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A single gene connects stiffness in glaucoma and the vascular system

Teresa Borrás

Department of Ophthalmology, University of North Carolina School of Medicine, 4109C Neuroscience Research Building CB 7041, 105 Mason Farm Road, Chapel Hill, NC 27599-7041, USA

Abstract

Arterial calcification results in arterial stiffness and higher systolic blood pressure. Arterial calcification is prevented by the high expression of the Matrix-Gla gene (MGP) in the vascular smooth muscle cells (VSMC) of the arteries' tunica media. Originally, MGP, a gene highly expressed in cartilage and VSMC, was found to be one of the top expressed genes in the trabecular meshwork. The creation of an *Mgp-lacZ* Knock-In mouse and the use of mouse genetics revealed that in the eye, *Mgp*'s abundant expression is localized and restricted to glaucoma-associated tissues from the anterior and posterior segments. In particular, it is specifically expressed in the regions of the trabecular meshwork and of the peripapillary sclera that surrounds the optic nerve. Because stiffness in these tissues would significantly alter outflow facility and biomechanical scleral stress in the optic nerve head (ONH), we propose MGP as a strong candidate for the regulation of stiffness in glaucoma. MGP further illustrates the presence of a common function affecting key glaucomatous parameters in the front and back of the eye, and thus offers the possibility for a sole therapeutic target for the disease.

Keywords

Calcification; Stiffness; Transgenic mice; Matrix Gla; Glaucoma

1. Introduction

1.1. Calcification and stiffness

Mineralization of the extracellular matrix (ECM) occurs when calcification deposits are formed into the extracellular space. Mineralization is initiated by the cell's release of membrane-bound matrix vesicles (MV) into the ECM, exposure to elevated Ca and Pi, and formation of calcium phosphate precipitates (Anderson, 2003; Kapustin et al., 2011, 2015). In the artery calcification, the majority of the calcification-competent extracellular vesicles are of exosomal origin. Once in the intercellular space, the released MVs associate with the ECM, interact with collagen and elastic fibrils, and by a still unknown mechanism serve as nucleation sites for the continuation of formation of hydroxyapatite nanocrystals (Kapustin

et al., 2011, 2015; Krohn et al., 2016). Calcification is now known to be a highly regulated process. These MVs contain mineralization inducing proteins, such as alkaline phosphatase (ALP), MMP2 and annexins, as well as potent mineralization inhibitors, such Matrix Gla (MGP) and Fetuin-A (Krohn et al., 2016; Schurgers et al., 2013). Alteration in the production of these proteins by the cell would result in different levels of them being loaded into the vesicles and as a consequence, in different extents of calcification. Absence of *Mgp* in the arterial wall of mice results in premature death caused by massive arterial calcification (Luo et al., 1997). Matrix vesicles from vascular smooth muscle cells (VSMC) treated with calcification media show a dramatic reduction of MGP 48 h after treatment. (Kapustin et al., 2011).

Stiffness of a tissue, or resistance to deformation by a mechanical force, occurs when its cells and ECM undergo a number of biological changes which would lead to an overall compromise of its flexibility and rigidity. In the cell, stiffness is a physiological response that is required to resist an exogenous force. Stiffness is also associated with pathological conditions in a number of diseases such cardiovascular, cancer, chronic kidney disease (CKD), diabetes and glaucoma (Braunger et al., 2015; Kerr and Guerin, 2007; Mattace-Raso et al., 2006; Shim et al., 2015; Tiago et al., 2016; Visontai et al., 2005). Increased stiffness can be caused by a variety of cellular, cell-ECM and ECM hardening mechanisms. Per example, in endothelial cells lining the blood vessels, a stiffness response has been show to occur by the activation of the RhoA signaling pathway which leads to changes in cytoskeleton and focal adhesions to withstand the force (Collins et al., 2012). In these cells, an ECM signaling mechanism through integrins is also known to influence the response of mechanosensitive proteins and induce stiffness. In the vessels, stiffness induced by the sheer stress generated by the blood flow has been associated with elastin fragmentation, collagen deposition, matrix protein cross-linking, advance glycation end products, oxidative stress, angiotensin, dietary salt and a number of other factors (Zieman et al., 2005). Mineralization of the ECM leads inexorably to an increased stiffness.

Given the recent attention to the fact that stiffness in glaucomarelated tissues is associated with most, if not all glaucomatous conditions of elevated IOP (Braunger et al., 2015; Girard et al., 2009; Last et al., 2011; Nguyen et al., 2013) in this review we like to bring the reader's attention to the involvement of a single gene which would be mediating stiffness in glaucoma, as well as stiffness in the systemic arterial system. The gene is Matrix Gla (*MGP*), a potent mineralization inhibitor, known to be responsible for the physiological softness of cartilage, ossification of bone, and the mineralization and calcification/stiffness of arteries in the vascular system (Schurgers et al., 2013). In the eye, this gene has been found to be very abundant, and active only in the trabecular meshwork and peripapillary sclera (Borrás et al., 2015b), two tissues highly relevant to the development of glaucoma. *MGP* expression levels, as well as those of its activating enzyme λ -carboxylase, are reduced in the trabecular meshwork of primary open angle (POAG) patients when compared to normal, age-sex matched controls (Xue et al., 2007). Transcription of *MGP* is also reduced in primary human trabecular meshwork cells (HTM) treated with glaucomatous agents TGF β 1 (Vittitow and Borrás, 2004), TGF β 2, and dexamethasone (Xue et al., 2007). Mechanical forces of elevated IOP and stretch also alter expression of the *MGP* gene in human perfused anterior segment organ cultures and porcine trabecular meshwork (Comes

and Borrás, 2009; Vittal et al., 2005; Vittitow and Borrás, 2004). In HTM cells, silencing the expression of *MGP* by siRNA, induces increase of the calcification marker ALP (Xue et al., 2007). When overexpressed in HTM cells, MGP reduced the calcification induction of BMP2 (Xue et al., 2006).

2. Association of vascular calcification and arterial stiffness

In the vascular system, calcification is associated with arterial stiffness. Early in 1997, before much of the calcification regulation was elucidated, experiments were conducted in rodents to demonstrate that accumulation of calcium in the media of the vessel wall would decrease aortic elasticity. The authors developed a rat model of calcium overload by treating rats with vitamin D and nicotine and produced 10–40-fold increase of calcium deposition in the elastic fibers of the media. After treatment, they measured stiffness of the arterial wall by elastic modulus, aortic pulse wave velocity (PWV) and isobaric elasticity. Their results showed that the stiffness of the arteries in the treated rats was significantly increased. Such increase was significantly correlated with the content of calcium in the wall (Niederhoffer et al., 1997). They therefore concluded that calcium overload on the medial elastic fibers is associated with decrease of aortic elasticity.

Association of calcification with stiffness is being nowadays observed in several major systemic diseases. Patients with CKD exhibit high mortality rate which does not seem to correlate with some of their risk factors such as elevated homocysteine, hypertension or diabetes, but rather with calcification of their blood vessels. Numerous reports indicate that, in CKD patients, medial calcification in the blood vessels occurs at an early age and with great severity. Measurement of stiffness in the calcified vessels of CKD patients as well as in those with end-stage renal disease by PWV demonstrated that not only the aortic stiffness was increased but that it was a strong predictor of cardiovascular mortality (Goodman et al., 2000; Kerr and Guerin, 2007; London et al., 2003; Robinson et al., 2005). Thus authors concluded that medial calcification and arterial stiffness play a major role in the development of CKD. In another disease, chronic obstructive pulmonary disease (COPD), patients were known to have increased aortic stiffness as measured by PWV. To investigate the relationship between aortic stiffness and calcification, a clinical study of 45 COPD patients absent of diabetes, renal or cardiovascular disease were subjected to thoraco-computed tomography for the determination of quantitative aortic calcium content. Their results showed a significant correlation of PWV values with aortic calcification. It was therefore concluded that aortic calcification was related to aortic stiffness in COPD patients (John et al., 2013).

3. Vascular dysfunction and arterial stiffness in glaucoma

The association of deficiencies in the vascular system with glaucoma, the vascular theory of glaucoma, has been around since the 19th century (Girkin, 2001). This theory proposes vascular health as an alternative risk factor to elevated IOP for glaucoma (Huck et al., 2014; Wirostko et al., 2009). Their thoughts are based on observations that some patients undergo glaucoma progression despite reduced IOP, while others do not develop glaucoma in the presence of elevated IOP (Heijl et al., 2002). Strong epidemiology data indicates that the

higher incidence of glaucoma in African Americans correlates with their higher incidence of cardiovascular disease (Huck et al., 2014). Several other clinical studies directly measuring systemic and ocular fluid dynamics of blood flow have reconfirmed the association of vascular dysfunctions with risk factors for glaucoma (Choi and Kook, 2015). Together, the findings would indicate that other than IOP factors, such as genetic individual response to elevated IOP (Comes and Borrás, 2009) and/or proper functioning of the arterial vessels are bound to be involved in the development of glaucoma (Resch et al., 2009).

Arterial stiffness, or loss of arterial elasticity, is an important risk factor for cardiovascular diseases (Mattace-Raso et al., 2006). Arterial stiffness in glaucoma, has been investigated in a number of clinical studies using different measuring parameters. Using a non invasive high precision ultrasound wall-tracking system, it was shown that the distensibility coefficient sensitivity of the common carotid artery was significantly reduced in the glaucoma group versus sex/age-matched controls, while the stiffness was significantly increased (Visontai et al., 2005). More recently, using carotid-femoral PWV (CF-PWV) as the stiffness marker, the carotid artery of a group of pseudoexfoliation glaucoma (PEXG) patients showed significantly higher stiffness than that of the controls (Turkyilmaz et al., 2014). Based on this association between CFPWV and PEXG, the authors proposed that the CF-PWV outcomes may be considered a risk factor for PEXG. Likewise, in a retrospective chart review, brachial-ankle PWV (baPWV) was analyzed in four groups: diabetes patients with POAG, POAG alone, normal tension glaucoma (NTG) and matched controls (Shim et al., 2015). Increased baPWV was positively associated with glaucoma in patients with diabetes, as well as in patients with only POAG and/or NTG. The authors proposed that arterial stiffness may contribute to the pathogenesis of glaucoma in diabetes patients.

4. Presence of stiffness in glaucomatous-relevant tissues

Elevated intraocular pressure (IOP) is the principal risk factor associated with the development of glaucoma (Kass et al., 2002). Physiological or elevated IOP is determined by the different resistances offered to the flow of aqueous humor by the trabecular outflow pathway (Rosenquist et al., 1989; Schuman et al., 1999). Dysfunction of this tissue by a variety of mechanisms would disrupt the facility of the aqueous humor flow and lead to pathological IOP and glaucoma. Investigators have determined the stiffness of the trabecular meshwork tissue from normal and glaucomatous patients by measuring the young elastic modulus using atomic force microscopy (AFM) (Last et al., 2011). They found that the glaucomatous trabecular meshwork was over 20-fold stiffer than the one from normal controls. Furthermore, subsequent studies by the same group identified that treatments of normal trabecular meshwork with factors/agents associated with elevated IOP and/or glaucoma, resulted also in a stiffer tissue. Dexamethasone, senescence and inhibitors of the Wnt pathway (see below), all led to higher trabecular meshwork cells stiffness (Morgan et al., 2015a, 2015b; Raghunathan et al., 2015). Other investigators have measured the specific stiffness in cells of the Schlemm's canal (SC) inner wall from normal and glaucomatous patients (Overby et al., 2014). Here the authors find that the difference in stiffness between normal and glaucomatous cells depended on the region probed with the AFM tip. When measuring with a sharp tip (20 nm tip radius), which probes the cell cortex layer, the authors find no difference in stiffness in normal versus glaucomatous cells. However, when probed

with the larger, spherical AFM tips (4.5 μm and 10 μm), which probe the subcortical cytoskeleton, they find systematic differences in stiffness between glaucomatous and normal SC cells. This discrepancy is attributed to the difference of just measuring the cell cortex (sharp tip), which is thicker and stiffer than the subcortical region, versus that of probing/measuring both the cortex and the underlying cytoskeleton (larger, rounded tip). Interestingly the authors also find a correlation between SC cell stiffness and a reduced number of pores in the glaucomatous cell monolayer. They therefore concluded increased stiffness of the SC to be a potential cause of reduced pore formation.

The second tissue of the eye where stiffness plays a role in the development of glaucoma is the peripapillary sclera surrounding the optic nerve head (ONH). The degree of stiffness of the sclera has great relevance in glaucoma (Nguyen et al., 2013). The sclera is the principal load-bearing structure of the eye and its biomechanical response to elevated IOP controls the extent of force applied to the ONH (Cone-Kimball et al., 2013; Grainger et al., 1998). Deformation of the ONH due to the IOP-stresses is a clinical landmark of glaucoma and affects axons and survival of the RGC cells. The stiffness of the posterior sclera has been evaluated in several animal models. In monkeys, investigators have used a custom built pressurized apparatus that allowed the calculation of various biomechanical properties such as strain, tangent modulus and stiffness (Girard et al., 2009). Their studies concluded that older monkeys exhibit a significantly stiffer sclera than younger ones. This stiffness would lead to higher stresses, influence ONH biomechanics and affect susceptibility to glaucoma in the older age. In the mouse, it was observed that the structure of the sclera changes with pressure elevation, showing alteration in fibrillar content and orientation. These structural changes would be consistent with an increased scleral stiffness (Cone-Kimball et al., 2013; Pijanka et al., 2014).

Altogether, experimental evidence in the anterior and posterior pole of the eye reveals that stiffness is a mechanical property which influences in a key manner the development of glaucoma. Identification of gene (s) that could modulate cellular stiffness would offer an attractive target for the management of the disease.

5. Common agents that induce arterial calcification, stiffness and glaucomatous conditions

A review of the literature of agents who contribute to vascular calcification and stiffness reveals a striking similarity with those agents which traditionally have been associated with glaucoma. Among them, TGF β , dexamethasone, the Wnt and RhoA pathways, transglutaminase 2 (TGM2) and connective tissue growth factor (CTGF).

5.1. TGF β 2

Members of the TGF β /bone morphogenetic protein 2 (BMP2) family have been described as potent inducers of osteogenic differentiation and calcification in mesenchymal and in vascular cells (Grainger et al., 1998; Shao et al., 2006). In our laboratory, early studies demonstrated that this property of TGF β 2 was also conserved in primary HTM cells. Treatment of HTM cells with 1 and 3 ng of TGF β 2 significantly induced the calcification

marker ALP, which was concurrent with a 2.5-fold reduction in *MGP* and *MGP*'s activating enzyme γ -carboxylase expression (Xue et al., 2007). Overexpression of another member of the TGF β family, BMP2, induced also calcification in HTM cells and in living rats, where it was coupled to elevated IOP (Buie et al., 2013). However, another member of the TGF β family, BMP7, has been shown to ameliorate rather than enhance vascular calcification in kidney disease (Mikhaylova et al., 2007). In an interesting parallel, BMP7 was shown to antagonize TGF β 2 and attenuate its effects in the trabecular meshwork (Fuchshofer et al., 2007). TGF β is also known to induce vascular stiffness (Zieman et al., 2005) and appeared activated in a proteomic study comparing normal and stiffer trabecular meshwork ECM (Raghunathan et al., 2015). The association of TGF β 2 with glaucoma is extensive (Gottanka et al., 2004; Lütjen-Drecoll, 2005; Tripathi et al., 1994).

5.2. Dexamethasone

Prolonged cell exposure to glucocorticoids has been found to be associated with the development of calcification in vascular and human mesenchymal cells (Mori et al., 1999; Mostafa et al., 2012). Using AFM and Arterial Wave Transit Time it was shown that treatment with steroids caused stiffening of blood cells in vitro, and in aortas and elastic arteries in a chronic model of inflammation in vivo (Ko et al., 2013; Lam et al., 2007). In the trabecular meshwork, HTM cells exposure to 0.1 μ M dexamethasone, significantly increased the presence of the calcification marker ALP starting at 3 days post-treatment (Xue et al., 2007). Independently, in a different laboratory, it was found that treatment with dexamethasone for 3 days resulted in a stiffness increase of 2-fold in the trabecular meshwork, and 4-fold in the laid-out ECM, when compared with the corresponding measurements in untreated cells (Raghunathan et al., 2015). It is well-established that topical ocular treatment with steroids induces elevated IOP in 30–40% of the population and in 90% of patients with POAG (Becker and Hahn, 1964; Tripathi et al., 1999).

5.3. Wnt pathway

The proteins of the Wnt signaling pathway are secreted, and transmit their signal by binding to frizzled (Frz) seven-transmembrane receptors. Upon binding, the signals are transmitted through several pathways. Activation of the Wnt canonical pathway results in de-phosphorylation and nuclear translocation of β -catenin which then interacts with transcription factors TCF/LEF for the activation of specific genes. Numerous studies support the notion that activation of the Wnt pathway is instrumental for diverting mesenchymal cells to osteoblasts (Corr, 2008; Gaur et al., 2005) and for vascular calcification (Johnson et al., 2008; Johnson and Terkeltaub, 2005; Marinou et al., 2012). For example, it was demonstrated that transglutaminase 2 (TGM2), a protein central to the calcification of VSMC, enhances calcification by activating the Wnt/ β -catenin pathway (see below). Antagonist of the Wnt pathway, such as secreted-related frizzle protein 1 (sFRP1) prevents calcification and stiffness in most systems. Thus, after gene ablation, transgenic KO mice (sFRP1^{-/-}) exhibit increased bone healing and mechanically stronger bone (Bodine and Komm, 2006; Gaur et al., 2005). Genes of the Wnt pathway and their inhibitors are also expressed and induced by elevated IOP in the trabecular meshwork (Comes and Borrás, 2009). Interestingly, contrary to what it is reported in the vascular and osteogenic fields (Bodine and Komm, 2006; Marinou et al., 2012), treatment of HTM cells with sFRP1 results

in increased stiffness rather than in the expected reduction (Morgan et al., 2015b). Furthermore, intravitreal adenoviral delivery of sFRP1 to mice induced elevated IOP, which was reversed by the topical administration of an inducer of the Wnt pathway (Wang et al., 2008). In the HTM cells, β -catenin appeared to be more phosphorylated in the sFRP1 treated cultures, indicating that the sFRP1 was interfering with the activation of Wnt/ β -catenin pathway which induces de-phosphorylation. However other non eye studies have shown that the sFRP1, initially seen as Wnt scavengers that prevented Wnt binding to the receptor, can bind directly to the extracellular Frz domain, and lead to the activation of the receptor (Schulte and Bryja, 2007). In addition, some members of the Wnt family, such as Wnt7b, counteract vascular calcification in atherosclerosis rather than enhancing it (Cheng et al., 2013), all to indicate that the regulation of calcification by the Wnt pathway is not fully understood. Despite this fact, it appears that the relevance of the Wnt pathway both in glaucoma and vascular diseases involves calcification and stiffness of the tissue.

5.4. RhoA pathway

RhoA is activated by force in endothelial vascular cells and this activation causes cellular stiffness (Collins et al., 2014). As in a feedback loop, RhoA activation also occurs in response to a stiffer ECM (Yeh et al., 2012) and activation of RhoA is known to be important for the cellular response to stiffness (Collins et al., 2014). The mechanism for the cellular stiffness appears to be mediated by the interaction of RhoA with integrins and the subsequent rearrangement of the cellular cytoskeleton and cellular adhesion complex (Guilluy et al., 2011). RhoA is also involved in the process of cell and ECM calcification. However, the initiation of this process by RhoA is not uniform and at times exhibits different outcomes. Thus in rat aorta organ cultures and bovine VSMC, treatment with RhoA siRNA induced ALP and calcification (Chen et al., 2010), while in human VSMC, treatment with the ROCK inhibitor Y-27632 inhibited ALP and calcification (Kizu et al., 2004). Transgenic mice overexpressing Cadherin-11 induce extensive calcification resulting in calcific aortic valve disease. Such calcification is mediated through upregulation of RhoA and it is attenuated by a ROCK inhibitor (Sung et al., 2016). Another study reports that mechanical strain promotes the production of spheroid mineralized structures in the aortic valve through a RhoA/ROCK mediated mechanism (Bouchareb et al., 2014).

In the eye, inhibition of RhoA blocked calcification induced by dexamethasone in primary HTM cells (Carabana et al., 2012). Cells treated with dexamethasone increased their endogenous ALP 4.8 ± 0.9 -fold over untreated controls. Treatment with an adenoviral vector carrying dominant-negative RhoA (AdnRhoA), which inhibited RhoA by overexpressing the GTP-binding site mutated form of the molecule, reverted the dexamethasone-induced ALP to 1.9 ± 0.4 - and 0.8 ± 0.9 -fold ($p \leq 0.003$) in a dose dependent manner. Calcification of HTM cells induced by overexpression of BMP2 (Buie et al., 2013) was also reverted by cell treatment with ROCK inhibitor Y-27632 (Carabana et al., 2012). Inhibiting the Rho pathway has been shown to reduce elevated IOP. In 1997, Y-27632 was demonstrated to be a potent smooth muscle relaxant and to correct hypertension in rats (Uehata et al., 1997). Subsequently, Y-27632 was demonstrated to reduce IOP in rabbits (Honjo et al., 2000), inhibit contraction of the trabecular meshwork (Thieme et al., 2000), increase outflow facility in rabbits and perfused porcine anterior segments (Honjo et al., 2001; Rao et al.,

2001) and increase SC permeability (Kameda et al., 2012). Recently, a long term gene delivery vector carrying dnRhoA prevented elevation of nocturnal IOP in rats for one month after a single injection (Borrás et al., 2015a). Currently, some ROCK inhibitors are being tested in clinical trials (Tanihara et al., 2008).

5.5. Transglutaminase 2

Transglutaminase 2 (TGM2) is a key regulator of Pi-induced calcification. It is being extensively studied in the cardiovascular field for its role in VSMC calcification, which is associated with mortality in several diseases, including diabetes and CKD (Chen et al., 2013). TGM2 is a calcium-dependent enzyme that catalyzes the crosslinking of most ECM proteins by post-translational transor de-amidation modifications. Inside the cell, TGM2 is activated by increased intracellular calcium. Through these modifications, TGM2 regulates osteoblast differentiation and matrix mineralization in culture (Al-Jallad et al., 2006). Studies from Nurminskaya's laboratory showed that purified TGM2 added to primary VSMC binds to the LRP5 Wnt receptor, activates the β -catenin pathway and induces the TCF/LEF transcription factors (Faverman et al., 2008). Addition of TGM2 also stimulated expression of RUNX2, a key osteogenic transcription factor, and downstream targets osteocalcin and bone sialoprotein. And, inhibition of TGM2 in these cells prevented warfarin-induced calcification (Beazley et al., 2012). In vivo, TGM2 modulates mineralization. VSMC from TGM2-null mice, cannot calcify in response to hyperphosphatemia (Johnson et al., 2008), and inhibiting TGM2 by administration of quercetin prevents calcification and elastocalcinosis (Beazley et al., 2013a) in the warfarin-induced calcification mouse model.

In the glaucoma field, TGM2 has been well-characterized. It was shown to be present in the trabecular meshwork cells and tissue, and elevated in glaucoma specimens when compared to age-matched controls (Fuchshofer et al., 2005; Tovar-Vidales et al., 2008, 2011; Welge-Lussen et al., 2000). TGM2 was also induced by TGF β 1 and TGF β 2 in trabecular meshwork and optic nerve astrocytes at the RNA, protein and enzymatic activity levels (Fuchshofer et al., 2005; Welge-Lussen et al., 2000). Because of the well-established fact that glaucoma is associated with increased ECM material in the glaucomatous trabecular meshwork, it has been proposed that TGM2 could be responsible for deterring ECM degradation by increasing cross-linking of its proteins (Welge-Lussen et al., 2000).

5.6. Connective tissue growth factor

Connective tissue growth factor (CTGF) is a complex, secreted matricellular protein involved in many different mechanisms (Lipson et al., 2012). Different stimuli can induce the expression of CTGF. When secreted, CTGF interacts with a variety of extracellular and cell surface proteins affecting their signaling pathways and cellular functions, all of which lead to tissue remodeling. CTGF induced osteogenic differentiation of mouse primary VSMC (Huang et al., 2013), and elevated levels of CTGF in the serum in patients of biliary atresia correlated with liver stiffness (elastography) and poor disease outcomes (Honsawek et al., 2013). Inhibition of CTGF reversed the process of fibrosis and a CTGF monoclonal antibody (FG-3019), which is in clinical development, prevents carotid artery vascular stiffness in a model of diabetic rats (Lipson et al., 2012).

In glaucomatous conditions, CTGF plays a key role. Numerous studies have demonstrated that elevated concentrations of TGF β 2, as it occurs in the aqueous humor of POAG patients, seem to be responsible for changes similar to those observed in the trabecular meshwork ECM in vivo. In vitro, it has been repeatedly shown that the observed changes in the ECM are mediated by the TGF β 2 downstream effector CTGF (Fuchshofer et al., 2011; Junglas et al., 2009). In vivo, transgenic mice overexpressing CTGF by adenoviral gene transfer or by generation of a β B1-crystallin driven CTGF cDNA exhibit elevated IOP and loss of optic nerve axons (Junglas et al., 2012). Also, SC cells from glaucoma patients which have increased levels of CTGF, were shown to be stiffer than those from matched healthy individuals (Overby et al., 2014).

Altogether, the combined evidence indicates that the same agents described to affect mineralization and stiffness in the vascular system have also been described as affecting IOP and glaucoma in the eye. The revised data findings thus support a potential central mechanism of stiffness in glaucoma which coincides with the current thinking in the field (Braunger et al., 2015). The evidence further point out that the stiffness observed in glaucomatous manifestations could be in part triggered by the induction of mineralization of the glaucoma associated tissues.

6. The MGP gene: a master regulator of calcification and stiffness

Matrix Gla (MGP) is a 10 kD secreted protein, highly abundant in cartilage chondrocytes and in VSMCs of the tunica media of arteries (Wallin et al., 2001). Activation of MGP occurs by a posttranslational modification where its glutamic acid residues (Glu) are converted to γ -carboxyglutamic acid (Gla) by a vitamin K-dependent γ -carboxylase enzyme (Price et al., 1983). In patients, *MGP* polymorphisms been linked to coronary artery calcification (Crosier et al., 2009; Herrmann et al., 2000). In mice, ablation of *Mgp* results in massive arterial calcification and death at about 5–6 weeks of age (Zieman et al., 2005). The mechanisms by which MGP inhibits calcification in the arteries are not totally understood. After being carboxylated, MGP undergoes a conformational change and binds calcium. This conformer of carboxylated MGP binds and sequesters BMP2, a potent inducer of bone formation (Ducy and Karsenty, 2000). It is believed that MGP acts as a negative regulator of BMP2. Also, *Mgp* colocalizes with elastin in the arterial elastic lamina and, in the KO mice, mineral deposition and calcification lesions are seen along the elastic lamella (elastocalcinosis). In this *Mgp* null mouse, calcification in the VSMC is preceded by elastin fragmentation (Beazley et al., 2013b) which appears to be the result of the induction of the elastase enzyme adipsin. An additional calcification mechanism of the *Mgp* KO appears to be associated with the presence of *Tgm2*. Mice which have ablated both genes, *Mgp* and *Tgm2*, live longer and exhibit less elastocalcinosis than the single null *Mgp*. *Tgm2* accumulates in the calcifying aorta of the *Mgp* null mice (Kaartinen et al., 2007) and is a key regulator of the transformation of VSMC into osteogenic cells (Johnson et al., 2008) (see above). Ablation of *Tgm2* then serves to ameliorate the calcification effects.

In cartilage, MGP prevents osteogenic transformation of chondrocytes. An autosomal recessive rare disease, Keutel syndrome, has been associated with several mutations in the *MGP* gene (Tüysüz et al., 2015). Patients develop abnormal cartilage calcification,

brachytelephalangism, peripheral pulmonary artery stenosis and in some, optic atrophy (Tüysüz et al., 2015). Less than 30 cases globally have been reported since its discovery in 1970 and phenotypes of the patients show clinical variability (Tüysüz et al., 2015). All the exams reported include young patients. Although very few ophthalmological exams have been described, in one case, a 6-year old patient had sudden loss of vision in both eyes at the age 3 and showed bilateral optic nerve atrophy (Hur et al., 2005). The severity of the disease is depending on the extent of the pulmonary involvement and limited reports seem to indicate that patients die at a younger age.

Inactive MGP is also associated with arterial stiffness. A recent clinical study enrolled 1001 patients in a family-based multicenter in Switzerland. Inactive MGP (dephospho-uncarboxylated MGP) was quantified in the plasma by ELISA and PWV was determined by applanation tonometry to measure stiffness. After adjusting for age and other cardiovascular factors, results showed that high levels of inactive MGP were positively associated with arterial stiffness (Pivin et al., 2015).

7. The MGP gene and MGP protein in the eye

7.1. In vitro studies in human trabecular meshwork

Earlier studies from our laboratory had identified *MGP* among the ten most highly expressed genes in the human trabecular meshwork (Gonzalez et al., 2000), a finding later confirmed by other investigators (Vittal et al., 2005; Wirtz et al., 2002). Subsequent studies demonstrated that trabecular meshwork in primary cells and perfused organ cultures had as much carboxylase activity as the VSMC, that the MGP was carboxylated and that the active protein was functional and able to reduce the calcification activity of BMP2 (Xue et al., 2006). Subsequently, a number of biological studies revealed that expression of *MGP* in primary HTM cells responded to glaucomatous insults. Thus, its expression was increased by elevated IOP at the time of homeostatic response (Vittitow and Borrás, 2004) and it was reduced by treatment with TGFβ1 (Vittitow and Borrás, 2004), TGFβ2 (Xue et al., 2007) and dexamethasone (Carabana et al., 2012). Most important, trabecular meshwork specimens from glaucoma patients exhibited decreased levels of *MGP* mRNA and higher levels of the calcification marker ALP when compared to age-sex matched controls (Xue et al., 2007). When the trabecular meshwork cells' *MGP* expression was blocked by *MGP* siRNA, the levels of ALP were increased (Xue et al., 2007).

7.2. In vivo studies in transgenic mice. Generation of *Mgp* KO and KI

7.2.1. *Mgp* Knock-Out mice, *Mgp*^{-/-}—As mentioned above, the *Mgp*-KO (Luo et al., 1997) is lethal and dies at 5–6 weeks of age. The *Mgp* KO provided a first glimpse on the effects of *Mgp* ablation in the eye but studies were limited due to the early death and compromised phenotype of the mice. Before their death at 5 weeks, mice exhibit a significant reduction in size, contorted shape and compromised breathing. Despite these limitations, unpublished experiments from our lab noted structural changes in the trabecular meshwork at the light and electron microscopy level, a moderate increase of IOP, and the increased expression of some calcification markers in the angle tissues. The detailed results of these experiments are in preparation.

7.2.2. Mgp-Cre Knock-In, Mgp^{CreKI/CreKI}—Localization and distribution of the *Mgp* expression in the eye are key to elucidate the mechanistic link between MGP and glaucoma. Attempts to determine the spatial and temporal patterns by in situ hybridization and immunohistochemistry were not indicative, due in part to unsuitability of the reagents used. This prompted us to use mouse genetics technology and to generate an *Mgp*-Cre Knock-In (*Mgp*-Cre KI) (Borrás et al., 2015b). In this mouse, an internal ribosomal entry site (IRES)-Cre recombinase expression cassette was inserted in between the translational *Mgp* STOP signal and its polyA. The Knock-In *Mgp*^{CreKI} was designed to express Cre recombinase only in cells where *Mgp* is expressed. The targeting vector is showing in Fig.1. When these mice are crossed with a mouse containing *loxP* sites flanking a STOP signal for a reporter gene, Cre recombination results in the deletion of the STOP and allows expression of the reporter gene. Histological assessment of the expressed reporter in their offspring does correspond to the expression of the gene driving the Cre. These Cre KI mouse lines are extremely valuable for the determination of localization of the desired gene expression in specific cell types, its temporal distribution during embryonic and adult development, and its regulation under disease conditions. An accurate determination of these parameters is essential to establish the role between MGP and glaucoma.

7.3. Localization of Mgp expression in the eye by using the Cre KI mouse line

7.3.1. Generation of Mgp^{CreKI/+}; R26R^{lacZ/+} Mgp-lacZ KI mouse line—Crosses of the *Mgp*-Cre KI mouse with the reporter mouse line *R26R-lacZ* (Soriano, 1999), resulted in the generation of *Mgp*^{CreKI/+}; *R26R*^{lacZ/+} (*Mgp-lacZ* KI) mouse line. The reporter here was the *LacZ* gene whose activity was measured by enzyme histochemistry on a X-gal substrate. In this study (Borrás et al., 2015b), a total of 43 experimental double heterozygous *Mgp-lacZ* KI showed functional Cre recombination in all eyes from ages 1–8 months obtained from different breeding pairs and from two founders. The blue β -gal staining indicative of high levels of Cre activity was intense and consistently present in just two areas of the eye globe, one on the anterior segment and the second in the posterior pole (Fig. 2).

7.3.2.. Expression in the anterior sement—The first area comprised the trabecular meshwork region, extending to the ciliary muscle and the supra-scleral zone above the SC. Given the function of the *MGP* gene in all other body tissues, the presence of this gene in the outflow pathway and outflow surrounding regions would suggest a role and a need to maintain the appropriate softness and elasticity required to facilitate aqueous humor flow. Its expression not only in the trabecular meshwork, but also on the ciliary muscle and immediate sclera above the canal further highlights the relevance of this gene in those tissues involved in the regulation of IOP.

7.3.3.. Expression in the posterior segment—The second main site of *Mgp* expression included the sclera tissue, in particular the peripapillary region surrounding the optic nerve. Blue cells are shown first on the mid-sclera and are restricted to the layer immediately underneath of the choroid (Borrás et al., 2015b). The expression increases towards the posterior sclera and exhibits the highest intensity and expansion at the peripapillary sclera supporting the optic nerve (Borrás et al., 2015b). These results seem to indicate that the peripapillary region appears to be in need of a strong protection against

becoming stiff/calcified, and that it is using the Mgp protein as one of the potential mechanisms to avoid higher stiffness. Thus, taking into account the localization of expression of the *Mgp* gene and its function, it could be suggested that the *Mgp* encoded protein could be affecting both the outflow facility and the biomechanical properties of the ONH region.

8. Sclera stiffness and myopia

The presence of *Mgp* expressing cells starting at the mid-sclera of the *Mgp-lacZ* mouse together with the fact of the high expression of this gene in cartilage, would agree with reports that the sclera of many vertebrates contains a cartilaginous cell layer (Gottlieb et al., 1990; O'Steen and Brodish, 1990; Yoshitomi and Boorman, 1990). Chondrocytes have been identified in the sclera of myopia models in chick (Kusakari et al., 1997, 2001). It would seem logical to think that a reduction of MGP in the sclera would tend to calcify the tissue, affect its rigidity and have an influence in the development of myopia. Interestingly sclera calcification has been amply reported in the literature since as early as 1958 by David Cogan (Cogan et al., 1958), and it has been associated with aging and disease conditions since then (Patrinely et al., 1982; Wong et al., 1979). In one example in Fisher rats, the scleral cartilage is converted to bone, and 95% of the rats show a calcified sclera (O'Steen and Brodish, 1990). The authors speculate then that scleral bone formations could compromise optic circulation and influence photoreceptor death during aging. The presence of the *Mgp* gene in the sclera is providing the first explanation of a likely mechanism responsible for the long observed eye sclera calcification.

In another turn, numerous population studies have associated high myopia as a risk factor of POAG (Marcus et al., 2011). Similarly, many studies have demonstrated that visual field loss progression is greater in POAG patients with high myopia, than in POAG patients without myopia (Lee et al., 2008). It would be an attractive idea to consider that expression of *MGP* could be beneficial for glaucoma not only by facilitating outflow and maintaining in check the stiffness of the ONH but also by ameliorating hardening of the sclera and high myopia.

9. Validation of the Cre-directed Mgp expression

Validation of the localization of expression of *Mgp* in the mouse eye was shown in an independent study where the *Mgp*-Cre KI mouse was crossed with a different reporter strain (*R26R-tdTomato*) to generate *Mgp*^{CreKI/+}; *R26R*^{tdTomato/+} (*Mgp*-tdTomato KI). The reporter mouse here carried the STOP signal for the red fluorescent protein variant (tdTomato) flanked by *loxP* sites. In this study, analysis of whole mounts of the eye of the double heterozygous showed a specific positive red fluorescence on the limbus, indicative of the trabecular meshwork fluorescent tissue underneath. Likewise, the red fluorescence was also observed on the posterior pole at the optic nerve head region (Fig. 3). In the fluorescent images of the whole animal, red fluorescent cartilage tissues could be also observed, further validating that the Cre activity is specifically directed to the established *Mgp* expressed tissues (Fig. 3). Because the half-life/photostability of the reporter proteins is 24–48 h for *lacZ* and 1 h for tdTomato (Martin et al., 2012; Smith et al., 1995), the protein marker expression observed in these studies represents the true transcription of the gene.

10. Concluding thoughts

Together, all evidence regarding calcification, stiffness, the role of MPG in the vascular system, its numerous glaucoma-associated properties and its localization to the most relevant eye tissues, supports that this protein is a strong candidate for the regulation of stiffness in glaucoma. The original findings of the gene's high abundance in the trabecular meshwork tissue coupled with the confirmation of the presence of its activating enzyme and the actual active protein, were the first signs that led us into the path of investigating its purpose in the eye and its involvement in modulating outflow. In vitro and ex-vivo work showed that all results, from regulation by elevated IOP to inverse correlation with calcification in the tissue from glaucoma patients, matched with those expected from a glaucoma relevant gene/protein. Thus, *MGP* was regulated by elevated IOP, and calcification markers were upregulated in the trabecular meshwork of POAG patients. *MGP* expression levels were reduced in the trabecular meshwork of the same POAG patients where calcification was increased. *MGP* levels were also reduced in trabecular meshwork in cells treated with TGF β and dexamethasone, and ablation of *MGP* by siRNA induced calcification. On the other side, the available morphology data appears to be inconsistent with the presence of calcification in the trabecular meshwork. Despite an extensive number of morphological studies in this tissue, there has been no reports on the presence of calcific plaques as those described in the sclera, albeit no similar calcification assays have been reported. In contrary, there are reports of increased stiffness in the trabecular meshwork. Although as described above, stiffness can occur independently of calcification, the high levels of an active calcification inhibitor in the tissue, and its reduction in glaucoma, makes logical to assume that calcification is at least in part responsible for the observed stiffness.

Continuing effort to support the relevance of *MGP* function's in glaucoma would need to come from experiments in vivo. In rats, induction of calcification of the trabecular meshwork results in elevation of IOP and degeneration of RCC, two landmarks of glaucoma. In mice, the generation of the *Mgp-Cre* KI strains reveals that *MGP* is not only localized to the trabecular meshwork region, but to the peripapillary sclera surrounding the ONH. Because of the relevance of the stiffness in this region to withstand the mechanical insults transmitted by elevated IOP, this second site localization raises the level of the importance of *MGP* in glaucoma. Although experiments with the *Mgp-KO* are limited due the early death of the mouse, conditional KO's would be able to elucidate whether a non lethal *Mgp* deletion in glaucoma-related tissues would significantly affect the development of the disease. Generation of such floxed *Mgp* mice are in progress in our laboratory. Lastly, the fact that a reduction in *MGP* would also affect ocular vascular stiffness and potentially myopia, indicates that this protein could address most the risk factors which have been associated with the disease in the clinical setting. The uniqueness of a single gene ameliorating the stiffness in the anterior, posterior segment as well as in the eye vasculature would make *MGP* a highly desirable therapeutic target for glaucoma.

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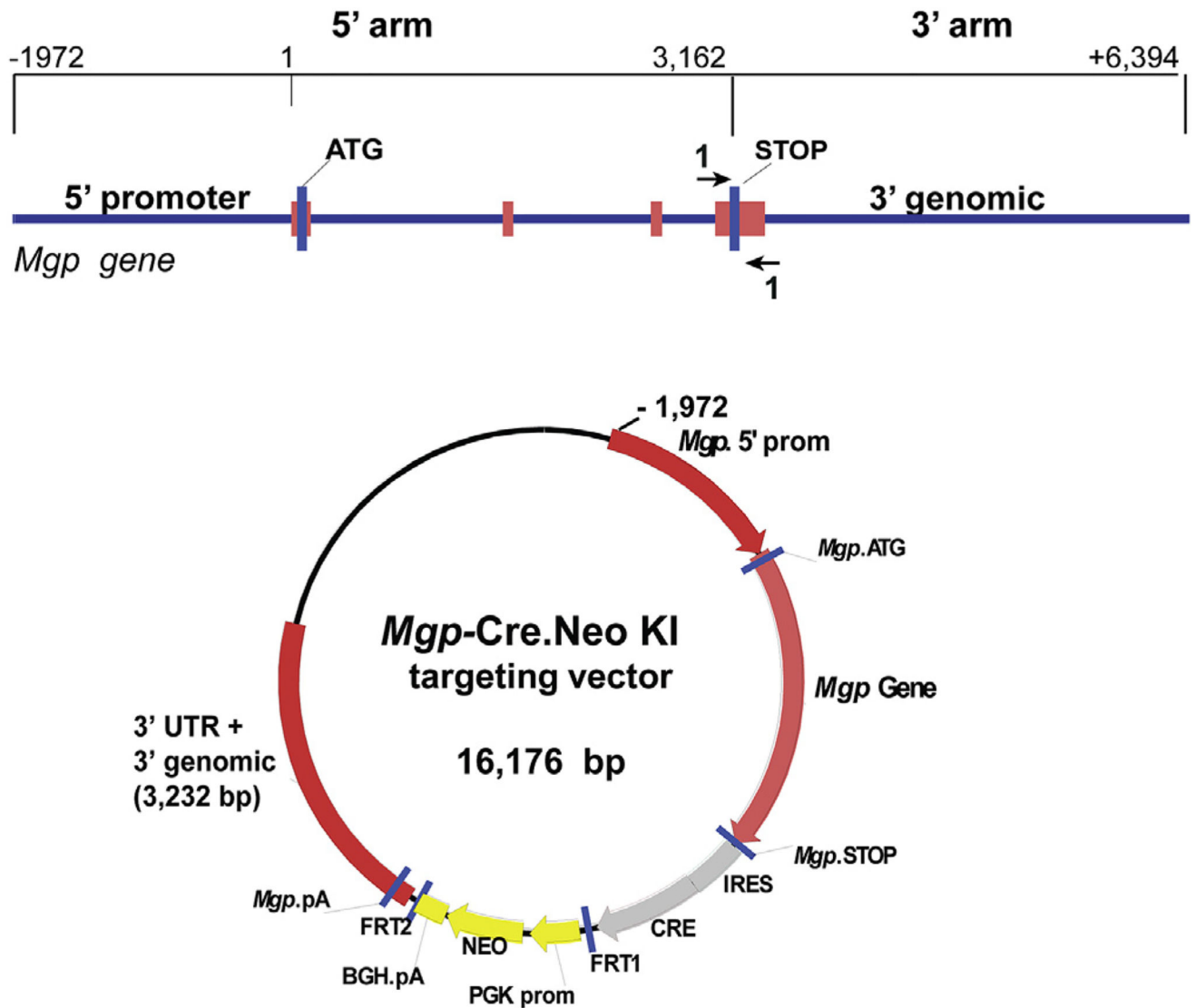


Fig. 1. Diagram of the targeting strategy to generate the *Mgp*-Cre Knock-In (*Mgp*-Cre KI) mouse. Targeting vector consists of homology arms to 1972 promoter bp, exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, exon 4 and 3022 bp of 3' genomic sequences (total 8.3 kb). Sequences containing the IRES-Cre-FRT-flanked neomycin resistance gene cassette were inserted between the *Mgp* STOP signal and the 3' UTR. The Neo cassette was later excised by crossing the mouse with the flippase (*FLP*) deleter mice followed by removal of the introduced *FLP* gene by intercrossing the heterozygous strains. The engineered mouse, *Mgp*-Cre KI, renders the Cre under the transcriptional control of the *Mgp* gene.

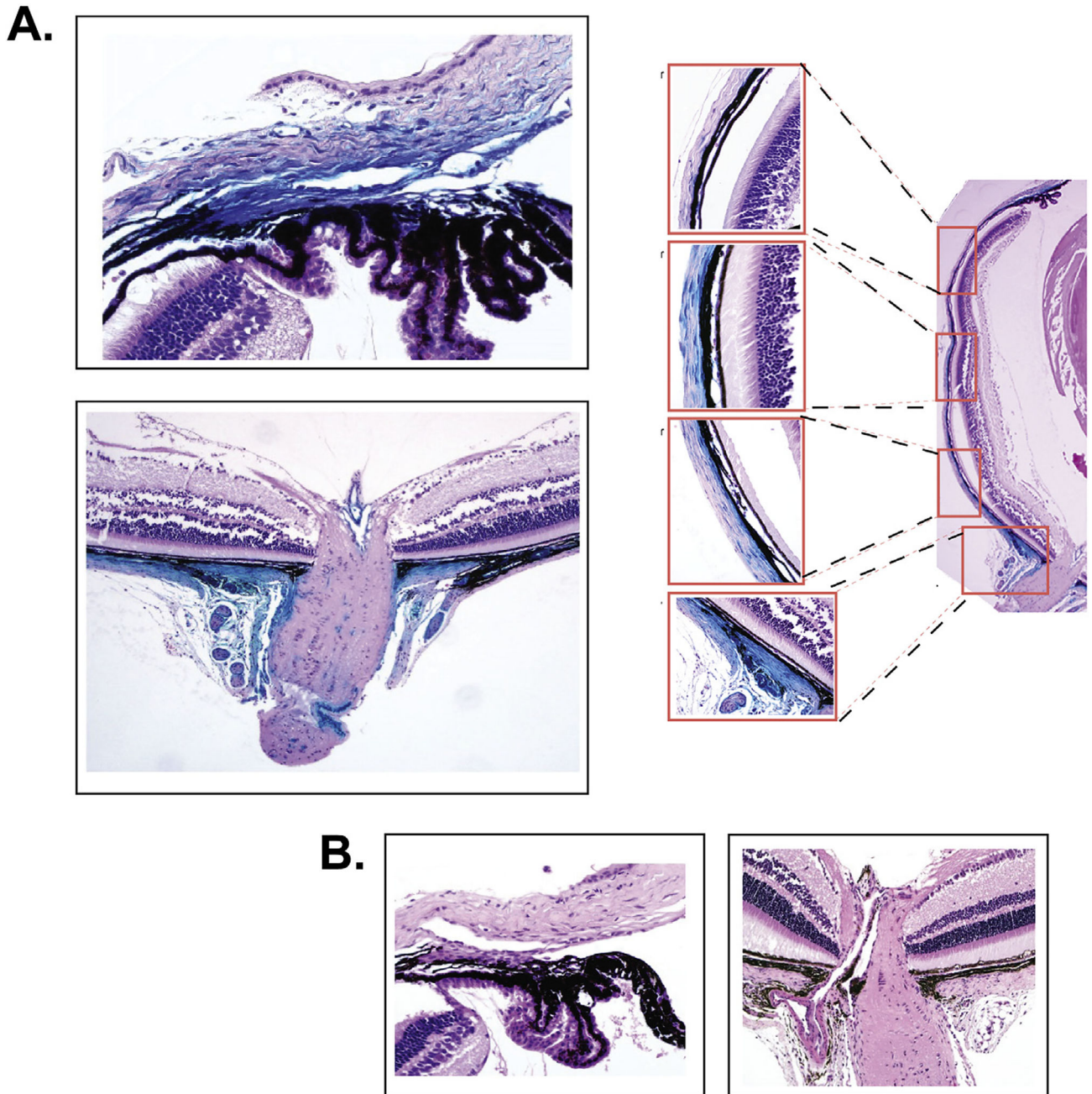


Fig. 2. *Mgp* Cre-mediated *lacZ* expression in the glaucoma associated tissues of the eye. A) *left*: meridional paraffin sections from the eyes of 4–6 weeks *Mgp-LacZKI* (*Mgp*^{CreKI/+}; *R26R*^{lacZ/+}) mice assayed for β -galactosidase activity and counterstained with hematoxylin and eosin. *Mgp* driven expression is very abundant in the trabecular meshwork region (TM) and peripapillary region of the ONH. *Right*: *Mgp* driven expression in the sclera is localized to the internal layer, increases from mid to posterior and reaches high levels at the peripapillary site around the ON. B) meridional paraffin sections from the eye of control mice (*Mgp*^{+/+}; *R26R*^{lacZ/+}).

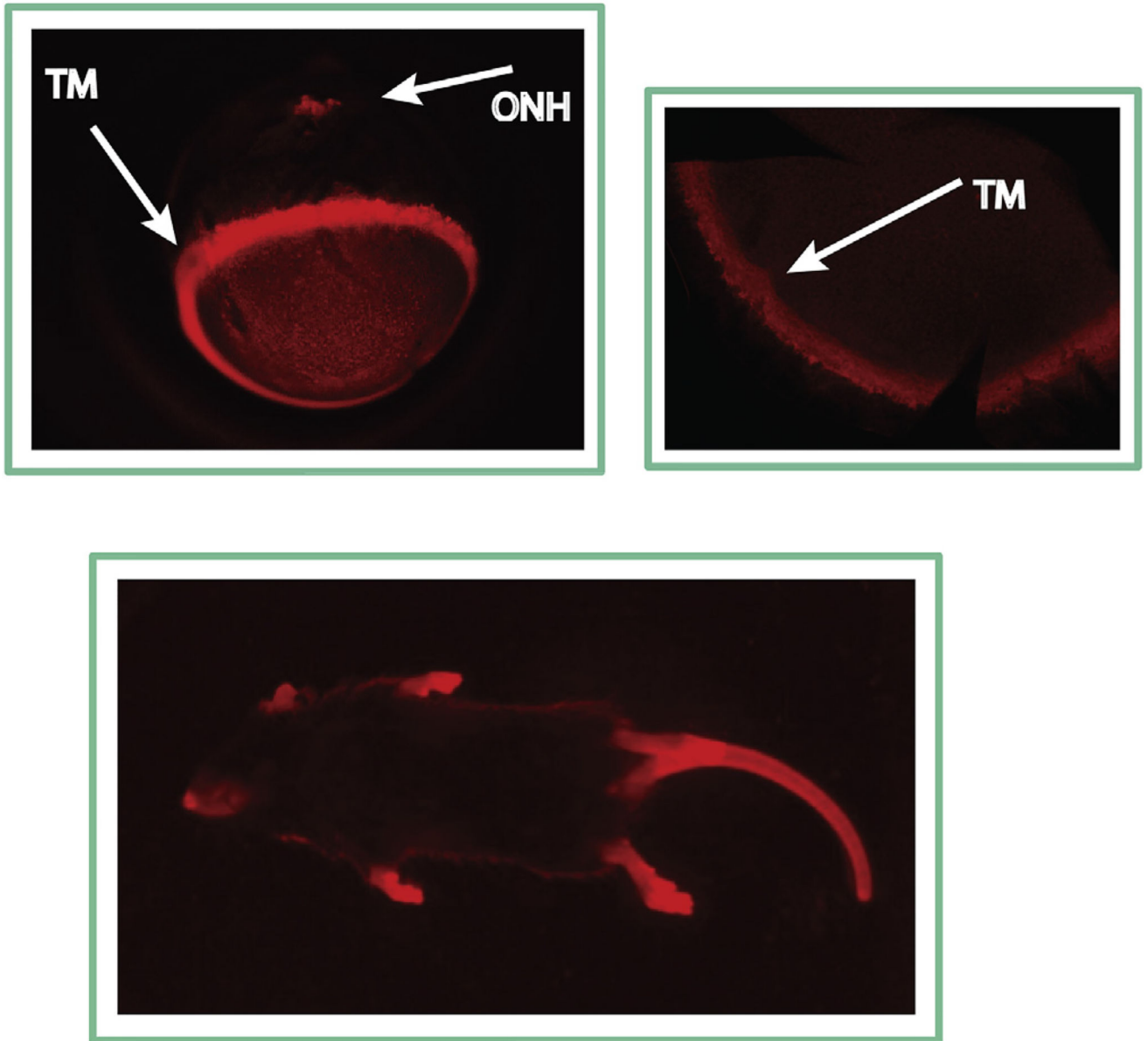


Fig. 3. *Mgp* Cre-mediated tdTomato expression in the *Mgp*-tdTomato KI (*Mgp*^{CreKI/+}; *R26R*^{tdTomato/+}) mice. Tissues with *Mgp* driven expression are fluorescent red. *Top*: Images from the whole eye (*left*) and dissected anterior segment (*right*) showing intense fluorescence on the trabecular meshwork and ONH area. *Bottom*: Image of a whole mouse photographed with a UV-transilluminator box. Cartilage tissues, where *Mgp* is abundantly expressed show intense red fluorescence, validating the *Mgp* driven expression.