLCE 2	10607-1-AP	…….	1 to 50	Protein tech Inc
Goat anti-rabbit Alexa-Fluor 488	A11008	…….	1 to 200	Molecular Probes
Rabbit IgG I-1000	U0304	……. ……	1.6mg/mL (PCOLCE1) 2.47mg/mL (PCOLCE2)	Vector Labs

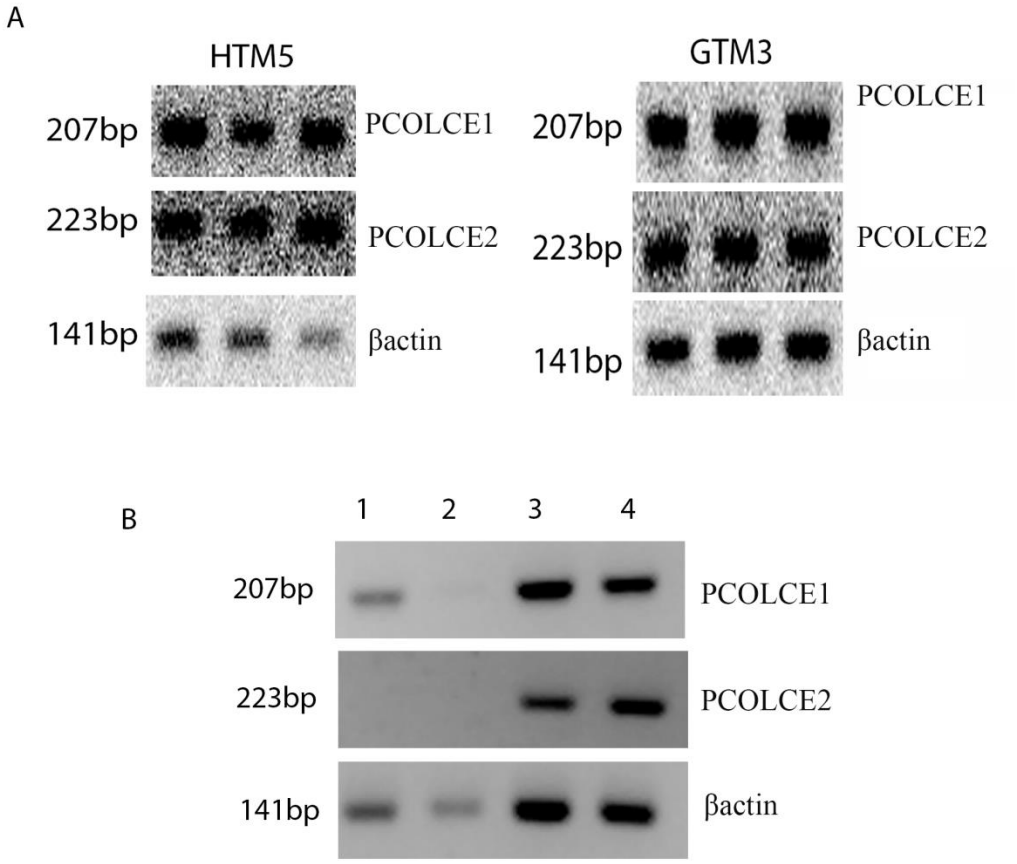

Figure 1

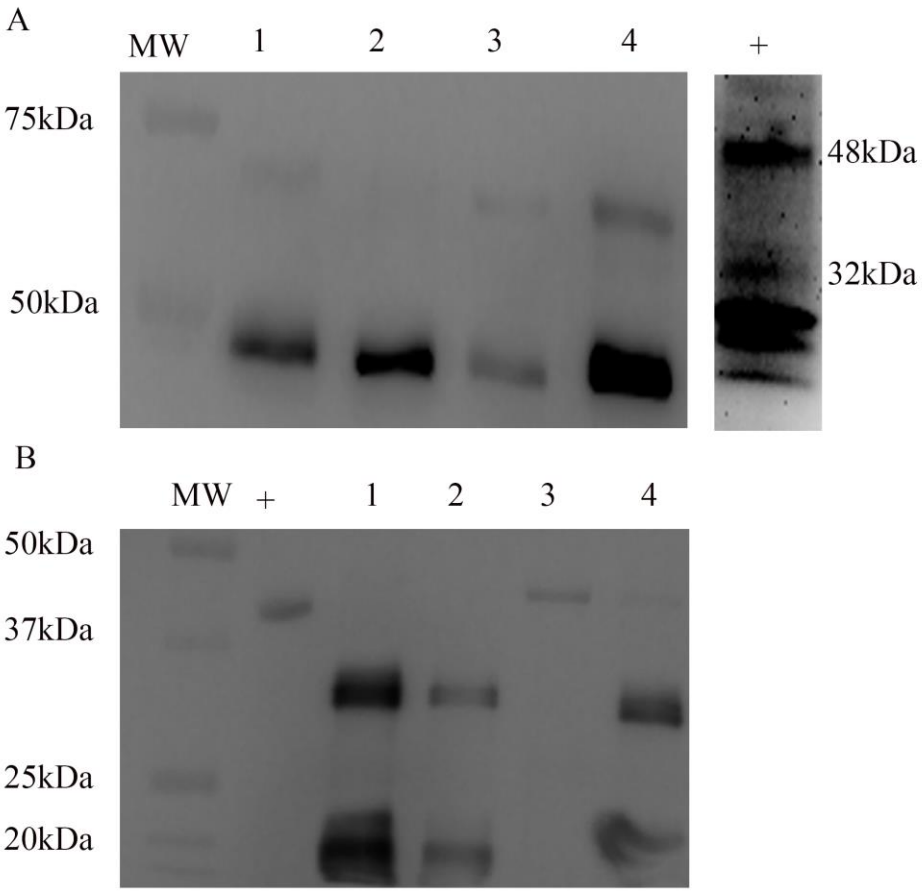
A
MW
1
2
3
4
+
75kDa
50kDa
48kDa
32kDa
B
MW
+
1
2
3
4
50kDa
37kDa
25kDa
20kDa

Figure 2

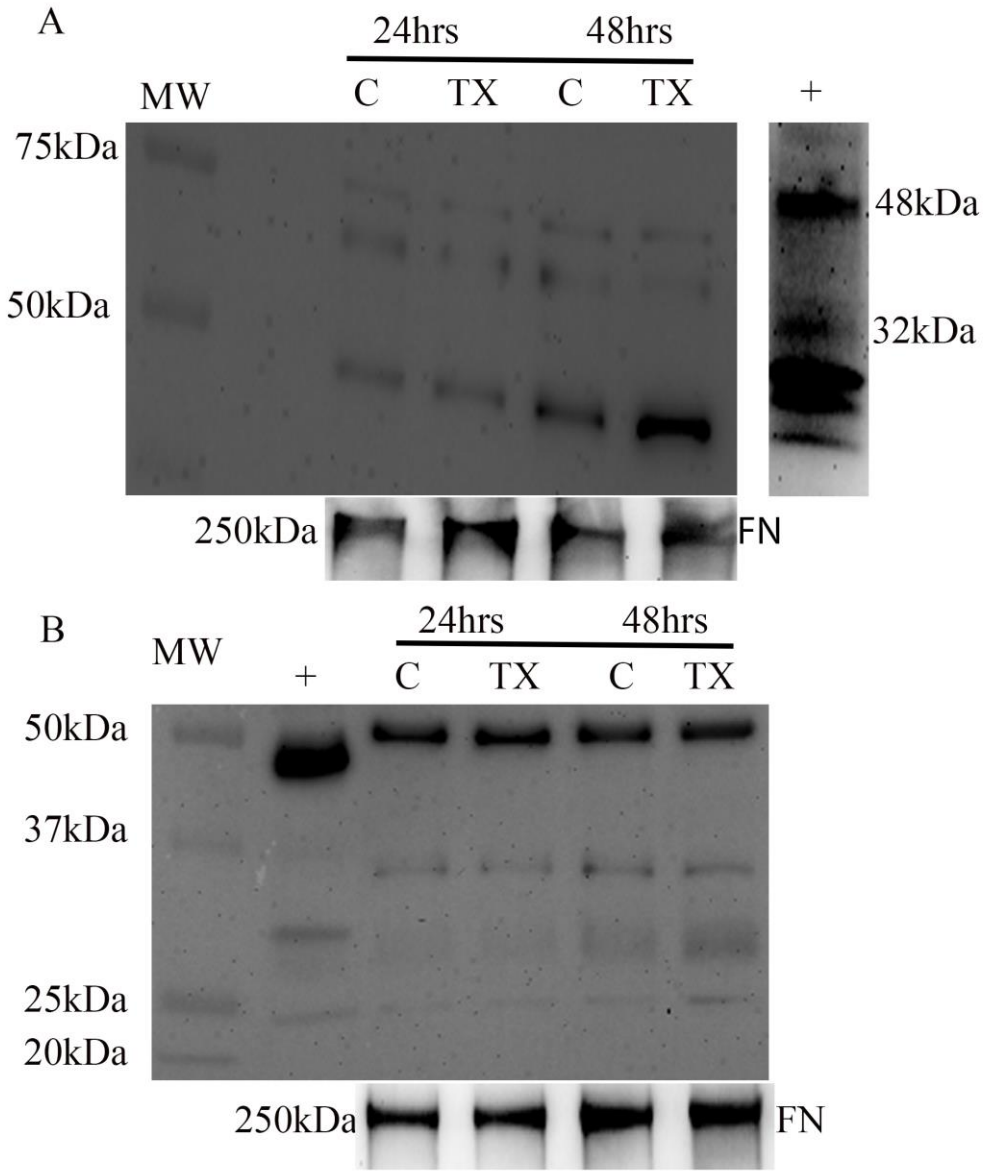
A
24hrs
48hrs
MW
C
TX
C
TX
+
75kDa
50kDa
48kDa
32kDa
250kDa
FN
B
MW
+
24hrs
48hrs
C
TX
C
TX
50kDa
37kDa
25kDa
20kDa
250kDa
FN

Figure 3

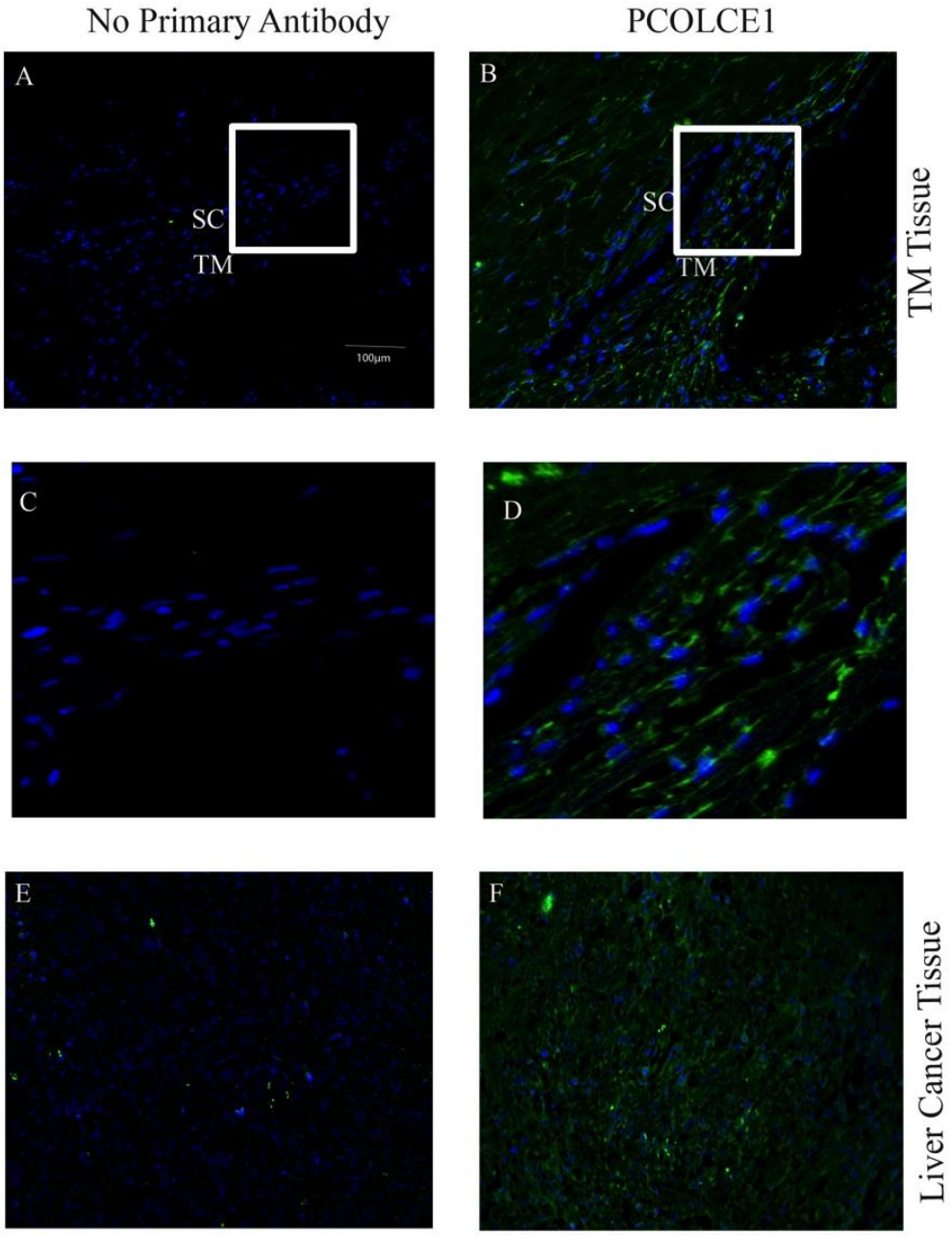
No Primary Antibody
PCOLCE1
A
B
SC
TM
100μm
SC
TM
TM Tissue
C
D
E
F
Liver Cancer Tissue

Figure 4

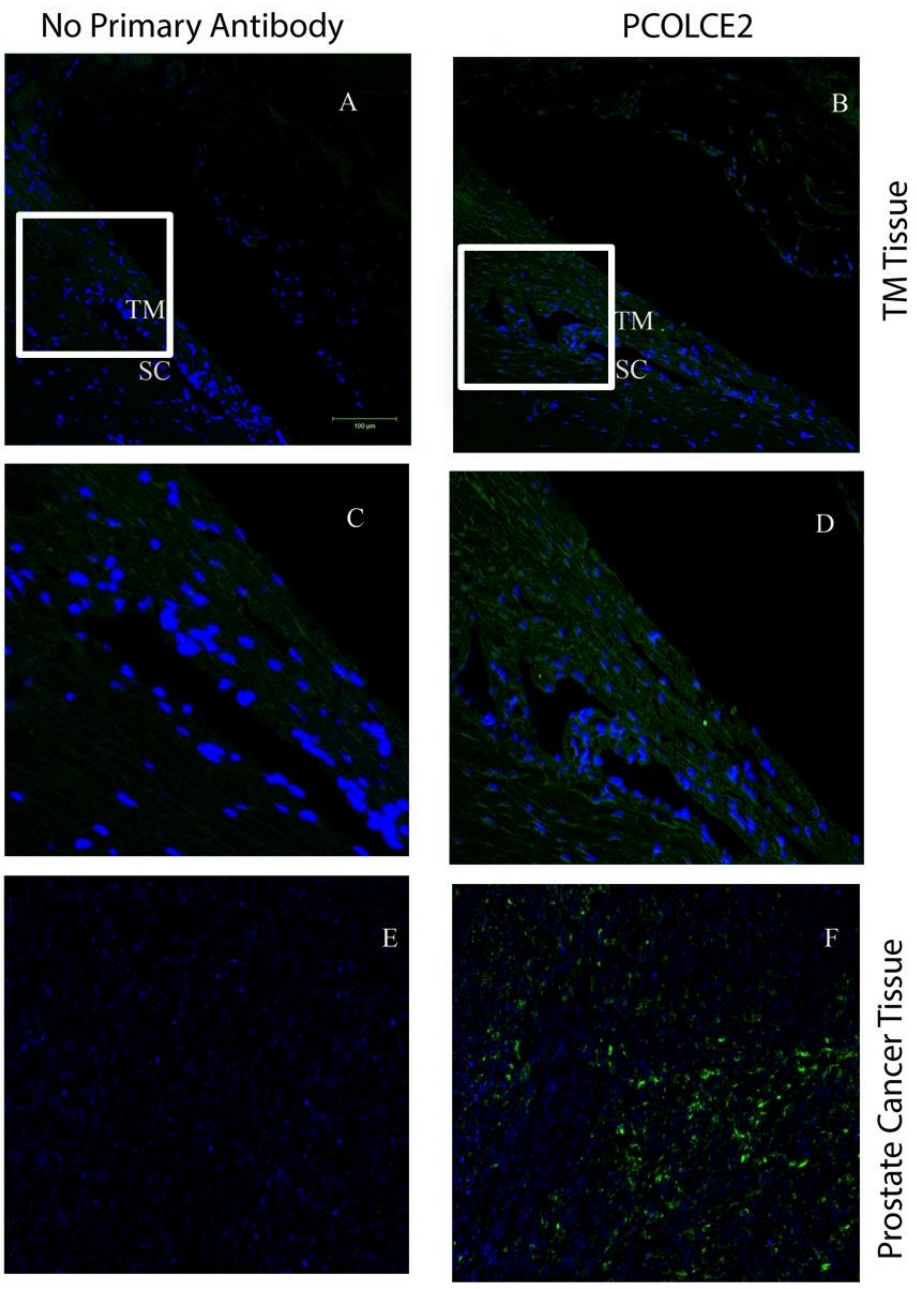
No Primary Antibody
PCOLCE2
A
B
TM
SC
TM
SC
TM Tissue
C
D
E
F
Prostate Cancer Tissue

Figure 5

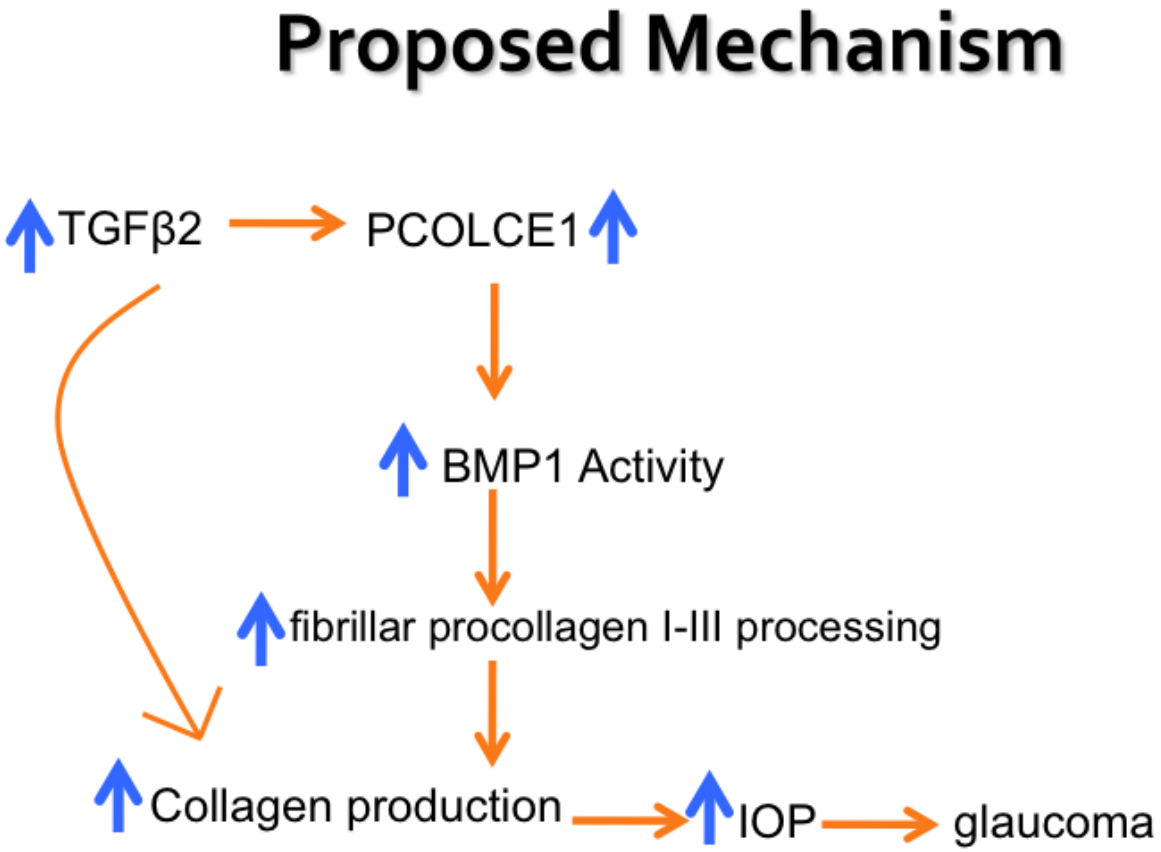
Proposed Mechanism
TGFβ2
PCOLCE1
BMP1 Activity
fibrillar procollagen I-III processing
Collagen production
IOP
glaucoma

Figure 6

References

1. Quigley HA. Glaucoma. *Lancet*. 2011;377(9774):1367-1377.

2. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog Retin Eye Res*. 1999;18(1):91-119.

3. Johnson M. 'What controls aqueous humour outflow resistance?'. *Exp Eye Res*. 2006;82(4):545-557. doi: 10.1016/j.exer.2005.10.011.

4. Ethier CR. The inner wall of schlemm's canal. *Exp Eye Res*. 2002;74(2):161-172. doi: 10.1006/exer.2002.1144.

5. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res*. 1994;59(6):723-727.

6. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol*. 2001;239(2):109-113.

7. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol*. 2002;46(3):249-253.

8. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol*. 2004;25(1):19-22.

9. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol*. 2001;239(3):199-207.

10. Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGFbeta/smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci*. 2011;52(8):5251-5259. doi: 10.1167/iovs.11-7587; 10.1167/iovs.11-7587.

11. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (LOX) genes in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2011;52(8):5240-5250. doi: 10.1167/iovs.11-7287; 10.1167/iovs.11-7287.

12. Zhao X, Ramsey KE, Stephan DA, Russell P. Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 2004;45(11):4023-4034. doi: 10.1167/iovs.04-0535.

13. Fuchshofer R, Welge-Lussen U, Lutjen-Drecoll E. The effect of TGF-beta2 on human trabecular meshwork extracellular proteolytic system. *Exp Eye Res*. 2003;77(6):757-765.

14. Fuchshofer R, Tamm ER. The role of TGF-beta in the pathogenesis of primary open-angle glaucoma. *Cell Tissue Res*. 2012;347(1):279-290. doi: 10.1007/s00441-011-1274-7; 10.1007/s00441-011-1274-7.

15. Tovar-Vidales T, Roque R, Clark AF, Wordinger RJ. Tissue transglutaminase expression and activity in normal and glaucomatous human trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2008;49(2):622-628. doi: 10.1167/iovs.07-0835; 10.1167/iovs.07-0835.

16. Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor-{beta}2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51(4):2067-2076. doi: 10.1167/iovs.09-4567; 10.1167/iovs.09-4567.

17. Bachmann B, Birke M, Kook D, Eichhorn M, Lutjen-Drecoll E. Ultrastructural and biochemical evaluation of the porcine anterior chamber perfusion model. *Invest Ophthalmol Vis Sci*. 2006;47(5):2011-2020. doi: 10.1167/iovs.05-1393.

18. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: Implications for intraocular pressure. *Invest Ophthalmol Vis Sci*. 2006;47(1):226-234. doi: 10.1167/iovs.05-1060.

19. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004;45(1):153-158.

20. Wozney JM, Rosen V, Celeste AJ, et al. Novel regulators of bone formation: Molecular clones and activities. *Science*. 1988;242(4885):1528-1534.

21. Urist MR. Bone: Formation by autoinduction. *Science*. 1965;150(3698):893-899.

22. Van de Putte KA, Urist MR. Osteogenesis in the interior of intramuscular implants of decalcified bone matrix. *Clin Orthop Relat Res*. 1965;43:257-270.

23. Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS. Bone morphogenetic protein-1: The type I procollagen C-proteinase. *Science*. 1996;271(5247):360-362.

24. Li SW, Sieron AL, Fertala A, Hojima Y, Arnold WV, Prockop DJ. The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci U S A*. 1996;93(10):5127-5130.

25. Kessler E, Adar R. Type I procollagen C-proteinase from mouse fibroblasts. purification and demonstration of a 55-kDa enhancer glycoprotein. *Eur J Biochem*. 1989;186(1-2):115-121.

26. Canty EG, Lu Y, Meadows RS, Shaw MK, Holmes DF, Kadler KE. Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. *J Cell Biol*. 2004;165(4):553-563. doi: 10.1083/jcb.200312071.

27. Prockop DJ, Kivirikko KI. Collagens: Molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem*. 1995;64:403-434. doi: 10.1146/annurev.bi.64.070195.002155.

28. Ge G, Zhang Y, Steiglitz BM, Greenspan DS. Mammalian tolloid-like 1 binds procollagen C-proteinase enhancer protein 1 and differs from bone morphogenetic protein 1 in the functional roles of homologous protein domains. *J Biol Chem*. 2006;281(16):10786-10798. doi: 10.1074/jbc.M511111200.

29. Petropoulou V, Garrigue-Antar L, Kadler KE. Identification of the minimal domain structure of bone morphogenetic protein-1 (BMP-1) for chordinase activity: Chordinase activity is not enhanced by procollagen C-proteinase enhancer-1 (PCPE-1). *J Biol Chem*. 2005;280(24):22616-22623. doi: 10.1074/jbc.M413468200.

30. Takahara K, Kessler E, Biniaminov L, et al. Type I procollagen COOH-terminal proteinase enhancer protein: Identification, primary structure, and chromosomal localization of the cognate human gene (PCOLCE). *J Biol Chem*. 1994;269(42):26280-26285.

31. Maruhashi T, Kii I, Saito M, Kudo A. Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase. *J Biol Chem*. 2010;285(17):13294-13303. doi: 10.1074/jbc.M109.088864; 10.1074/jbc.M109.088864.

32. Inomata H, Haraguchi T, Sasai Y. Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation. *Cell*. 2008;134(5):854-865. doi: 10.1016/j.cell.2008.07.008; 10.1016/j.cell.2008.07.008.

33. Scott IC, Blitz IL, Pappano WN, Maas SA, Cho KW, Greenspan DS. Homologues of twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. *Nature*. 2001;410(6827):475-478. doi: 10.1038/35068572.

34. Vadon-Le Goff S, Kronenberg D, Bourhis JM, et al. Procollagen C-proteinase enhancer stimulates procollagen processing by binding to the C-propeptide region only. *J Biol Chem*. 2011;286(45):38932-38938. doi: 10.1074/jbc.M111.274944; 10.1074/jbc.M111.274944.

35. Goldsmith EC, Bradshaw AD, Spinale FG. Contributory pathways leading to myocardial fibrosis - moving beyond collagen expression. *Am J Physiol Cell Physiol*. 2012. doi: 10.1152/ajpcell.00347.2012.

36. Baicu CF, Zhang Y, Van Laer AO, Renaud L, Zile MR, Bradshaw AD. Effects of the absence of procollagen C-endopeptidase enhancer-2 on myocardial collagen accumulation in chronic pressure overload. *Am J Physiol Heart Circ Physiol*. 2012;303(2):H234-40. doi: 10.1152/ajpheart.00227.2012; 10.1152/ajpheart.00227.2012.

37. Kessler-Icekson G, Schlesinger H, Freimann S, Kessler E. Expression of procollagen C-proteinase enhancer-1 in the remodeling rat heart is stimulated by aldosterone. *Int J Biochem Cell Biol*. 2006;38(3):358-365. doi: 10.1016/j.biocel.2005.10.007.

38. Ogata I, Auster AS, Matsui A, et al. Up-regulation of type I procollagen C-proteinase enhancer protein messenger RNA in rats with CCl4-induced liver fibrosis. *Hepatology*. 1997;26(3):611-617. doi: 10.1002/hep.510260312.

39. Moali C, Font B, Ruggiero F, et al. Substrate-specific modulation of a multisubstrate proteinase. C-terminal processing of fibrillar procollagens is the only BMP-1-dependent activity to be enhanced by PCPE-1. *J Biol Chem*. 2005;280(25):24188-24194. doi: 10.1074/jbc.M501486200.

40. Steiglitz BM, Keene DR, Greenspan DS. PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1. *J Biol Chem*. 2002;277(51):49820-49830. doi: 10.1074/jbc.M209891200.

41. Xu H, Acott TS, Wirtz MK. Identification and expression of a novel type I procollagen C-proteinase enhancer protein gene from the glaucoma candidate region on 3q21-q24. *Genomics*. 2000;66(3):264-273. doi: 10.1006/geno.2000.6229.

42. Williamson RA, Panagiotidou P, Mott JD, Howard MJ. Dynamic characterisation of the netrin-like domain of human type 1 procollagen C-proteinase enhancer and comparison to the N-terminal domain of tissue inhibitor of metalloproteinases (TIMP). *Mol Biosyst*. 2008;4(5):417-425. doi: 10.1039/b717901d; 10.1039/b717901d.

43. Bekhouche M, Kronenberg D, Vadon-Le Goff S, et al. Role of the netrin-like domain of procollagen C-proteinase enhancer-1 in the control of metalloproteinase activity. *J Biol Chem*. 2010;285(21):15950-15959. doi: 10.1074/jbc.M109.086447; 10.1074/jbc.M109.086447.

44. Adar R, Kessler E, Goldberg B. Evidence for a protein that enhances the activity of type I procollagen C-proteinase. *Coll Relat Res*. 1986;6(3):267-277.

45. Ricard-Blum S, Bernocco S, Font B, et al. Interaction properties of the procollagen C-proteinase enhancer protein shed light on the mechanism of stimulation of BMP-1. *J Biol Chem*. 2002;277(37):33864-33869. doi: 10.1074/jbc.M205018200.

46. Kessler E, Adar R, Goldberg B, Niece R. Partial purification and characterization of a procollagen C-proteinase from the culture medium of mouse fibroblasts. *Coll Relat Res*. 1986;6(3):249-266.

47. The human protein atlas. http://www.proteinatlas.org/search/pcolce2. Accessed 2/10/2013, 2013.

48. Kessler E, Mould AP, Hulmes DJ. Procollagen type I C-proteinase enhancer is a naturally occurring connective tissue glycoprotein. *Biochem Biophys Res Commun*. 1990;173(1):81-86.

CHAPTER III: CONCLUSION AND FUTURE DIRECTIONS

Conclusion

In conclusion, our results indicate that PCOLCE1 and PCOLCE2 are expressed in normal human TM cells and tissues. This baseline information in the normal TM is important in order to examine their expression in glaucomatous TM cells and tissues to determine if their expression is altered. If this is true, then we can investigate their role in regulation of BMP1 activity, the processing of procollagen to collagen and collagen accumulation in the glaucomatous TM, we will also study their effects in regulating IOP to determine whether these proteins are important players in pathogenesis of glaucoma. This then will expand our current knowledge of the molecular mechanism of the pathogenesis of glaucoma in the TM and can allow for the development of new therapeutic targets for the TM so that better treatment can be provided to glaucoma patients. The following are some of the potential future studies to address these concerns and questions.

Limitations

While the current studies focused on the expression of PCOLCE1 and PCOLCE2 in TM cells and tissues and whether or not TGFβ2 increases their expression, there are some limitations that need to be addressed for this study.

The first limitation is that the current study only looked at expression of PCOLCE1 and PCOLCE2 in normal TM cells and tissues, but not at their expression in glaucomatous TM cells and tissues. Therefore, studies should be conducted to determine if PCOLCE1 and PCOLCE2 expression are increased in glaucomatous TM cells and tissue so their roles in pathogenesis of glaucoma can then be evaluated. A second limitation in this study is associated with the

immunolocalization of PCOLCE1 and PCOLCE2 in normal TM tissue. To make a comparison of which protein (PCOLCE1 or PCOLCE2) has greater localization in TM tissue, factors need to be taken into consideration which include the following: antibody affinity for PCOLCE1 or PCOLCE2 in the TM tissue and area/region of TM that is examined as the TM extends around the anterior segment of the eye. To support the idea that PCOLCE1 or PCOLCE2 is greater in localization in TM tissue, TM tissue can be extracted for western immunoblot studies and densitometry analysis. Another way to measure protein level expression is with immunostaining that will focus on the different area/regions in the TM. These TM regions would then be averaged for PCOLCE1 or PCOLCE2 protein expression and thus can indicate that PCOLCE1 and/or PCOLCE2 is more prominent in TM tissue and if the role of these proteins is worth evaluating in glaucoma pathogenesis.

Another limitation with the immunostaining is determining where exactly PCOLCE1 or PCOLCE2 is localized within the TM tissue. Even though it may appear that PCOLCE1 is within or along the TM beams and PCOLCE2 is diffused in the TM, the immunostaining does not allow precise localization in TM tissue. Thus PCOLCE1 or PCOLCE2 localization cannot be exactly pinpointed. Therefore, immunolocalization at the electron microscopy level should be used to determine precise PCOLCE1 or PCOLCE2 localization in the TM tissue.

The last limitation in this study is associated with TGFβ2 induction on PCOLCE1 or PCOLCE2 expression. TGFβ2 induction on PCOLCE1 or PCOLCE2 expression was only studied at the protein but not at the mRNA level. Therefore, mRNA studies at different time points with treatment of 5ng/ml of TGFβ2 in TM cells can be conducted to further support TGFβ2 induction on PCOLCE1 or PCOLCE2 expression.

Future Studies

Expression of PCOLCE1/PCOLCE2 in glaucomatous TM cells and tissues

To confirm expression of PCOLCE1/PCOLCE 2 in glaucomatous TM cells and tissues, glaucomatous TM cells and tissues will be obtained for mRNA and protein studies as well as immunohistochemistry studies (see methods section in Chapter 2). A comparison will be made between normal and glaucomatous TM cells and tissues to determine if PCOLCE1/PCOLCE2 expression is altered in glaucomatous TM cells and tissues.

Overexpression of PCOLCE1/PCOLCE2 in human TM cells

To study the role of PCOLCE1 and PCOLCE2 in the regulation of BMP1 activity, it is important to (a) generate a transient TM cell line that overexpresses PCOLCE1 or PCOLCE2 and (b) establish a siRNA knockdown of these genes. For overexpression studies, human TM cells will be transfected with commercially available Green Fluorescent Protein (GFP) tagged mammalian vector pCMV6-AC-GFP-PCOLCE 1 or with pCMV6-AC-GFP-PCOLCE2 from OriGene (Technologies Rockville, MD) using an empty mammalian vector. To confirm that the transfected cells are overexpressing PCOLCE1 or PCOLCE2 successfully, conditioned medium (CM) will be obtained and be used for immunoblotting studies with an anti-tGFP specific antibody from Origene which will then be visualized using the Fluor Chem™ 8900 imager (Alpha Innotech, San Leandro, CA) using ECL detection reagents (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology).

Knockdown of PCOLCE1/PCOLCE2 in human TM cells

For knockdown studies, siRNA of PCOLCE1 or PCOLCE2will be purchased from Origene. A Trilencer-27 Universal Scrambled siRNA will serve as a negative control. Fluorescent-labeled duplexes such as HPRT siRNA (for demonstration of successful suppression of a control gene using a known effective siRNA) will be used as a positive control at a concentration range of 0.1 to 50 nM will be used to determine maximum knockdown.

For confirmation of maximum knockdown of PCOLCE1 or PCOLCE2, cell lysates will be obtained and be used for WB analysis to verify the functionality of the knockdown protein expression of these proteins. In addition RNA will be harvested from transfected cells and be used in quantitative RT-PCR to determine the loss of gene expression of PCOLCE1 or PCOLCE2.

Regulation of BMP1 Activity in human TM cells

Next, enhancement of BMP1 activity would be measured using a BMP1 activity assay (Recombinant BMP1 protein (R&D Systems) and a fluorogenic peptide substrate Mca-Y-V-A-D-A-P-K (Dnp)-OH (R&D Systems)). Supernatant will be collected from both overexpression and knockdown studies of PCOLCE1 or PCOLCE2 and will be used for this assay. These studies would confirm that overexpression of PCOLCE1 or PCOLCE2 will lead to an increase in BMP1 activity. Increased BMP1 activity cleaves the fluorogenic substrate and results in an increase in fluorescence. In the knockdown studies, there should be a decrease in BMP1 activity leading to decreased cleavage of the fluorogenic substrate and ultimately a decrease in fluorescence.

Procollagen Processing and Collagen Deposition

To understand procollagen processing in human TM cells, CM will be collected from overexpression/knockdown experiments and will be used for WB studies against C propeptide or the anti-α1 (I) C-telopeptide region of fibrillar procollagens (I-III). If it is found that there is increase in BMP1 activity, then we should expect increase in procollagen processing. If there were an increase in procollagen processing, ultimately that would lead to accumulation of collagen in the ECM of the TM, similar to what is seen in glaucoma. Therefore, to measure collagen deposition content in vitro, the sircol collagen assay will be used and instructions will be followed as provided by Biocolor Ltd (United Kingdom).

Ex-vivo examination of the role of PCOLCE1 or PCOLCE2 in regulating IOP

Perfusion-cultured human anterior segments have been used as an *ex vivo* model to study glucocorticoid-induced glaucoma [1-3]. This model would be appropriate to examine the effect of recombinant proteins PCOLCE1 or PCOLCE2 in regulating IOP since it has been found that both proteins are expressed in human TM cells and tissues [4].

The perfused bovine anterior segment POC model has been previously described [4]. Fresh bovine eyes will be dissected and sealed on a custom-made acrylic dish with an O-ring. Perfusion medium will be infused by a syringe pump at a constant infusion rate of 2.5 μL/min. The human anterior segments will then be perfusion cultured for one to three days until the IOP becomes stable. Then recombinant proteins PCOLCE1 or PCOLCE2 will be infused into human eyes. Control eyes will be transduced with PBS pH7.4-7.5. The other way that PCOLCE1 or PCOLCE2 can be introduced into human anterior segments in the POC model is via (a) overexpression of PCOLCE1 or PCOLCE2 or (b) knockdown of PCOLCE1 or PCOLCE2. In the

overexpression study, one eye will be transduced with Ad5PCOLCE1 or Ad5PCOLCE2 while the contralateral eye will be induced with an empty viral vector. IOP will be monitored and then TM tissue will be obtained from the eyes and be analyzed for changes in BMP1 expression as well fibrillar collagen (type I-III) deposition via immunohistochemistry and western blot. For the knockdown study, one eye will be transduced with Ad5-siRNAPCOLCE1 or Ad5-siRNAPCOLCE2, while the contralateral eye will be transduced with empty viral vector. IOP will then be measured and then TM tissue will be obtained from the eyes and analyzed for changes in BMP1 expression as well as fibrillar collagens (type I-III) deposition via immunohistochemistry and western blot.

The proposed studies will give us a better understanding of the role of PCOLCE1 or PCOLCE2 in the regulation of BMP1 activity, procollagen processing and IOP in TM cells and tissues and will offer more insight into ECM structural changes, (i.e. collagen deposition), increased outflow resistance and elevated IOP observed in glaucoma.

References

1. Clark AF, Steely HT, Dickerson JE,Jr, et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2001;42(8):1769-1780.

2. Clark AF, Wilson K, de Kater AW, Allingham RR, McCartney MD. Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci*. 1995;36(2):478-489.

3. Johnson DH, Bradley JM, Acott TS. The effect of dexamethasone on glycosaminoglycans of human trabecular meshwork in perfusion organ culture. *Invest Ophthalmol Vis Sci*. 1990;31(12):2568-2571.

4. Mao W, Tovar-Vidales T, Yorio T, Wordinger RJ, Clark AF. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. *Invest Ophthalmol Vis Sci*. 2011;52(11):8068-8075. doi: 10.1167/iovs.11-8133; 10.1167/iovs.11-8133.