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EVIDENCE FOR A CALCIFICATION PROCESS IN THE TRABECULAR MESHWORK

Teresa Borrás and Núria Comes

Department of Ophthalmology, University of North Carolina School of Medicine, Chapel Hill, NC

Abstract

The human trabecular meshwork (TM) expresses many genes that have been associated with physiological (bone, cartilage, teeth) and pathological (vascular systems, kidney) calcification. In particular, the TM highly expresses the inhibitor of calcification Matrix Gla (MGP) gene, which encodes a vitamin K-dependent protein that requires post-translational activation to inhibit the formation of calcium precipitates. TM cells have high activity of the activating γ -carboxylase enzyme and produce active MGP. Silencing MGP increases the activity of alkaline phosphatase (ALP), an enzyme of the matrix vesicles and marker of calcification. Overexpressing MGP reduces the ALP activity induced by bone morphogenetic 2 (BMP2), a potent inducer of calcification. In this review we gathered evidence for the existence of a mineralization process in the TM. We selected twenty regulatory calcification genes, reviewed their functions in their original tissues and looked at their relative abundance in the TM by heat maps derived from existing microarrays. Although results are not yet fully conclusive and more experiments are needed, examining TM expression in the light of the calcification literature brings up many similarities. One such parallel is the role of mechanical forces in bone induction and the high levels of mineralization inhibitors found in the constantly mechanically stressed TM. During the next few years, examination of other calcification-related regulatory genes and pathways, as well as morphological examination of knockout animals would help elucidate the relevance of a calcification process to TM overall function.

Keywords

Human trabecular meshwork; Perfused anterior segments, primary HTM cells, Calcification genes; Microarrays; Heat maps

1. Introduction

The trabecular meshwork (TM) is a soft spongiform tissue whose primary function is that of maintaining a physiological resistance to the flow of aqueous humor. Failure to regulate such resistance results in an elevation of intraocular pressure (IOP), the major risk factor for the development of glaucoma (Kass et al., 2002). The TM has also a very unique architecture. It contains cells, beams and fibrils, open extracellular spaces and extracellular material, all arranged in a characteristic flexible layer-like structure. Maintenance of the softness nature of

*Corresponding author: Teresa Borrás, Ph.D., Department of Ophthalmology, University of North Carolina School of Medicine, 6109 Neuroscience Research Building CB 7041, 103 Mason Farm Road, Chapel Hill, NC 27599-7041, Tel. 919-843-0184, Fax. 919-843-0749, e-mail: E-mail: tborras@med.unc.edu.

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such structure is of highest relevance to the TM's function. For that, the TM needs to have in place molecular mechanisms that would maintain the physical properties of elasticity, tension and softness.

The aqueous humor flows continuously in the anterior chamber and exits the eye wandering in between the TM's cells and extracellular matrix (ECM). Because of this continuous flow, the TM is the target of numerous biochemical and mechanical factors, which in turn trigger differential expression of its genes. The TM transcriptome is quite diverse and reflects the variety of mechanisms used by the tissue to counteract insults and ensure a physiological outflow facility. Genes expressed in the TM reveal that secretion, cell-matrix interactions, cytoskeletal reorganization and especially, ECM functions are key for its proper operation.

Expression of genes that affect ECM properties, in particular those affecting calcification in other tissues has been observed in the TM (Borrás, 2008a; Borrás, 2008b; Gonzalez et al., 1999; Gonzalez et al., 2000; Vittitow and Borrás, 2004). The most striking similarity is the presence of the gene encoding the inhibitor of calcification Matrix Gla (MGP) which was shown to be one of the highest expressed genes in the tissue (Borrás, 2008a). The fact that MGP has been confirmed as an inhibitor of calcification (Proudfoot and Shanahan, 2006) and that overexpression and silencing of this gene in the TM results in alterations of calcification markers (Xue et al., 2006; Xue et al., 2007) is a strong indication that biomineralization processes might be ongoing in this tissue and need to be counteracted.

Mineralization is the result of the formation of a calcium-phosphate precipitate (hydroxyapatite crystal). Formation of the first hydroxyapatite crystal takes place in matrix vesicles which are generated in the plasma membrane of the cells and released upon uploading calcium and Pi (Anderson, 2003). The matrix vesicles contain alkaline phosphatase (ALP) activity which produces a local increase of free phosphate and results into the formation of the apatite crystal (Ali et al., 1970). In the ECM, the matrix vesicle releases the crystal to the extracellular compartment where it gets deposited on collagen fibrils and continues to grow by a thermodynamic process using the first hydroxyapatite crystal as a template (Anderson, 2003; Yang et al., 2004).

Contrary to the original belief that calcification was due to spontaneous precipitation of hydroxyapatite, it is now known that mineralization is a highly regulated process (Steitz et al., 2001). Physiological precipitation of calcium-phosphate crystals occur in the ECM of bone, cartilage and teeth. The mineralization process is controlled by local cells via the secretion of mineral-binding ECM proteins and proteoglycans, the transport of inorganic phosphate to the extracellular compartment, and by enzymes of the alkaline phosphatase family, which can also degrade mineralization inhibitors (Addison et al., 2007; Giachelli, 2005).

Calcification of soft tissues occurs during pathological conditions and has important clinical implications. Ectopic calcifications are well known to occur in cardiovascular diseases, atherosclerosis, arthritis, end-stage kidney disease and cancers. The molecular mechanisms governing ectopic calcification are pretty similar to those regulating physiological calcification, and mineral formation in the soft tissues depends on a balance of "procalcific" and "anticalcific" regulatory molecules (Giachelli, 2005). Very recently it has also been shown that in addition to the mineralization occurring in the ECM, calcium-phosphate mineral deposits can occur intracellularly (Azari et al., 2008). Such deposits have been observed in electron-dense vesicular structures in Purkinje cells during cerebellum calcification (Ando et al., 2004).

There is extensive evidence that vascular smooth muscle cells (VSMC), which originate from pluripotent mesenchymal cells, transdifferentiate into chondrocytes and undergo chondrogenic commitment leading to vascular calcification. TM cells derived from the mesenchymal cells

of the neural crest and exhibit many of the characteristics of VSMCs (Lütjen-Drecoll and Rohen, 1996). Since they contain key calcification markers, it has been proposed that TM cells under certain conditions, could undergo the same fate (Xue et al., 2006; Xue et al., 2007).

In this article we review the current evidence of the presence of calcification in the TM with a particular focus in the presence and relative abundance of calcific and anticalcific regulatory proteins (Figure 1a and b). We review the function that these mineralization associated genes play in physiological calcification and in ectopic, pathological mineralization in their established tissues and discuss the meaning of their presence or absence in the TM.

2. Alkaline phosphatase (ALP)

ALP is an enzyme that hydrolyzes a variety of substrates that lead to increases of Pi. It is an essential component of matrix vesicles where it leads to increased orthophosphates required for the growing hydroxyapatite crystal. ALP is activated when a pluripotent mesenchymal cell commits to differentiate into a bone-forming cell (osteoblast) and it is significantly elevated in matured committed osteoblasts (Hashimoto et al., 1998; Pittenger et al., 1999). ALP also allows inactivation of mineralization inhibitors (Magne et al., 2005). The disease hypophosphatasia, characterized by defective bone mineralization, is linked to a mutation in the ALP gene (Mornet, 2000). ALP is a well established early marker of osteogenesis (Hashimoto et al., 1998; Luo et al., 2004) and vascular calcification (Jono et al., 2000a; Shanahan et al., 2000; Tanimura et al., 1986). Under normal conditions, ALP is not expressed in the TM (Figure 1a and b). However, ALP activity was reported increased in HTM cells aged in culture (Xue et al., 2006) and after treatment with DEX, TGF β 2 and MGP siRNA (Xue et al., 2007). ALP activity was also significantly higher in the intact TM tissues from glaucomatous patients (Xue et al., 2007).

3. Osteocalcin (BGLAP/BGP)

Also known as Bone Gla protein, osteocalcin is expressed in osteoblasts and in osteoblast-like calcifying vascular cells. Osteocalcin is highly expressed during the last stages of bone formation and is a characteristic component of the bone matrix of differentiated osteoblasts. Osteocalcin is highly induced by vitamin D3 (Viereck et al., 2002), a potent differentiation factor for osteoblasts (Owen et al., 1991), and considered essential for the calcification of the bone matrix. However, the involvement of osteocalcin in mineralization is complex. Osteocalcin null mice have increased bone density (Ducy et al., 1996) indicating that osteocalcin functions to suppress excess calcification without affecting initial mineralization. Although osteocalcin has not been reported as induced in the TM (Borrás, 2003; Rozsa et al., 2006; Vittal et al., 2005; Zhao et al., 2004), it appears expressed at low levels in microarrays of normal cells and tissues (Figure 1A and 1B).

4. Biglycan (BGN)

Biglycan (alias dermatan sulfate proteoglycan) forms part of the small leucine-rich repeat family of proteoglycans (SLRPs), which are associated with the commitment to the osteogenic phenotype in osteoblast differentiation (Balint et al., 2003). It binds to collagen type I (Schönherr et al., 1995) and TGF β (Hildebrand et al., 1994) and is present in atherosclerotic plaques associated with collagen type I and III. Disruption of the biglycan gene in mice leads to an osteoporosis-like phenotype with reduced bone strength (Xu et al., 1998), while its upregulation is associated with osteoarthritis progression in mice (Watters et al., 2007). Biglycan is closely related to Fibromodulin and Decorin. Together with elastic and collagen fibers, dermatan sulfate proteoglycans form part of the core of the TM beams (Lütjen-Drecoll and Rohen, 1996). In the TM, biglycan is moderately expressed (Figure 1a and b) and it is altered by mechanosensitive insults of pressure and stretch (Borrás, 2008a).

5. Bone morphogenetic protein 2 (BMP2)

BMP2 belongs to the superfamily of TGF β proteins. Of all the BMPs, only BMP2 and BMP4 have been shown to induce osteoblast differentiation. BMP2 by itself has the full potential to initiate bone formation and to induce the differentiation of multipotent mesenchymal progenitor cell lines to the osteogenic lineage (Ducy and Karsenty, 2000; Riley et al., 1996). Osteogenesis signaling of BMP2 involves the SMAD proteins and a number osteoblast-specific transcription factors such as Hoxc-8, RUNX2 and osterix (Matsubara et al., 2008; Viereck et al., 2002; Yang et al., 2000). BMP2-induced osteogenesis is known to require activation of the canonical Wnt signaling pathway (Rawadi et al., 2003; Vaes et al., 2005), which plays a key role in the regulation on bone remodeling (Goldring and Goldring, 2007). As well, BMP2 induces osteogenesis-like characteristics in primary human TM (HTM) cells (Xue et al., 2006). Overexpression of BMP2 induces ALP activity in primary HTM cells and its encoded protein colocalizes with the calcification inhibitor Matrix Gla (MGP) (Xue et al., 2006). However, BMP2 expression was reported downregulated in HTM DEX-treated cells (Rozsa et al., 2006). BMP2 osteogenic action can be inhibited by its binding to MGP in various cell types, including the TM (Boström et al., 2001; Wallin et al., 2000; Xue et al., 2006).

6. Cadherin 11 (CDH11)

Also known as Osteoblast Cadherin, cadherin 11 is a calcium-dependent cell-cell adhesion protein which is highly expressed in osteoblasts. It was originally isolated as a protein expressed specifically in osteoblasts (Okazaki et al., 1994) and then found to be critical for BMP2 induced osteogenic differentiation (Cheng et al., 1998). Cadherin 11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo- and chondro-lineages (Kii et al., 2004). Cadherin knockout mice exhibit reduced bone density (Kawaguchi et al., 2001). Cadherin 11 is quite abundant in the TM (Borrás, 2003; Tomarev et al., 2003) and is upregulated in primary HTM cells versus intact tissue (Figure 1a and b).

7. Collagen type I (COL1A1)

Collagen type I is an integral component of calcified matrix vesicles (Glimcher, 1990; Katz and Li, 1973; Yang et al., 2004) and the type I collagen fibril plays a critical role in bone mineralization. The mineral in bone is located primarily within the fibril; during mineralization the collagen fibril is formed first and then water within the fibril is replaced with mineral. In ectopic calcification, VSMCs cultured on collagen type I enhance calcification (Watson et al., 1998). Mutations in collagen type I are associated with osteogenesis imperfecta (Kuivaniemi et al., 1997). In the TM, collagen type I is an important structural component of the ECM and constitutes part of the core of the TM beams (Lütjen-Drecoll and Rohen, 1996). Collagen type I is the substrate of matrix metalloproteinase 1 (MMP1), a regulator of outflow facility (Bradley et al., 1998), and it is induced by ascorbic acid (Zhou et al., 1998), a potent inducer of calcification. Collagen type I mRNA is induced in primary HTM cells versus perfused intact tissue (Figure 1a and b).

8. Connective Tissue Growth Factor (CTGF)

CTGF plays a major role in angiogenesis, chondrogenesis, osteogenesis, tissue repair, cancer and fibrosis (Shi-Wen et al., 2008). Mice deficient in CTGF die soon after birth because of their inability of their rib cage to ossify properly (Ivkovic et al., 2003). In vitro, treatment of primary osteoblast cultures with recombinant CTGF (rCTGF) causes an increase in cell proliferation, ALP activity and calcium deposition (Safadi et al., 2003). In vivo, CTGF promotes endochondral ossification and regeneration of damaged cartilage (Kikuchi et al., 2008). Application of the CTGF in a gelatin hydrogel base to a rat femur resulted in induction of osteoblastic mineralization within 2 weeks. Delivery of rCTGF into the femoral marrow

cavity of rats induced osteogenesis (Safadi et al., 2003). CTGF was also shown to be upregulated during fracture healing in a mouse model (Kikuchi et al., 2008). This gene is highly abundant in the TM (Tomarev et al., 2003), is elevated in the aqueous humor of patients with pseudoexfoliation glaucoma (Ho et al., 2005), and shows significant induction in microarrays of samples subjected to mechanical stress and DEX (Rozsa et al., 2006; Vittal et al., 2005).

9. Decorin (DCN)

Decorin is another member of the SLPRs and contains both chondroitin and dermatan sulfate glycosaminoglycans. It is highly expressed in bone cells and co-localizes to the mineralized ECM of bones (Waddington et al., 2003). Decorin co-localizes with collagen, aids in the assembly of collagen fibers and regulates hydroxyapatite crystal growth (Azari et al., 2008; Boskey et al., 1997; Giachelli, 1999). Mice lacking both decorin and biglycan exhibit an osteoporetic phenotype (Corsi et al., 2002). Decorin also induces calcification of arterial smooth muscle cell cultures and co-localizes to mineral deposition in human atherosclerotic plaque (Fischer et al., 2004). Decorin was recently reported to play a role in intracellular pathological calcification of soft tissues (Azari et al., 2008). Decorin is highly abundant in the TM (Gonzalez et al., 2000; Tomarev et al., 2003) and one of the most abundant of all calcification related genes selected for this review (Figure 1a and b). Decorin is one of the main proteoglycans of the matrix of the TM (Borrás, 2003; Ueda et al., 2002). Its mRNA was reported both up- and downregulated in the HTM cells after DEX (Borrás, 2008a; Ishibashi et al., 2002; Rozsa et al., 2006).

10. Fibromodulin (FMOD)

Fibromodulin is an ECM keratan sulfate proteoglycan and another member of the SLPRs. It interacts with collagen type I fibrils and has been shown to regulate collagen fibrillogenesis (Ameye and Young, 2002). Fibromodulin binds to collagen type I at the same site of lumican which is a different binding site than that of the decorin (Svensson et al., 2000). It also binds to TGF β (Hildebrand et al., 1994). The binding of fibromodulin to collagen impairs the fibril growth and creates thinner collagen fibrils (Font et al., 1998). Mice deficient in fibromodulin have weak tendons and ligaments causing osteoarthritis (Ameye and Young, 2002; Svensson et al., 1999). The expression of this SLRP in the TM is moderate (Figure 1a and b), but it is significantly upregulated by mechanical stimuli (Borrás, 2008a; Vittal et al., 2005).

11. Bone Sialoprotein (IBSP)

Bone Sialoprotein or Integrin-binding Sialoprotein, is an extracellular glycoprotein synthesized by osteoblasts and osteoclasts (Bianco et al., 1991; Fisher et al., 1983). It is a major noncollagenous, structural protein of the bone matrix and other mineralizing connective tissues (Chen et al., 1992). Studies on rat development showed that it has a specific role in the initial stages of connective tissue mineralization (Chen et al., 1992). Bone sialoprotein binds to collagen (Fujisawa et al., 1995) and it also has an RGD (Arg-Gly-Asp) domain (Oldberg et al., 1988). IBSP was reported to be one of the Wnt pathway proteins to be significantly downregulated in an array analysis of osteoarthritic bone (Hopwood et al., 2007). To date this protein does not appear to be very relevant for the TM. It is not (or barely) expressed (Figure 1a and b) and there are not reports about its induction in the tissue.

12. Matrix Gla (MGP)

Matrix Gla (MGP) is a vitamin K-dependent protein found most abundantly in bone and cartilage. MGP is activated post-translationally by conversion of its glutamic acid residues to γ -carboxylglutamic (Gla) by the enzyme γ -carboxylase (Price et al., 1983). Once activated, MGP regulates calcium deposition by binding to calcium and calcium crystals, inhibiting

BMP2 and binding to ECM components, such as elastin and vitronectin (Proudfoot and Shanahan, 2006). MGP was initially discovered in demineralized extracts of bone matrix (Price and Williamson, 1985) but it is highly expressed also in VSMCs (Shanahan et al., 1993). MGP knockout mice develop extensive calcification of elastic arteries and cartilage, and die at two months due to blood-vessel rupture (Luo et al., 1997). Today it is well established that the protection of soft tissue calcification is partly because of the complex of calcium to inhibitors of calcification, such as MGP (Proudfoot and Shanahan, 2006).

MGP is one of the most expressed genes in the human TM tissue (Borrás, 2008a; Gonzalez et al., 2000; Tomarev et al., 2003) and its expression is affected by insults associated with glaucoma such elevated pressure (Vittitow and Borrás, 2004) and mechanical stretch (Vittal et al., 2005). Cells of the TM have also been shown to have high carboxylase activity and to produce active MGP (Xue et al., 2006). Expression of MGP and γ -carboxylase activity is decreased in HTM cells aged in culture (Xue et al., 2006) and in glaucomatous TMs where the ALP activity marker is significantly increased (Xue et al., 2007). Overexpression of MGP reduces ALP activity in a model of BMP2-induced osteogenesis (Xue et al., 2006) while silencing of MGP increases ALP activity (Xue et al., 2007). In humans, a rare inherited disease, Keutel syndrome (Munroe et al., 1999; Teebi et al., 1998), characterized by abnormal calcification of cartilage and stenosis of pulmonary arteries has been linked to a defective MGP gene. Ophthalmological evaluation in several patients described decreased vision and optic nerve atrophy (Hur et al., 2005; Teebi et al., 1998). However, a comprehensive TM and IOP study in Keutel patients is lacking, in part because to date only about 20 cases have been reported worldwide (Hur et al., 2005).

13. Osteoglycin (OGN)

Osteoglycin, also known as Mimecan and Osteoinductive Factor, is another extracellular protein member of the SLPRS family of proteoglycans, and thus associated with the commitment to the osteogenic phenotype in osteoblast differentiation (Balint et al., 2003). Osteoglycin shares about 30% homology with decorin and biglycan. It is highly expressed in osteoblasts, calcifying vascular cells and cornea (Balint et al., 2003; Shanahan et al., 1997; Tasheva et al., 2002). Recently it was shown also to be highly expressed in the cochlea (Williamson et al., 2008). Mice lacking osteoglycin show an increase in bone density (Ducy et al., 1996) as well as in collagen fibril diameter, suggesting a role in fibrillogenesis (Tasheva et al., 2002). Osteoglycin was identified in atherosclerotic plaques (Fernandez et al., 2003). Implantation of osteoglycin plus TGF β type 1 or 2 into subcutaneous tissues of rats induces ectopic formation of bone at the implantation site (Kukita et al., 1990). Osteoglycin expression is altered by mechanical insults in a variety of tissues, including the TM (Borrás, 2008a; Patel et al., 2007b; Wong et al., 2003). A proteomic analysis has identified the presence of this gene in normal and glaucomatous TM tissues (Bhattacharya et al., 2005) while a differential expression microarray found osteoglycin upregulated in tissues from primary open angle glaucoma patients (Diskin et al., 2006).

14. Osteomodulin (OMD)

Osteomodulin, also named Osteoadherin, is a keratan sulfate proteoglycan that belongs to the SLRP's family (Sommarin et al., 1998). It was found expressed in bovine mature osteoblasts and in odontoblasts, leading to the suggestion that it may be implicated in bone mineralization (Buchaille et al., 2000). By microarray analysis, osteomodulin was found to be associated with BMP2-induced differentiation of premyoblasts to the osteogenic lineage (Balint et al., 2003). Moreover, in vitro overexpression of osteomodulin in osteoblasts resulted in increased ALP activity and mineralization while silencing it by siRNA reduced the calcification process (Rehn et al., 2008). Recently osteomodulin has been proposed as an osteoblast differentiation marker

which is induced by osteoclast activity (Ninomiya et al., 2007). Osteomodulin has been identified as a mechanosensitive gene in osteoblasts (Patel et al., 2007b) and in the TM (Vittitow and Borrás, 2004).

15. Osteoclast stimulating factor (OSTF1)

Osteoclast-stimulating factor 1 is an intracellular protein produced by osteoclasts to enhance osteoclast activity and bone resorption (destruction) (Reddy et al., 1998). The stimulatory osteoclast activity is exerted through the binding of the osteoclast specific factor to other proteins. Binding of OSTF1 to survival motor neuron gene product (SMN) induces secretion of soluble osteoclast stimulators (Shanmugarajan et al., 2007). Mutations in SMN result in spinal muscular atrophy, a children's disease which involved motor neuron dysfunction and congenital bone fractures (Shanmugarajan et al., 2007). This gene is present but expressed at low levels in the TM (Figure 1a and b) and there are not reports on its induction.

16. Periostin (POSTN)

Periostin, also known as Osteoblast-specific Factor 2 (OSF2), is a secreted protein by osteoblasts (Horiuchi et al., 1999). Periostin is expressed in collagen rich tissues that are subjected to constant mechanical stresses. It is required to mediate and cushion mechanical forces exerted during mastication in teeth (Rios et al., 2005). Periostin-deficient mice have skeletal defects (Rios et al., 2005) and have revealed that periostin can regulate collagen type I fibrillogenesis and viscoelastic properties of connective tissue (Norris et al., 2007). Recently, periostin was found to be a new member of the vitamin K-dependent, γ -carboxylated proteins. It was localized to bone nodules in bone marrow-derived mesenchymal stromal cells and suggested to have a role in mineralization (Coutu et al., 2008). Microarray studies in the human TM showed that periostin expression was increased in perfused organ cultures by elevated pressure (Vittitow and Borrás, 2004), and in cultured TM cells by mechanical stretch (Vital et al., 2005) and TGF β (Vital et al., 2005; Zhao et al., 2004).

17. Parathyroid hormone-like hormone (PTHLH)

Parathyroid hormone-like hormone, a secreted peptide also known as Parathyroid hormone-related protein (PTHrP), is involved in calcium and phosphate homeostasis. It was discovered as a humoral factor implicated in the cause of hypercalcemia in lung malignancy (Suva et al., 1987). It is a local regulator of calcification and addition of the exogenous peptide to VSMCs inhibits ALP and calcification (Jono et al., 1997). PTHLH is downregulated by vitamin D3 (Jono et al., 1998; Okazaki et al., 2003), a physiological mediator of bone formation (Chapuy et al., 1992), through an interaction of the vitamin D receptor with a promoter negative element in PHTLH. PTHLH is thus considered an inhibitor of calcification (Okazaki et al., 2003). In the TM, PTHLH is moderately expressed (Figure 1a and b) and it was reported as downregulated by DEX in primary HTM cells (Rozsa et al., 2006). Curiously, in primary HTM cells, PTHLH is increased after overexpression of wild-type TIGR/MYOC while it is downregulated after overexpression of the Q368STOP mutant (our laboratory, unpublished).

18. Osteoblast specific factor 2 (RUNX2)

Osteoblast-specific Transcription Factor 2, also known as Core-binding Factor α 1 (Cbfa1), is a member of RUNX family of transcription factors (Ducy et al., 1997). It was isolated as the factor binding to an osteoblast-specific promoter element in osteocalcin (Ducy et al., 1997) and then found to regulate the expression of key osteoblast specific genes (Ducy et al., 1997). ALP, osteocalcin and osteopontin contain Runx2/Cbfa1 binding sites. First believed to be expressed only in cells of osteogenic lineage, RUNX2/Cbfa1 is induced when VSMCs gain an osteogenic phenotype and lose their lineage markers smooth muscle 22 α and smooth muscle

α -actin (Steitz et al., 2001). This transcription factor is moderately expressed in the TM (Figure 1A and 1B) and there are no reports of its induction.

19. Osteonectin (SPARC)

Osteonectin, also called SPARC (Secreted Protein Acidic and Rich in Cysteine) and BM-40 (Basement membrane 40), is a calcium and collagen binding ECM glycoprotein (Lane and Sage, 1994). It was first identified as highly abundant in mineralized tissues (Termine et al., 1981). Subsequent studies showed that osteonectin also belongs to a group of regulatory ECM molecules known as matricellular proteins, which modulate cell-matrix interactions but do not have a structural role in the matrix (Bornstein and Sage, 2002). Osteonectin is expressed at sites of active skeleton and ECM remodeling (Lane and Sage, 1994). Null mice have decreased osteoblast and osteoclast formation resulting in decreased bone density (Delany et al., 2000). As a matricellular protein, osteonectin contains modular domains that can function independently to bind cells and ECM and consequently, it has been shown to have multiple biological activities. Recently, it has been proposed to be a collagen chaperone, enhancing collagen stability intracellularly and regulating collagen fibrillogenesis extracellularly (Martinek et al., 2007). SPARC is very abundant in the TM (Figure 1A and 1B) where it has been shown to be a mechanosensitive gene that responds to elevated pressure and stretch (Borrás, 2008a; Vittal et al., 2005).

20. Osteopontin (SPP1)

Osteopontin, also known as Secreted Phosphoprotein 1, is a noncollagenous phosphoprotein associated with biomineralization in bone tissue as well as with ectopic calcification (Jono et al., 2000b). Osteopontin binds tightly to hydroxyapatite and inhibits hydroxyapatite crystal growth and calcification in vivo and in vitro (Steitz et al., 2002). Experiments using a mouse model of subcutaneous implants of biopsy punches of aortic valves showed that osteopontin is a natural inhibitor of ectopic calcification in vivo where not only inhibits mineral deposition but also actively promotes its dissolution (Giachelli, 2005; Steitz et al., 2002). Osteopontin also has a multidomain structure and functions as a matricellular protein (Giachelli and Steitz, 2000); it binds to cell receptors through an integrin-binding site (RGD) as well as to extracellular proteins.

Experiments in osteopontin-deficient mice showed that osteopontin is not involved in normal bone development but is important in bone remodeling by influencing the resorption of bone by osteoclasts (Franzen et al., 2008). After reduction of mechanical stress, osteopontin knockout mice reverted the bone loss displayed by the wild-type due in part to a deficit in bone resorption (Ishijima et al., 2001). Osteopontin is highly expressed in human TM tissue (Gonzalez et al., 2000; Tomarev et al., 2003) and is downregulated in HTM cells in culture (Gonzalez et al., 1999) (Figure 1A and 1B).

21. Osteoprotegerin (TNFRSF11B)

Osteoprotegerin is a member of the Tumor Necrosis Factor receptor gene superfamily. Osteoprotegerin is secreted by osteoblasts and binds specifically to Osteoprotegerin Ligand, an osteoclast differentiation factor also known as RANKL (receptor activator of NF κ B). Binding of the two proteins inactivates the ligand, which is an essential factor for osteoclast development (Theoleyre et al., 2004). Thus, osteoprotegerin regulates bone remodeling by decreasing osteoclast, bone destruction activity and increasing bone strength. Mice deficient in OPG-L, or that overexpress OPG, lack osteoclast activity and develop severe osteopetrosis (increased bone density) (Kong et al., 1999; Simonet et al., 1997). In contrast, mice lacking OPG develop osteoporosis and show high rate of pathological fractures and arterial calcification (Bucay et al., 1998). Recently Osteoprotegerin has been shown to inhibit

apoptosis-induced by TRAIL (TNF-related apoptosis inducer ligand) (Holen and Shipman, 2006) and to be regulated by the Wnt/ β catenin pathway (De Toni et al., 2008). Osteoprotegerin is moderately expressed in the TM and it is significantly downregulated by DEX (Rozsa et al., 2006).

22. Calcium deposition and ALP activity in the trabecular meshwork

In order to assess the presence of a calcification process in the TM calcification, studies in HTM cells and intact perfused tissue have been conducted under different aging and glaucomatous conditions using chemical, biological and histological calcification assays. Xue et al. (2006) reported that primary HTM cells aged in cultured for four weeks had higher levels of calcium, increased ALP activity and formed calcification nodules which stained with alizarin red (Xue et al., 2006) (Figure 2). The authors also reported that treatment with DEX likewise resulted in elevated cellular calcium concentration, increased ALP activity and the appearance of calcification nodules (Xue et al., 2007) (Figure 3). In a similar study, perfused TM tissue from glaucoma donors showed higher ALP activity than aged-matched controls while the mRNA expression of MGP and its activation enzyme, γ -carboxylase was reduced (Xue et al., 2007). Treatment with TGF β 2 induced the activity of the calcification marker ALP and reduced the expression of the inhibitor MGP, and silencing MGP resulted also in an increased of activity of the ALP enzyme (Xue et al., 2007).

Taken together these studies are very suggestive but not yet determinative. Although evaluation of ALP is the most widely used calcification marker in the physiological (bone) and pathological (vascular calcification) fields, other proteins involved in the regulation of biomineralization (such osteocalcin, osteopontin, decorin, biglycan etc) would need to be tested. Although calcium deposition experiments are very encouraging, a greater number needs to be performed. As well, morphological examination of the TM of the knockout mice for the key bone associated genes would be very informative.

23. Concluding remarks

Our intent in writing this review was not only to report on the calcification studies done in the TM, but to expose the eye community to the vast literature of physiological and pathological calcification. At the gene level, the similarities with the TM are at times staggering (Figure 1A and B). Definitely more studies are needed, but the evidence so far suggests that calcification, or its prevention, might be a very important processing for the TM. Nature does not waste its own resources and a cell would not be expending its transcription machinery in abundantly transcribing a gene (like MGP) whose function would not be used. Further, as we learn by the calcification literature, mechanical stimuli such as tension or pressure are key to building stronger bones and teeth (Patel et al., 2007a; Wong et al., 2003). It would make sense that a soft tissue like the TM, which is continually subjected to mechanical pressure, would need to protect itself from such happening. Nonetheless, quite a few issues need to concur and be resolved. It is for instance curious that, even if it has not been specifically addressed, none of the extensive electron microscopy studies available has ever reported on the presence or absence of calcification in the electron dense structures of the human trabecular meshwork. Also, although MGP conserves the inhibition of calcification role in the TM (Xue et al., 2006), the function of additional osteogenic genes in the outflow tissue is not yet known. As many of the genes described here, such as biglycan, SPARC or fibromodulin, have other functions than calcification, their main functional role in the TM could be different and no calcification related. Experiments like those conducted with MGP, would need to be performed. In conclusion, maintaining TM's ECM softness by preventing calcification might be an important mechanism to influence outflow facility. Such a mechanism could be an active

part of the homeostasis processes that tend to adjust the elevated pressure insult and therefore, the development of glaucoma.

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Figure 1. Heat map of twenty calcification regulatory genes in the human TM
Human U133Plus2.0 Affymetrix Genechips analyzed with the GeneSpring software package. Expression signals in each chip are compared to a median value. Change from the median is visually represented by a color assignment (scale at right). The custom calcification gene list was generated from the Gene Ontology lists on Gene Spring. A: human TMs from perfused post-mortem eyes used as control in several experiments. Ages of donors were between 62–92 years old (all Caucasians and all but one males). Perfusion was conducted at constant flow for 3 to 6 days under standard, serum free conditions. Chips 112 – 113 were untreated; chips 116–129 were treated with a control recombinant adenovirus carrying no gene (AdNull). B: primary HTM cells from control cultures. Primary cells were all from the same line (donor 43

year old Caucasian male), at passage 4 and grown on 10% FBS. Each chip's RNA originated from an independent experiment. Chips 13–15 were untreated; chips 17–23 were treated with a control recombinant adenovirus carrying no gene (AdNull).

Figure 2. Evidence of calcification in aged HTM cells in cultures

Measurements of normalized calcium content (top) and alkaline phosphatase activity (middle) in young (1 week in culture) and old (4 weeks in culture) primary HTM cells at passage 6. Bottom panel shows alizarin red staining of calcification nodules on cells aged in culture for 12 weeks (Xue et al., 2006).

Figure 3. Evidence of calcification in HTM cells treated with Dexamethasone (DEX)

Measurements of normalized calcium content (top, 10 days post-treatment) and alkaline phosphatase activity (middle) in untreated and DEX-treated (0.1μM) primary HTM cells. Bottom panel shows alizarin red staining of calcification nodules on DEX-treated cells for 4 days. (Xue et al., 2007 and unpublished).