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The Microfibril-Associated Glycoproteins (MAGPs) and the Microfibrillar Niche

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Abstract

The microfibril-associated glycoproteins MAGP-1 and MAGP-2 are extracellular matrix proteins that interact with fibrillin to influence microfibril function. The two proteins are related through a 60 amino acid matrix-binding domain but their sequences differ outside of this region. A distinguishing feature of both proteins is their ability to interact with TGFβ family growth factors, Notch and Notch ligands, and multiple elastic fiber proteins. MAGP-2 can also interact with αvβ3 integrins via an RGD sequence that is not found in MAGP-1. Morpholino knockdown of MAGP-1 expression in zebrafish results in abnormal vessel wall architecture and altered vascular network formation. In the mouse, MAGP-1 deficiency had little effect on elastic fibers in blood vessels and lung but resulted in numerous unexpected phenotypes including bone abnormalities, hematopoietic changes, increased fat deposition, diabetes, impaired wound repair, and a bleeding diathesis. Inactivation of the gene for MAGP-2 in mice produced a neutropenia yet had minimal effects on bone or adipose homeostasis. Double knockouts had phenotypes characteristic of each individual knockout as well as several additional traits only seen when both genes are inactivated. A common mechanism underlying all of the traits associated with the knockout phenotypes is altered TGFβ signaling. This review summarizes our current understanding of the function of the MAGPs and discusses ideas related to their role in growth factor regulation.

Keywords

Microfibril; Microfibril-associated glycoprotein; MAGP; diabetes; osteoporosis; obesity; TGFβ

Introduction: The microfibrillar niche

Extracellular matrix microfibrils are 10–15 nm filaments traditionally viewed as static extracellular matrix (ECM) fibers that provide strength to tissues, facilitate elastin assembly and sequester latent TGFβ. We now know that microfibrils are complex polymers

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specifically modified by ancillary proteins that associate with fibrillin to address specialized needs. The ability to tailor microfibril activity provides a dynamic mechanism for altering growth factor signaling, controlling morphogenic gradients, and influencing direct cell-matrix interactions.

The major structural proteins of the microfibrils are the fibrillins (FBN1, FBN2 & FBN3), which are large modular glycoproteins consisting of repeating calcium-binding epidermal growth factor (EGF)-like domains interspersed between unique 8-cysteine domains [1–3]. These proteins arose >600 million years ago and remained relatively unchanged over that period [4]. With the appearance of vertebrates, there emerged a unique set of microfibril-associated proteins (MAGPs, Fibulins-4 & -5, and EMILINs) [5,6] that interact with fibrillin to lend new functions to microfibrils. Their abundance in the ECM of both developing and mature tissues points to the role that microfibrils play in creating an extracellular environment, or niche, that influences cellular phenotypes. It is now clear that this "microfibrillar niche" is a major regulator of tissue development and repair.

Microfibrils provide information to cells through direct interactions with cell-surface receptors and by regulating growth factor signaling—particularly growth factors in the TGFβ family. Microfibrillar proteins that interact directly with integrins include the fibrillins, tropoelastin, EMILIN1, MAGP-2, and fibulin-5 [7–13]. MAGP-1 and MAGP-2 are able to signal through notch receptors [14], and tropoelastin interacts with cell surface heparan sulfate proteoglycans and can modulate signaling through IGF and PDGF receptors [15,16]. In terms of growth factor signaling, fibrillin's ability to interact with and sequester the bone morphogenic proteins (pro-BMPs) and the large latent complex of TGFβ (TGFβ-LLC) is well-established [17,18], but other microfibrillar proteins also interact with TGFβ family members. EMILIN1, for example, interacts with proTGFβ and prevents its activation [19]. The MAGPs interact with active but not latent TGFβ as well as with several members of the BMP family [20]. Clearly, the proteins that contribute to the microfibrillar niche have the potential to stimulate or modulate the major extracellular signaling pathways.

This review will focus on the two proteins belonging to the **M**icrofibril **A**ssociated **G**lyco**P**rotein (MAGP) family and will summarize our current understanding of their structure, functional properties, and how they interact with fibrillin to define the characteristics of the intact microfibril. MAGP-1 was the first protein in this family to be characterized followed shortly thereafter by MAGP-2. Three other proteins, MFAP-1, -3, and -4, [21–24] were grouped together with MAGP-1 and MAGP-2 because of their small size and localization to microfibrils. However, recent findings show that MFAP-1 and MFAP-3 are not extracellular matrix proteins but reside in the nucleus where MFAP-1 participates in RNA splicing [25,26] and MFAP-3 functions as a nuclear kinase [27]. Even though these three proteins have no structural or sequence homology with MAGP-1 or MAGP-2 or with each other, they, as well as the MAGPs, were assigned the gene name *MFAP*. This nomenclature is unfortunate since MFAP-1, -3, and -4 are clearly not in the same gene family as the MAGPs and the gene names are often mixed up with protein names. For example, MAGP-1 and MAGP-2 were given the gene names *MFAP2* and *MFAP5*, respectively, with *MFAP2* often being mistaken for MAGP-2 in the literature and online databases. For the sake of clarity, this review will adopt the gene nomenclature of *MAGP1* in

place of *MFAP2* for MAGP-1 and *MAGP2* in place of *MFAP5* for MAGP-2. Information about MFAP-1, -3, and -4 can be found in a previous review of the MAGPs and other non-fibrillin microfibril proteins [28].

MAGP-1

Microfibril-associated glycoprotein-1 (MAGP-1) was initially isolated from the elastin-rich bovine ligamentum nuchae using a rigorous biochemical extraction protocol [29–31]. Within the extract that contained MAGP-1 was a smaller molecular weight protein (MP25) that, when characterized, was found to have some structural similarity to MAGP-1 and was given the name MAGP-2 [32]. MAGP-1 has the widest distribution of the MAGPs and appears to be a constituent protein of most, if not all, vertebrate microfibrils. Figure 1A shows MAGP-1 localized to elastic fiber microfibrils in ligament tissue using electron microscopy and immunogold labeling. Microfibrils extracted from tissues using crude collagenase or chaotropic agents have a beads-on-a-string appearance with a ~50 nm spacing between the beads [33]. Immunogold labeling localized MAGP-1 in these fibers to the bead region (Figure 1B) with the suggestion of multiple MAGP-1 molecules at each bead locus [34]. The same ~50 nm distribution was visualized on native (nonextraced) microfibrils with anti-MAGP-1 immunogold labeling. Note that the bead structures are unique to extracted microfibrils and are not evident in native fibers (Figure 1C). MAGP-2 has a distribution similar to MAGP-1 (Figure 1D) suggesting that both proteins bind to similar sites on the fibrillin molecules (discussed in detail below). The regular spacing of the anti-MAGP antibodies suggests that every MAGP binding site is occupied in a normal microfibril.

Although enriched in cysteine, MAGP-1 lacks the cysteine-containing epidermal growth factor-like (EGF) repeating motifs found in the fibrillins, fibulins, and LTBPs [35]. The N-terminal half of the protein is acidic, enriched in proline, and contains a cluster of glutamine residues. The C-terminal half contains all thirteen cysteine residues and has an overall net positive charge (Figure 2). The molecular mass of MAGP-1 calculated from its mRNA is ~20kDa, although the protein isolated from tissues migrates at ~30 kDa on SDS-PAGE [35]. This is also true for MAGP-1 produced by cell-free translation or by bacterial expression systems, suggesting that its anomalous migration on gels is a function of its primary structure and not post-translational modifications. Purified MAGP-1 has a propensity to form dimers and higher molecular weight multimers that do not dissociate with denaturation and disulfide bond reduction. Functional mapping studies identified the conserved glutamine-rich motif as contributing to self-association of the protein, most likely through formation of extremely stable parallel β-pleated “tape structure” [36,37].

MAGP-1 gene structure, alternative splicing, and tissue expression

The origin of the MAGP-1 gene dates to the beginning of the vertebrate lineage 650–800 My ago [6,38] and predates both tropoelastin [39,40] and MAGP-2 [6]. MAGP-1 in humans is encoded by a single copy gene, *MFAP2, (MAGP1 in this review)* located on chromosome 1p36.1- p35 [41]. This is a region with conserved synteny to mouse chromosome 4, where the orthologous *Magp1* gene maps [42]. The human, mouse, and bovine genes are divided into one noncoding and eight coding exons whose splice sites, codon splice phase, and

protein coding sequence are highly conserved. The gene in human and mouse contains a single transcription start site and exon 1 is untranslated [43]. Sequences upstream of the transcription start site lack TATA boxes in both genes, but they do contain multiple Sp1 binding sites and binding sites at roughly conserved positions in both sequences for AP-2, AP-4, NF-**k** B, and c-ETS transcription factors [43,44]. Protein diversity is generated from the single MAGP-1 gene through alternative splicing [43], with one transcript encoding an intracellular form of the protein [45].

MAGP-1 mRNA is widely expressed in mouse tissues, mainly in mesenchymal and connective tissue cells, where it is easily detected as early as day 8.5 of development [42]. Figure 3 compares the temporal expression profile of MAGP-1, MAGP-2, fibrillin-1 and fibrillin-2 in developing mouse aorta. A similar profile has been reported in lung [46]. Expression of MAGP-1 is highest in the fetal and neonatal period, and lowest in the adult. MAGP-2 shows the opposite expression pattern, being lowest in the fetal period and rising throughout the neonatal period to highest levels in adult tissue. Interestingly, fibrillin-1 has an expression pattern like MAGP-2 whereas fibrillin-2 follows the pattern of MAGP-1. It should be noted, however, that expression of the MAGPs is relatively high compared to other proteins at all stages of development.

Posttranslational modifications: Glycosylation, transglutamination, and tyrosine sulfation

MAGP-1 is a glycoprotein [31] with O-linked mucin type carbohydrates [35]. At least three glycosylation sites in the amino terminal half of the molecule were identified and mutational studies showed that glycosylation occurs at more than one of these sites [36] (Figure 2). There are several other modifications in the amino terminus that influence MAGP1-function. For example, the conserved glutamine at position 20, two residues away from the amino terminus following cleavage of the signal peptide, is a major amine acceptor site for the transglutaminase reaction. A transglutaminase crosslink stabilizing interactions between MAGP-1 and other microfibrillar proteins is an interesting possibility because both fibrillin-1 and tropoelastin are substrates for transglutaminase [47,48]. Another interesting functional modification of MAGP-1 is sulfation of tyrosine residues that lie in a conserved sulfation consensus sequence (DYYDY) [36] (Figure 2). Sulfation increases the negative charge in a region of MAGP-1 that is already negatively charged due to enrichment in acidic amino acids. The result is the potential for a stronger electrostatic interaction with positively charged binding proteins, of which tropoelastin and TGFβ are classic examples. In general, tyrosine sulfation can alter the affinity and selectivity of protein-protein interactions and can modulate the oligomerization state of the sulfated protein [49]. The functional significance of tyrosine sulfation in MAGP-1 is unclear.

ECM and growth factor binding sites in the N-terminus of MAGP-1

The N-terminal portion of MAGP-1 interacts noncovalently with proteins through the anionic acidic and sulfotyrosine residues (Figure 2). The first protein shown to specifically bind to MAGP-1 was tropoelastin, the elastin precursor molecule [47,48,50]. A series of studies using inhibiting peptides and blocking antibodies identified a tropoelastin-binding sequence near the N-terminus of MAGP-1 (residues 21-38) [47,50,51]. MAGP-1's ability to bind tropoelastin suggested that it played a role in elastic fiber assembly by bridging elastin

interactions with microfibrils [51]. It is now clear from gene knockout studies in the mouse that neither MAGP-1 nor MAGP-2 is required for elastin assembly. Elastic fibers form normally when one or both MAGPs are deleted in mice (discussed below) and cells that do not make MAGP-1 (e.g., RFL6 fibroblasts) are some of the best at forming insoluble elastic fibers in culture [52]. Other ECM components that interact with MAGP-1 include the α3 chain of collagen VI [50], decorin [53], and biglycan [54]. MAGP-1 does not bind to collagens I, II, and V [50].

In addition to binding various ECM molecules, the highly interactive charged N-terminus of MAGP-1 also binds active forms of TGFβ1 and TGFβ2 as well as BMP2, BMP4, and BMP7 [20,55]. All of these growth factors bind noncovalently and with high affinity. There is specificity to this interaction since MAGP-1 does not bind TGFβ3, FGF9, FGF21 or the cytokines TNFα, IL-4, IL-13, and RANKL [56]. Interestingly, MAGP-1 does not bind LTBP1, which indicates that MAGP-1 binds active TGFβ but not the large latent complex [57].

The C-terminal matrix-binding domain and ShK homology domain

The odd number of cysteine residues (13) in MAGP-1 ensures a free sulfhydryl that can interact to form covalent MAGP-1 dimers or covalent bonds with other molecules. Disulfide bonding between cysteine residues of MAGP-1 and other microfibril components appears to be important given that reducing agents are required to extract MAGP-1 from tissues [31]. Indeed, a 54 amino acid sequence containing the first seven cysteine residues (encoded by exons 7 & 8) defines a matrix-binding domain that is sufficient for the deposition of MAGP-1 into the ECM [58] (Figure 2). The spacing between the cysteines ($CX_6CX_{12}CX_4CX_{14}CX_4$ $CX_{10}C$) does not conform to the consensus for any known cysteine-rich repeat, and it may represent a unique invention of the MAGPs. The high number of cysteines within this sequence suggests the formation of a highly structured domain and mutational studies identified every cysteine as critical for maintaining functionality [58]. The matrix-binding domain is the only conserved structural motif shared with MAGP-2 (see below) and is conserved in all species of MAGP-1 and MAGP-2.

The matrix-binding domain has a strong binding preference for tandem EGF-like motifs and has been shown using direct binding assays or yeast two-hybrid screens to interact with fibrillins, fibulins, Jagged1, Jagged2, Delta1, Notch1, and multiple EGF-like domain protein 6 (MEGF6), all of which contain EGF domain repeats [59–63]. Other molecules recognized by the matrix-binding domain include Von Willebrand factor, glycogenin, and high mobility group protein 14 (HMG14) [60]. An interesting use of the matrix-binding domain sequence is to take advantage of its matrix-binding potential to target and anchor growth factors or other proteins to the ECM of cultured cells [64].

Outside of the matrix-binding domain, homology/structure predictions of the sequence containing the last five cysteines suggest a ShK motif [also called SXC (six cysteine) motif] at the C-terminal end of the protein (Figure 2). ShK domains are common in the astacin family of metalloendopeptidases, which includes BMP1/Tolloid, meprins, and MMP23 as examples. The ShK motif in astacins is thought to mediate protein-protein interactions [65].

MAGP-1 interaction sites on fibrillin

A major binding site for MAGP-1 on fibrillin-1 was localized using direct binding studies to the fibrillin-1 N-terminal region encoded by a fragment that contains exons 1-8 (Figure 4). Because binding between the two molecules is strongly calcium-dependent, the two calcium binding EGF-like domains encoded by exons 7 and 8 were implicated in contributing to the binding site [48,66]. Other studies, however, placed the MAGP-1 binding site further towards the N-terminus near the furin cleavage site [67].

Yeast two-hybrid, ligand blotting, and solid phase binding assays mapped a MAGP-1 binding site to the third 8-cysteine motif in fibrillin-2 encoded by exon 24 (Figure 4). Interestingly, no binding was detected to sequences near the N-terminus of fibrillin-2 where MAGP-1 interacts with fibrillin-1 [59]. The internal MAGP-1 binding site was confirmed in fibrillin-1, although binding is weaker than to the fibrillin-1 N-terminal region [66,68]. The third 8-cysteine domain in fibrillin lies in the region of the gene where mutations produce the most severe phenotypes associated with neonatal Marfan syndrome (fibrillin-1) and congenital contractural arachnodactyly (fibrillin-2). It is possible that these fibrillin mutations alter the ability of fibrillin to bind MAGP-1, which may contribute to the severity of the disease. The biological relevance of two MAGP-1 binding sites on fibrillin is not understood, nor is it known whether both sites are occupied at the same time. MAGP-1 is not required for fibrillin assembly or for stabilizing lateral interactions of fibrillin molecules, since microfibrils form normally without MAGP-1 or MAGP-2 [69].

MAGP-1 regulation of growth factor availability

A major function of microfibrils outside of providing structural support to tissues is to control the availability of growth factors—particularly members of the TGFβ superfamily[70]. Fibrillin is considered the major functional protein in this regard, but the MAGPs also have a role to play in controlling growth factor signaling [20,56]. All three TGFβs are secreted as an inactive latent dimer bound to a member of the latent TGFβ-binding protein (LTBP) family. This large latent complex (TGFβ-LLC) binds covalently to fibrillin molecules in microfibrils, thereby creating a growth factor reserve in the ECM that can be activated and mobilized when needed. Many BMPs (BMP2, 4, 5, 7 & GDF5) also interact with fibrillins via a prodomain that confers latency to the growth factor [71,72]. Like TGFβ-LLC, pro-BMPs must be activated to generate growth factor activity, which usually involves the separation of the growth factor domain from the prodomain [73]. Binding studies with fibrillin fragments have mapped a high affinity-binding site for pro-BMPs and TGFβ-LLC to the N-terminal region of fibrillin-1 encoded by exons 4-7 [18,57,74]. Because MAGP-1 also binds to this region of fibrillin-1 [48,67], a possible mechanism whereby MAGP-1 can influence growth factor activity is by blocking binding of latent growth factors to the microfibril (see Figure 5A–B). Massam-Wu et al [57] showed using protein-binding assays that MAGP-1 blocks the binding of LTBP1 to a fragment of fibrillin-1 that contains the LTBP1 binding site. Conversely, LTBP1 reduces the interaction between MAGP-1 and fibrillin-1. Thus, the binding of MAGP-1 and LTBP1 (and hence, TGFβ-LLC) to fibrillin-1 appears to be mutually exclusive. When added to fibroblast cultures, MAGP-1 strongly stimulated TGFβ-receptor dependent Smad2 phosphorylation, indicating that MAGP-1 can release TGFβ-LLC from assembled microfibrils to generate active TGFβ [57]. If MAGP-1

is absent, as in MAGP-1 knockout animals, this scenario predicts more TGFβ-LLC bound to microfibrils and, hence, a larger pool of TGFβ available for activation.

Another mechanism whereby MAGP-1 might influence growth factor signaling is through its ability to directly bind active TGFβ or BMPs (Figure 5C). Here, fibrillin and MAGP-1 are working together in synergistic ways to regulate growth factor availability, although there is an important functional difference: The latent form of TGFβ that covalently binds to fibrillin requires activation and release through interactions with proteases or specific cell-surface integrins [75] (Figure 5C, red arrow). The form of TGFβ bound to MAGP-1, in contrast, is already activated and noncovalently bound (green arrow). This model provides a way for the ECM to store a pool of active TGFβ that can be easily mobilized when needed (blue arrow). Or, alternatively, MAGP-1 could serve to suppress TGFβ signaling by removing excess TGFβ from the immediate membrane microenvironment analogous to a decoy receptor (green arrow). The absence of MAGP-1 would lead to an increase in TGFβ signaling due to the inability of the microfibrillar matrix to sequester the active growth factor.

MAGP-1 knockdown in zebrafish

Important insight into MAGP-1 function came from gene inactivation studies in fish and mice. In zebrafish, morpholino knockdown of MAGP-1 resulted in morphologically normal embryos except for enlarged caudal fins. Examination of vascular integrity found dilated vessels in the brain and the eyes, irregular lumens in axial vessels, and a dilated caudal vein with altered venous plexus formation. The fish were viable with these defects and the phenotypes were incompletely penetrant [76]. Transiently expressed MAGP-1 protein rescued the vascular abnormalities, confirming that the vascular phenotypes are specific to the loss of the MAGP-1. Similar phenotypes (vessel dilation) were observed when MAGP-1 was overexpressed in zebrafish embryos indicating that a critical balance of MAGP-1 protein level is required for proper vascular morphogenesis [76]. Interestingly, fibrillin-1 morphant embryos and MAGP-1 morphant embryos exhibited overlapping vascular defects, supporting synergistic effects of these microfibrillar proteins. Fragmented elastic fibers in large vessels in the MAGP-1 morpholino fish suggest a role for MAGP-1 in elastin assembly or maintenance [76]. MAGP-1 must have a different function in the veins and small resistance vasculature, however, because these vessels lack significant amounts of elastin.

Changes in vascular patterning were also seen in a second study focused on zebrafish retinal vasculature formation. Knockdown of MAGP-1 resulted in arrested development of the hyaloid vasculature characterized by defects in angiogenic remodeling and failure of vessels to coalesce into defined branches [77]. A characteristic of the phenotype was an aggregate of vascular endothelial cells at the posterior lens and an extremely reduced number of branches that appear thicker, poorly patterned and stagnated compared to hyaloid vasculature of wild-type (WT) larvae or control morphants [77]. Interestingly, immunofluorescence analysis of the developing mouse eye showed that capillaries on both the posterior and anterior surface of the postnatal day one lens are embedded in a loose ECM that is enriched in MAGP1 [78].

Reduced caudal and hyaloid vascular branching in *MAGP-1* morphant embryos suggests a role for MAGP-1 in vascular patterning. Whether MAGP-1 works through direct cell-matrix interaction or by regulating growth factor availability is not know. Alternatively, defective plexus formation may be secondary to MAGP-1's ability to regulate Notch signaling (discussed below), which is an important pathway in vessel sprouting and angiogenesis. While more studies are required to completely understand the role MAGP-1 plays in maintaining vessel patterning or vessel wall integrity, both zebrafish studies confirm that MAGP-1 is not required for the specification of vascular cell fate.

MAGP-1 gene inactivation in mice

Inactivation of the *Magp1* gene in mice [20] resulted in numerous unexpected phenotypes that were more widespread than in fish. Surprisingly, tissues rich in elastin (skin, vasculature, and lung) were relatively unaffected. There was no apparent skin laxity and no noticeable defect in lung structure. Vascular development was normal and blood pressure and vascular compliance of large elastic vessels were similar to WT animals. Ultrastructural analysis of the aortic wall of *Magp1*$^{-/-}$ mice in a mixed genetic background found no difference in fiber orientation, elastin density, or lamellar number when compared to controls. Instead, MAGP-1 deficiency manifested as bone abnormalities, hematopoietic changes, increased fat deposition, diabetes, impaired wound repair, and a bleeding diathesis [20]. Taken together, these findings suggest that MAGP-1 is dispensable for elastic fiber assembly in the mouse but important for other processes of tissue homeostasis or differentiation. The severity and penetrance of the phenotypes were influenced by mouse genetic background, but most persisted in pure, mixed, and outbred strains [20].

Bone defects

Microfibrils are abundantly expressed in bone, and can be found in the stromal vasculature, periosteal matrix, surrounding osteocytes, chondrocytes, and osteons, on the endochondral surface, and within the trabecular matrix [79,80]. *Magp1*$^{-/-}$ mice in a mixed genetic background demonstrated a spectrum of bone abnormalities including infrequent mild kyphosis and scoliosis, abnormal rib cage formation, and lesions of the long bones [20]. In a pure outbreed Black Swiss (BSw) background, kyphosis, scoliosis, and rib cage abnormalities were seen infrequently whereas the long bones lesions persisted with complete penetrance. No syndactyly, polydactyly, or arachnodactyly were seen in any background and tibial length measurements demonstrated only a slight overgrowth. Otherwise, skeletal development was normal.

Consistent with a weakened bone suggested by the appearance of spontaneous bone fractures, femurs from *Magp1*$^{-/-}$ mice on a pure BSw background were found to have reduced whole bone strength and rigidity when subjected to mechanical testing. When examined by longitudinal dual energy x-ray absorptiometry (DEXA) and microcomputed tomography (μCT), *Magp1*$^{-/-}$ animals were found to have an age-dependent osteopenia associated with reduced bone mineral density in the trabecular and cortical bone [55] (Figure 6A & B). In addition, the trabecular microarchitecture was more fragmented and the diaphyseal cross-sectional area significantly reduced. This defect was not due to an

osteoblast deficiency since *Magp1*$^{-/-}$ mice exhibited normal osteoblastogenesis, osteoblast number, mineralized bone surface, and bone formation rate. Instead, these mice had ~60% more osteoclasts compared to WT mice, suggesting that increased bone resorption was responsible for the osteopenia seen in MAGP-1-deficient animals [55]. Osteoblasts from *Magp1*$^{-/-}$ mice had substantially elevated expression of receptor activator of nuclear factor κB ligand (RANKL) [56] (Figure 6C), a protein that is essential for directing the formation of osteoclasts from their bone marrow macrophage precursors [81]. Expression of osteoprotegerin, a RANKL decoy receptor that blocks RANKL binding was unchanged. Interestingly, bone marrow macrophages from *Magp1*$^{-/-}$ mice showed a higher propensity than did WT cells to differentiate to osteoclasts in response to RANKL, suggesting that they are primed to respond to osteoclast-promoting signals [55]. Elevated RANKL expression was normalized when cultured *Magp1*$^{-/-}$ osteoblasts were treated with a neutralizing antibody targeting free TGFβ [56] (Figure 6C). These studies show increased RANKL expression associated with MAGP-1 deficiency and provide a link to altered TGFβ signaling as the causative signaling pathway regulating RANKL expression in MAGP-1-deficient osteoblasts.

Bleeding phenotype and hemostasis

The initial indication of a bleeding defect in *Magp1*$^{-/-}$ mice was a prolonged bleeding time when tail snips were harvested for genotyping. Follow-up studies using a carotid artery injury/flow assay confirmed a ~2-fold delay in thrombotic occlusion in MAGP-1-deficient mice compared to WT controls [82] (Figure 7A). Mice heterozygous for the MAGP-1 deletion showed intermediate occlusion values, indicative of a gene dosage effect. The extended bleeding times in the tail vein and carotid artery indicate that the absence of MAGP-1 affects hemostasis in both high (arterial) and low (venous) blood pressure systems. Interestingly, injection of recombinant MAGP-1 into the tail vein five minutes before carotid injury reversed the extended occlusion times in the MAGP-1-deficient animals [82] (Figure 7B).

Clotting parameters were normal in the MAGP-1-deficient mice, confirming that the clotting pathways were unaffected by the absence of MAGP-1. Platelets showed normal aggregation properties in response to various agents, but platelet number was ~30% lower in the deficient animal. In protein binding assays, MAGP-1 interacted directly with von Willebrand factor (VWF) and fibrinogen, but did not modulate the interaction between von Willebrand factor and platelets [82].

The pattern of blood flow in the carotid injury assay suggested that MAGP-1 deficiency diminishes the ability of the thrombus to adhere to the injured blood vessel wall, resulting in thrombi that form and break away (Figure 7A). This finding suggests that MAGP-1 exerts its stabilizing effects by direct interactions between the proteins in the thrombus and components of the vascular matrix. MAGP-1's ability to act as a substrate for transglutaminase may provide a mechanism for covalently anchoring fibrin clots to the vessel wall concurrent with fibrin stabilization by the plasma transglutaminase factor XIIIa.

In arteries, microfibrils and elastic fibers produced by endothelial cells [83] and smooth muscle cells [84,85] are organized into elastic sheets, or lamellae, that are oriented

circumferentially between the cell layers. In both muscular and conducting arteries, the subendothelial matrix consists of a thin endothelial cell basement membrane in close association with the internal elastic lamina. Upon endothelial injury or denudation, the internal elastic lamina is exposed and becomes a major surface for thrombus attachment. MAGP-1-containing microfibrils are at the surface of elastic lamellae where they can easily interact with platelets and clotting proteins [86–89].

Hematopoiesis and monocytopenia

The bleeding defect, low platelet count, and increased bone TGFβ levels in *Magp1*$^{-/-}$ mice suggests that the hematopoietic stem cell niche may be affected by MAGP-1-deficiency. Initial evidence supporting MAGP-1-related changes in hematopoiesis came from studies of *Magp1*$^{-/-}$ mice exposed to cigarette smoke in a smoke-induced emphysema model (manuscript in preparation). The extent of lung damage after 6 months of smoke exposure was significantly less in *Magp1*$^{-/-}$ mice compared to smoke-exposed WT controls (Figure 8). When the extent of inflammation was characterized, the number of monocytes/macrophages was lower in both the lung tissue and lavage of the smoke-exposed knockout animals. Macrophages secrete potent proteolytic enzymes that lead to the development of emphysema through destruction of the lung ECM [90]. Hence, the modest lung damage in the MAGP-1-deficient mice following cigarette smoke exposure is consistent with the lower macrophage burden in these animals. Evaluation of monocyte populations in blood and bone marrow showed that MAGP-1-deficient mice had a monocytopenia characterized by fewer circulating monocytes and fewer monocytes in the bone marrow (Figure 9). It is interesting that single nucleotide polymorphisms in and around the *MAGP1* gene were associated with lung function in a genome-wide association study (GWAS) [91].

Wound healing

The injury response in smoke-exposed lungs suggests that tissue repair is altered in *Magp1*$^{-/-}$ mice. This was confirmed using a excisional dermal wound model that showed delayed wound closure in knockout mice compared to WT, with a lag period of ~7 days [20] (Figure 10A). Why repair was delayed is not clear, but possible mechanisms were suggested by several observations. First, there were fewer macrophages in the wound area at early stages of wound closure, which is consistent with the monocytopenia in MAGP-1-deficient animals. Second, scratch wound assays using dermal fibroblasts from MAGP-1-deficient mice showed a ~2-fold delay in wound closure, indicative of slower *Magp1*$^{-/-}$ fibroblast migration (Figure 10B).

Obesity and metabolic disease

One phenotype that shows complete penetrance in *Magp1*$^{-/-}$ mice regardless of genetic background is increased adiposity [20,92]. This characteristic is particularly interesting in light of studies associating obesity and diabetes traits in humans to a locus on chromosome 1p36 that includes the gene for MAGP-1 (*MAGP1*) [93–96]. The role of MAGP-1 and the ECM in metabolic homeostasis was recently reviewed [97]. *Magp1*$^{-/-}$ mice on normal chow show elevated whole-body adiposity by ~10 weeks of age [92] (Figure 11A). This change is not due to either increased caloric intake or reduced ambulatory activity, but is associated with reduced metabolic efficiency. *Magp1*$^{-/-}$ mice have elevated serum and tissue

triacylglycerol as well as elevated serum cholesterol levels when compared with WT mice. Insulin and glucose tolerance tests established reduced insulin sensitivity and impaired glucose clearance leading to elevated blood glucose levels. *Magp1*$^{-/-}$ mice were also more susceptible to the adverse metabolic effects of high fat diet where weight gain, hyperglycemia, hyperinsulinemia, and insulin resistance were significantly accentuated compared to control animals. Further, ectopic lipid accumulation in the liver is substantially elevated in the knockout animals compared to WT mice fed the high fat diet [92].

The defect leading to increased lipid accumulation in *Magp1*$^{-/-}$ mice is related to elevated fatty acid (FA) uptake in a background of normal lipolysis or lipid catabolism [92]. Basal FA uptake in *Magp1*$^{-/-}$ white adipose tissue was elevated compared to WT tissue and did not respond to stimulation by insulin, suggesting abnormalities in the uptake pathway. In muscle, a major energy-using tissue, key lipid uptake/storage genes were elevated compared to WT when mice were exposed to high fat diet. Genes for lipolysis and lipid catabolism, in contrast, remained at WT levels.

Brown adipose tissue (BAT) is a specialized fat that generates heat by burning lipid [98]. BAT uniquely expresses uncoupling protein-1 (UCP-1), a mitochondrial membrane protein that “uncouples” energy produced from cellular respiration to generate heat. When UCP-1 function is compromised, thermogenesis is adversely affected, resulting in lower body temperature and difficulty in maintaining body temperature in response to cold. In *Magp1*$^{-/-}$ mice on both normal chow and a high fat diet, brown fat mass is increased because lipid is not metabolized efficiently. Measurement of core body temperature found *Magp1*$^{-/-}$ mice to be colder than control animals (Figure 11C) and these animals had significantly greater body temperature loss when exposed to cold. Consistent with altered thermoregulation, *Magp1*$^{-/-}$ mice were not as efficient as WT mice in upregulating both body temperature and expression of the BAT-associated genes *Ppargc1α* and *Ucp1* following cold exposure. Brown fat was not the only fat type affected in the knockout animals. Cold challenge induces adipocyte browning and expression of brown fat genes in white subcutaneous fat. Induction of these cells, called inducible brown fat, brite, or beige fat, was significantly blunted in *Magp1*$^{-/-}$ subcutaneous fat [92]. Collectively, these studies indicate that MAGP-1 deficiency results in inefficient fatty acid metabolism in brown fat and suppresses the acquisition of brown fat features in white fat. These changes support a model where adiposity is increased in *Magp1*$^{-/-}$ mice because of increased lipid uptake and reduced energy expenditure in the form of heat production.

A comparison of TGFβ activity in white adipose tissue from *Magp1*$^{-/-}$ and WT mice showed Smad2 phosphorylation to be significantly increased in the knockout animals, indicative of elevated TGFβ activity (Figure 11D). Fibrosis and inflammation, downstream consequences of increased TGFβ signaling, were also elevated in knockout white adipose tissue [92]. A role for TGFβ in orchestrating the MAGP-1-obesity phenotype was confirmed by treating *Magp1*$^{-/-}$ and WT mice with a neutralizing antibody to TGFβ. After five weeks of treatment, adiposity and body temperature of *Magp1*$^{-/-}$ mice were both near WT levels [92] (Figure 11E–F). Thus, MAGP-1 supports energy expenditure and protects against excess lipid accumulation by regulating the availability of TGFβ. These findings are in

agreement with numerous studies showing that TGFβ has a negative effect on thermogenesis while supporting white adipose tissue expansion and insulin resistance [99,100].

In summary, MAGP-1 deficiency predisposes mice to hyperlipidemia, hyperglycemia, hyperinsulinemia, ectopic lipid accumulation and impaired glucose metabolism, suggesting that MAGP-1 serves a protective role against metabolic disease. The underlying mechanism leading to excess lipid accumulation in *Magp1*$^{-/-}$ animals is increased lipid uptake coupled with altered thermogenesis. MAGP-1 is involved in regulating thermogenesis and the browning of white adipocytes through a TGFβ-mediated pathway that influences expression of the browning proteins PGC-1α and Ucp1, as well as other genes that participate in energy dissipation through fatty acid oxidation. As with the bone phenotype discussed above, MAGP-1's ability to regulate TGFβ activity underlies the complex metabolic traits that serve as protection against metabolic stress.

Comparison of MAGP-1 and fibrillin-1 loss-of-function mutations

It is interesting to compare the MAGP-1-deficiency phenotypes with fibrillin-associated mutations in mice and humans. *Magp1*$^{-/-}$ mice have overlapping skeletal phenotypes with mice harboring loss-of-function mutations in fibrillin-1, the major binding partner of MAGP-1. These animals share osteopenia and increased RANKL expression as a common skeletal phenotype [79,101,102]. Furthermore, enhanced RANKL production and increased osteoclastogenesis are connected to improper TGFβ signaling in both mutant genotypes [70,103,104]. The similar skeletal phenotype suggests that both proteins contribute to a common mechanism of bone cell regulation that involves TGFβ.

The obesity phenotype associated with MAGP-1 deficiency, however, is opposite what is seen with fibrillin mutations in humans that lead to Marfan syndrome. Many of the traits associated with Marfan syndrome (fibrillin-1 mutations) are attributed to TGFβ gain-of-function because disrupted microfibrils no longer efficiently sequester the latent TGFβ complex [105,106]. Given that active TGFβ supports adipocyte hypertrophy and suppresses thermoregulation, individuals with Marfan syndrome should be predisposed to obesity and diabetes, similar to what occurs with MAGP-1 deficiency. However, most individuals with Marfan syndrome have reduced adiposity [106,107] and obesity has not been reported in mice with fibrillin mutations. Thus, while there are similarities in the bone phenotypes in MAGP-1 and fibrillin-1 mutant mice, the obesity phenotype is different, which illustrates the overlapping and distinct functions of the two proteins.

MAGP-2

The second member of the MAGP family is MAGP-2, a 25 kDa glycoprotein exclusively associated with microfibrils but with a more restricted tissue distribution than MAGP-1 [108]. It has little similarity with other known proteins with the exception of the matrix-binding domain of MAGP-1 (Figure 12). MAGP-2 is rich in serine and threonine, contains a RGD motif that binds the αvβ3 integrin, and has a basic cysteine-rich C-terminal half [9,32,109]. The many differences between MAGP-1 and MAGP-2 proteins and genes

suggest that the two proteins are functionally diverse, which was recently confirmed by gene knockout studies in mice showing little overlap between phenotypes [69].

MAGP-2 gene structure, alternative splicing

The human MAGP-2 gene, *MFAP5*, (*MAGP2 in this review*) is 11 kbp in size and is located on chromosome 12 at 12p12.3-13.1 [32]. It consists of 10 exons, 9 of which contain coding sequence for a total of 173 amino acids. The translation initiation codon is in exon 2. The gene contains only one major transcription initiation site and the upstream putative promoter region is AT-rich and lacks TATA, CAAT, and other common regulatory elements [109].

The mouse MAGP-2 gene also contains 10 exons in a gene spanning 16 kb located on the distal region of chromosome 6 [110]. The placement of all intron/exon junctions within the coding region is identical to that of the human gene. Exon 2 contains the translation initiation codon and, in contrast to the human gene, there are three transcription initiation sites in the mouse. The mouse promoter also lacks a TATA box as well as consensus sites for Sp1 or CAAT-binding factors [110]. Human MAGP-2 has three insertions of four, three, and two amino acids, not found in the mouse or bovine gene, spread throughout the N-terminal half of the protein. Otherwise, the MAGP-2 gene coding regions are highly conserved between mouse and human while the untranslated regions are not. There is no evidence of alternative splicing in the human gene [109], although a splice variant lacking exon 6 has been identified in the mouse [69]. Two mRNA species of 0.9 kb and 1.6 kb seen in mouse tissues arise from alternative polyadenylation site usage [110].

Comparison of the MAGP-1 and MAGP-2 genes shows that structural conservation is confined to the size and splice junction alignment of two penultimate exons 8 and 9 in the MAGP-2 gene and 7 and 8 in the MAGP-1 gene (Figure 12). These two exons encode the first 6 of the 7 precisely aligned cysteine residues that make up the matrix-binding domain, described in detail above [109]. A common feature of proteins recognized by the MAGP-2 matrix-binding domain is the presence of tandem EGF-like repeats [60].

MAGP-2 has a more restricted pattern of tissue expression than MAGP-1, being absent from the microfibrils of the ocular zonule and the elastic fibers of the aortic media and having a restricted distribution in tissues such as kidney and skin. High-level expression of MAGP-2 was seen in skeletal muscle, lung, mammary gland, thymus, uterus and heart in human and mouse tissue [108,110]. Overexpressing MAGP-2 in cultured cells suggested that MAGP-2 could facilitate both collagen I and elastin assembly without influencing gene expression [111,112].

Posttranslational modification of MAGP-2 and structural comparison to MAGP-1

The gene for MAGP-2 evolved from the MAGP-1 gene through a multigene duplication at the MAGP-1 locus approximately 525–600 My ago [6]. Even so, there are few similarities between MAGP-1 and MAGP-2 outside of the matrix-binding domain. The N-terminal region of MAGP-2 is rich in serine and threonine residues and lacks the proline, glutamine, and tyrosine-rich sequences found in MAGP-1. The specific residues in MAGP-1 that make up the elastin/collagen IV binding site are different in MAGP-2, but the sequence retains the acidic character through an enrichment in aspartic acid that facilitates tropoelastin and TGFβ

binding. There are consensus sites for N- and O-linked glycosylation in MAGP-2, but the motifs for tyrosine sulfation and transglutamination found in MAGP-1 are absent. The C-terminal region of MAGP-2 contains eight cysteines in contrast to the 13 found in MAGP-1, but it is this region of the molecule that shows precise alignment between the first seven cysteines in the matrix-binding domain of MAGP-1 [32,58] (Figure 12).

Immediately downstream of the matrix-binding domain sequence in MAGP-2 is a proprotein convertase cleavage site that can be acted upon by multiple proconvertase family members to release a 20 amino acid C-terminal fragment. A similar proconvertase cleavage site is not found in MAGP-1. Both cleaved and uncleaved forms of MAGP-2 were detected extracellularly but only full-length protein was identified in cell lysates, suggesting that proteolytic processing occurs mainly outside the cell [113]. The functional role of proconvertase cleavage is unknown. It is not required for MAGP-2 secretion and *in vitro* studies show that processing promotes, but is not required, for incorporation of MAGP-2 into a fibrillin-rich ECM [61].

MAGP-2 interactions with fibrillin and role in matrix assembly

It is now clear that MAGP-2, like MAGP-1, is covalently and periodically located along the fibrillin-containing microfibril [68]. The MAGP-2 matrix-binding domain mediates binding to fibrillin-1 and fibrillin-2 and different studies have localized binding within fibrillin to sequences near the N-terminus, the C-terminus, and in the middle of the molecule. Using a yeast two-hybrid assay with the MAGP-2 matrix-binding domain as bait, Penner at al [60] identified a conserved region at the C-terminus of fibrillin-1 and fibrillin-2 containing seven tandem calcium binding EGF-like repeats that conferred binding of MAGP-2. No MAGP-2 binding to N-terminal sequences of fibrillin-2 was observed, but it should be noted that a putative MAGP-1 binding site in exons 1-4 of fibrillin-1 were not included in constructs used in the confirmatory screen [60].

In a different study using solid phase binding assays and affinity blotting techniques, the specific interaction of MAGP-2 was identified with two fragments from the N-terminal half of fibrillin-1 (containing domains 1-14 & 13-30) and the N-terminal region of fibrillin-2 (containing domain 1-30). Interestingly, no MAGP-2 binding was detected to the C-terminal fragments identified in the previous study [68]. MAGP-1 was found to bind to the same fibrillin fragments as MAGP-2 and in competitive binding assays, MAGP-1 blocked binding of MAGP-2 to the domain 13-30 fragment of fibrillin-1 but not to fibrillin-1 fragment 1-14 or fibrillin-2 1-30. These findings indicate that MAGP-2 has a binding site distinct from that of MAGP-1 on the most N-terminal fragment of fibrillin-1, but that the two MAGPs share the same site in the 13-30 region. In addition, MAGP-2 has at least one binding site in the fibrillin-2 fragment 1-30 that is not shared with MAGP-1. It is interesting to note that the fibrillin-1 13-30 and fibrillin-2 1-30 fragments contain exon 24 that was shown by Werneck et al [59] to be a high affinity binding site for MAGP-1. Further support for the internal MAGP-2 binding site was provided by Lamaire et al [114], who showed elevated levels of MAGP-2 in skin and cells from a tight skin (tsk) mouse that expresses a fibrillin-1 mutation with a large in-frame duplication of exons 17-40. The tsk fibrillin contains a duplication of

the exon 24 site but not the N-terminal or C-terminal regions identified as MAGP-2 binding sites in other studies.

Immunogold labeling of elastic tissue showed that MAGP-2 had a regular covalent and periodic (about 56 nm) association with fibrillin-containing microfibrils (Figure 1D). Analysis of isolated microfibrils indicated that MAGP-2 was attached at two points along the microfibril substructure—the first site on the beads and a second site at the shoulder of the interbead region close to where the two arms fuse. In contrast, MAGP-1 was localized exclusively to the beads. Comparison of the MAGP-2 binding data with known fibrillin epitope maps of the microfibrils showed that the first site correlated with the N-terminal MAGP-2 binding region and the second site with the more central MAGP-2 binding region on the fibrillin-1 molecule [68].

Interactions of MAGP-2 and MAGP-1 with Notch and Notch Ligands

One of the ligands recognized by the matrix-binding domain of MAGP-2 is Jagged1 [60,63], an activating ligand of Notch receptor signaling. By interacting with Jagged1, MAGP-2 induces its shedding from the cell surface and the two proteins remain associated in the extracellular space where MAGP-2 may regulate the activity of shed Jagged1 [63]. MAGP-2-induced shedding of Jagged1 is metalloproteinase-dependent and can be inhibited by metalloproteinase inhibitors. MAGP-2 can also interact with Jagged2 and Delta1 but without shedding from the cell surface. MAGP-1 also interacts with Jagged1, but does not augment shedding. Thus, induction of Jagged1 shedding appears to be restricted to MAGP-2 [63].

MAGP-1 and MAGP-2 also interact with the EGF-like repeats of Notch1 [14] and lead to cell surface release of Notch1 extracellular domain and subsequent activation of Notch signaling. Notch1 extracellular domain release is dependent on formation of the Notch1 heterodimer by a furin-like cleavage, but does not require the subsequent ADAM metalloproteinase cleavage necessary for production of the Notch1 signaling fragment. In contrast to protease-induced shedding of Jagged 1, the MAGPs induce a protease-independent dissociation of the Notch1 heterodimer. MAGP-2 binding to Notch1 does not inhibit binding of other Notch ligands or their ability to induce receptor activation [14]. Unlike Notch canonical ligands (e.g. Delta1, Jagged1), Notch and MAGP-2 are required to be in the same cell for Notch signaling; Notch activation does not occur when MAGP-2 is added to the culture medium of Notch signaling-competent cells or when these cells are co-cultured with MAGP-2 expressing fibroblasts [14]. This co-expression requirement is surprising since MAGP-2 is a secreted protein that is not retained on the cell surface and is capable of interacting with Notch ligands in fluid phase assays.

MAGP-2 and angiogenesis

An unexpected property of MAGP-2 is its ability to influence endothelial cell sprouting and angiogenesis. MAGP-2 was identified as a pro-angiogenic factor in a screen of differentially expressed genes associated with endothelial cell angiogenesis [115]. In endothelial cell Notch activity assays, MAGP-2 was found to inhibit Notch1 signaling by antagonizing Jagged1's ability to induce Notch1 cleavage and activation [62]. Notch signaling is known to

antagonize angiogenic sprouting [116], which means that by interacting with Jagged1 and suppressing Notch signaling, MAGP-2 acts as a novel activator of sprouting of endothelial cells and angiogenesis. It is important to note that the inhibitory effects of MAGP-2 on Notch signaling are cell-type specific since MAGP-2 increases notch signaling in non-endothelial cell lines [62].

MAGP-2 in cancer and inflammation

Elevated MAGP-2 expression has been linked to poor outcome in neck squamous cell carcinomas [117] and in ovarian cancer [118–120], and has been proposed as an independent prognostic biomarker of survival and chemosensitivity [120]. MAGP-2 is expressed at high levels by cancer stromal fibroblasts and promotes tumor cell survival as well as endothelial cell motility and survival via the αvβ3 integrin receptor. *In vitro* studies demonstrated a significant increase in ovarian cancer cell motility and invasion potential but not cell proliferation after treatment with recombinant MAGP-2. These properties were associated with calcium release inside the cell that influenced cytoskeletal changes and mediated MAGP-2's motility and invasion promoting effects [120]. There was also a correlation between increased MAGP-2 expression and microvessel density, suggesting that the proangiogenic role of MAGP-2 may lead to increased tumor growth *in vivo* [118].

MAGP-2 has also been shown to have anti-inflammatory activity. When administered to mice subjected to endotoxemic shock, MAGP-2 reversed the cytokine storm and provided a significant survival benefit comparable to treatment with anti-inflammatory anti-TNF-α [121]. MAGP-2 attenuated inflammation by stimulating endogenous secretion of IL-10. IL-10 is a pleiotropic cytokine that down regulates Th1 cytokine expression and macrophage activation and is the master suppressor cytokine secreted by regulatory T cells [122].

MAGP-2 Knockout mice

Phenotypes associated with inactivation of the gene for MAGP-2 in mice demonstrate that MAGP-1 and MAGP-2 have shared as well as unique functions. *Magp2*$^{-/-}$ mice appear grossly normal, are fertile, and have no reduction in life span. Cardiopulmonary development is normal. The animals are normotensive and vascular structure and vascular mechanics are comparable to WT animals [69]. Together, these findings are indicative of normal, functional elastic fibers in the MAGP-2 knockout.

Mice lacking MAGP-2 did not show the osteopenia that was evident in MAGP-1-deficient mice. Spontaneous fractures were not observed and the skeleton of *Magp2*$^{-/-}$ mice appeared normal. μCT showed trabecular and cortical bone to be comparable in all aspects with WT controls. The obesity phenotype that is completely penetrant in the MAGP-1-knockout animals is absent in *Magp2*$^{-/-}$ mice [69]. Together, these findings suggest that loss of MAGP-2 by itself has a minimal effect on bone or fat character and, unlike MAGP-1, MAGP-2 has little or no role in normal bone and adipose homeostasis.

An unexpected trait associated with MAGP-2-deficiency is decreased levels of neutrophils in the circulation and in the spleen. Neutrophil number, based on GR1 antibody positivity and expressed as a percentage of total peripheral blood and spleen cells, was decreased by ~50%

in the circulation and ~40% in the spleen. However, *Magp2*$^{-/-}$ mice did not have the monocytopenia observed in *Magp1*$^{-/-}$ mice, suggesting that MAGP-1 and MAGP-2 have discrete functions in hematopoiesis. MAGP-2 is secreted in the bone marrow niche by bone marrow mesenchymal stromal cells where it can interact with hematopoietic stem cells and influence cell fate [121].

MAGP-1;MAGP-2 Double knockout mice

MAGP-1;MAGP-2 double knockout mice (*Magp1*$^{-/-}$;*Magp2*$^{-/-}$) are viable and fertile [69]. Their elastic fibers in skin, lung, and blood vessels are normal in appearance and function. The mice have phenotypes characteristic of each individual knockout as well as several interesting traits only seen when both genes are inactivated. The neutropenia seen with MAGP-2 deficiency and the monocytopenia associated with MAGP-1 deficiency are both present in the double knockout [69], indicating that the two proteins have non-overlapping roles in the bone marrow niche that influences hematopoiesis.

The double knockout mice do not develop spontaneous fractures and they lack the severe osteopenia associated with MAGP-1 deficiency. This suggests that loss of MAGP-2 in tandem with loss of MAGP-1 is protective against bone density loss. These animals do, however, share the smaller marrow cavity found in cortical bone of *Magp1*$^{-/-}$ mice [55]. Why loss of MAGP-2 is protective against bone loss may lie in MAGP-2's ability to manipulate Notch signaling. MAGP-1 and MAGP-2 both have the ability to bind Notch1 and several Notch ligands (see above). Both proteins mediate ectodomain shedding of Notch1 and activate the Notch intracellular domain, but MAGP-2 has the added ability to activate Notch signaling through release of jagged1 from the cell surface. Notch is an important regulator of osteoclasts [123] and inhibition of Notch signaling increases osteoblast differentiation, thereby increasing bone formation. Thus, increased osteoblast function in response to decreased Notch signaling associated with MAGP-2 deficiency may serve to counter MAGP-1-induced increases in osteoclastogenesis in double knockout mice.

One phenotype that is only apparent when both MAGP genes are inactivated is aortic dilatation [69]. Vascular compliance studies showed that the diameter of the ascending aorta in 6-month-old double knockout animals is significantly increased compared to WT, *Magp1*$^{-/-}$, or *Magp2*$^{-/-}$ mice. Vessel dilation was age dependent and progressive as animals at 4 months of age had normal vessel diameter. Aortic dissections were not observed in any of the 6-month-old animals and studies of older animals were not conducted. Double knockout animals at three months of age were normotensive with systolic, diastolic, and mean blood pressures comparable to age-matched WT animals. Vessel dilation is the only trait that overlaps with studies in zebrafish where loss of MAGP-1 alone causes distension of cranial and caudal blood vessels during development [76,77]. To date, inactivation of MAGP-2 in zebrafish has not been reported.

Relevant to the vascular changes seen in the double knockout mice is the recent finding that *MAGP2* loss-of-function mutations in humans are involved in the physiopathology of thoracic aortic aneurysms and dissection (TAAD) [124]. Mutation screening of a large number of TAAD-affected individuals identified two mutations in different parts of the

MAGP-2 molecule that segregated with the disease. Interestingly, all exons of the gene for MAGP-1 were also screened and no variations that could account for the disease were identified, indicating that MAGP-1 does not contribute to TAAD in this group. Functional analysis showed that the two *MAGP2* mutations caused pure or partial haploinsufficiency. When the effect of *MAGP2* loss-of-function mutations on the TGFβ pathway were examined, increased nuclear phosphorylated Smad2/3 was identified in the aorta from the affected individuals [124]. These findings are consistent with MAGP-2's ability to bind and sequester TGFβ [69].

It is interesting that *MAGP2* heterozygosity in humans leads to a severe aortic dilation when no vascular dilatory phenotype was evident in either heterozygous or homozygous *Magp2*$^{-/-}$ mice. The requirement that genes for both MAGP-1 and MAGP-2 be inactivated in mice before aortic dilation occurs, and that vessel dilation in fish occurs when knocking down MAGP-1 alone, suggests that the location or overall function of the two MAGPs within the vessel wall may be different between fish, mice, and humans, or that known differences in cardiovascular hemodynamics act as biological modifiers. In mouse aorta, MAGP-2 is highly expressed in the intima and adventitia whereas MAGP-1 is mostly expressed in the media associated with elastic fibers [69]. A similar pattern has been shown in bovine aorta [108]. Hence, inactivation of both genes would have more pronounced effects on vessel wall integrity than modification of each gene individually. The spatial expression of MAGP-2 in human and fish aorta has not been reported.

MAGPs as matricellular proteins

The spectrum of MAGP functional properties and knockout phenotypes resembles, in many ways, what has been described for matricellular proteins [125–127]. This family of proteins serves primarily a cell modulatory rather than a direct structural role in the ECM. Some members of this family include SPARC (osteonectin or BM-40), the thrombospondins (TSPs), and the tenascins [128]. MAGP-1 resembles the thrombospondins (TSPs) in its ability to bind multiple proteins and its role in angiogenesis, hemostasis, and wound healing. Even the knockout animals share similarities in that mice that lack TSP-1 have enhanced thrombus embolization caused by defective thrombus adherence to the injured blood vessel wall [129], similar to the MAGP-1-deficient mouse. TSP2 mice have a bleeding diathesis that manifests as a prolonged bleeding time.

Of all the matricellular proteins, functions attributed to MAGP-1 most resemble those of SPARC [130]. Like SPARC, MAGP-1 is a substrate for transglutaminase and both proteins have a functional intracellular form that arises through alternative splicing [45,131]. Both proteins modulate the activity of TGFβ, bind multiple ECM proteins, alter the shape of cells and influence cell adhesion. Mice depleted of each gene have several phenotypes in common, including osteopenia, increased fat, altered dermal wound healing, and reduced infiltration of macrophages into tissues. It will be interesting going forward to determine whether the rapidly increasing knowledge of matricellular proteins can inform our understanding of MAGP function.

Conclusions

Fibrillin-containing microfibrils are found in the extracellular matrix (ECM) of all tissues. They are compositionally complex, consisting of the fibrillins and MAGPs as core protein components. Findings from knockout fish and mice show that the MAGPs play an important biological role in multiple organs, including bone, fat, blood vessels, hemostasis, and the immune system. Mechanistic studies provide strong evidence for MAGP regulation of the TGFβ pathway as one of its major functional roles in tissues. Because fibrillin also regulates TGFβ signaling, fibrillin and MAGP-1 must be working synergistically to regulate growth factor availability. The close relationship between these two proteins raises the interesting question of how much of the pathology associated with fibrillin mutations is directly linked to changes in MAGP-fibrillin interactions and subsequent MAGP-dependent function. Conversely, do mutations that result in the absence of MAGP from the microfibril impart gain-of-function properties to fibrillin? These are complex questions that are difficult to answer when dealing with a polymeric structure comprising multiple components that influence each other in defining overall fiber function.

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Abbreviations used

BAT	brown adipose tissue
BSw	black Swiss
DEXA	longitudinal dual energy x-ray absorptiometry
ECM	extracellular matrix
EGF	epidermal growth factor
LTBP	latent TGFβ binding protein
MAGP	microfibril associated glycoprotein
My	million years
TGFβ-LLC	TGFβ large latent complex
μCT	microcomputed tomography
SPARC	secreted protein acidic and rich in cysteine
TAAD	thoracic aortic aneurysms and dissection
UCP-1	uncoupling protein-1
VWF	von Willebrand factor

WT	wild-type
tsk	tight skin mouse
N-terminal	amino-terminal
C-terminal	carboxy-terminal

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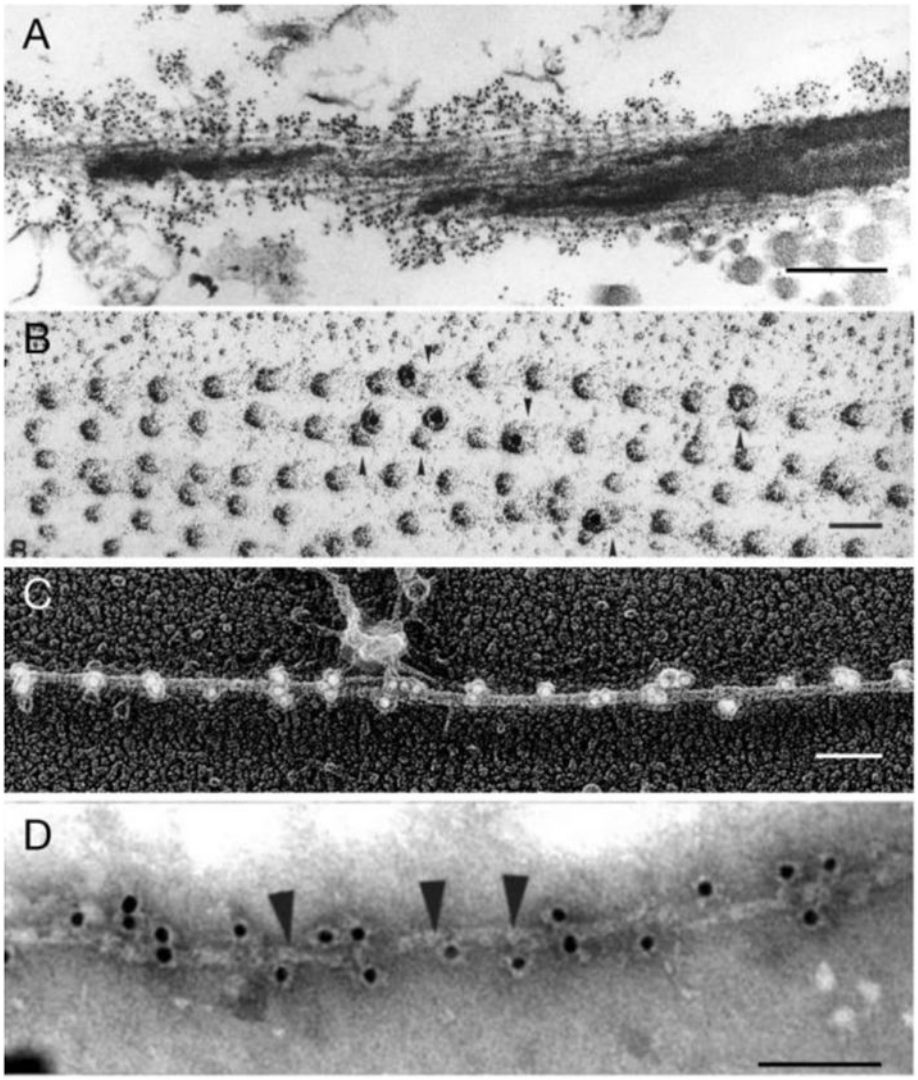

Figure 1.

Ultrastructural localization of MAGP-1 in elastic fiber microfibrils. A) MAGP-1-directed immunogold labeling of microfibrils surrounding the elastin core of a developing elastic fiber in ligament. B) Rotary shadowing and immunolabeling of extracted microfibrils showing anti-MAGP-1 gold binding to the bead component. C) Deep etch electron micrograph of a single native microfibril decorated with anti-MAGP-1 gold showing a regular distribution along the microfibril and multiple gold particles at most binding sites. D) Immunogold localization of MAGP-2 on isolated microfibrils negatively stained. Arrowheads indicate the position of the beads. Bar = 500 nm (A), 50 nm (B & C), and 100 nm (D). The image in panel A was kindly provided by Dr. Douglas Keene, Oregon Health and Science University, Portland, OR. Images in panels B and D are from [34] and [68], respectively. Used with permission.

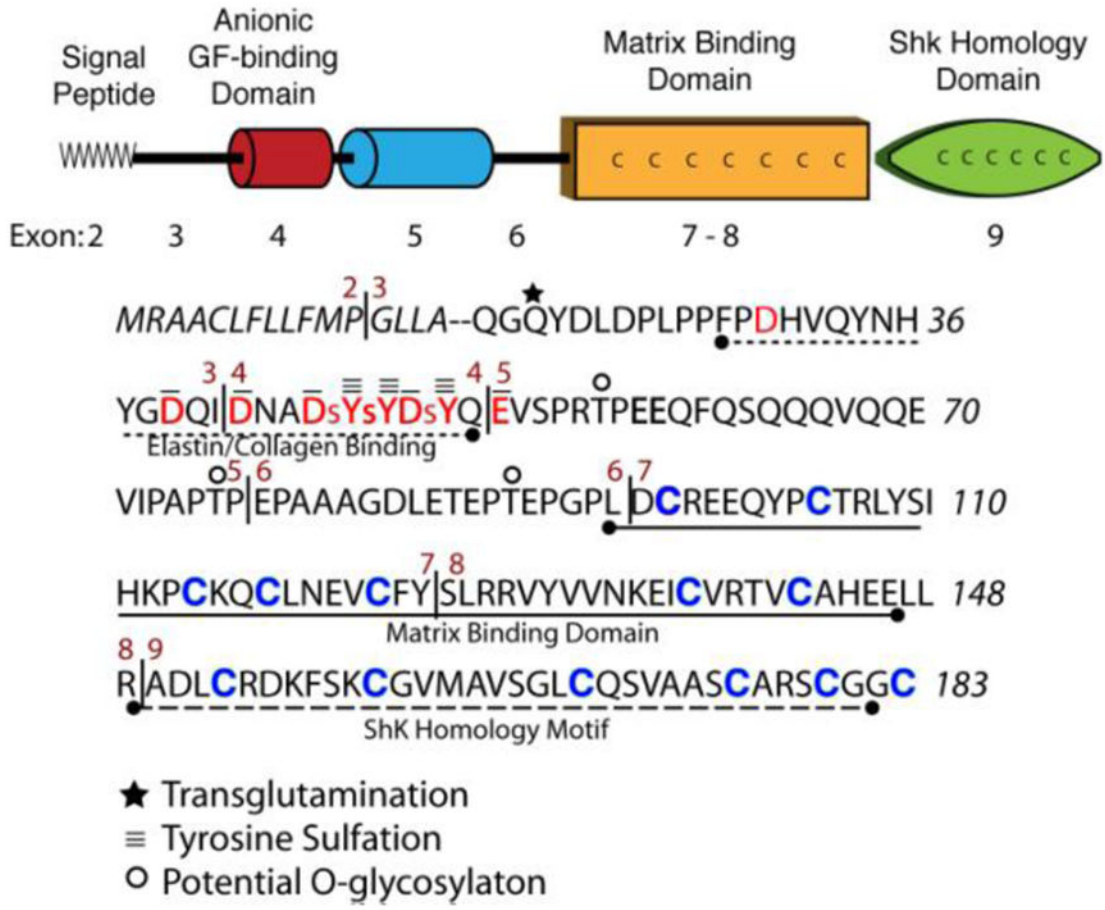

Figure 2.

Mouse MAGP-1 sequence showing exon boundaries (red numbers) and known binding domains. The acidic and sulfotyrosine residues in the protein-binding domain are shown as red letters. Threonine residues predicted as sites of O-glycosylation are indicated with circles. The 13 cysteine residues are in blue. The glutamine residue involved in transglutamination is indicated with a star. The dotted underline indicates the region that binds tropoelastin, collagen, and TGFβ. The solid underline is the matrix-binding domain and the dashed underline highlights the ShK homology motif.

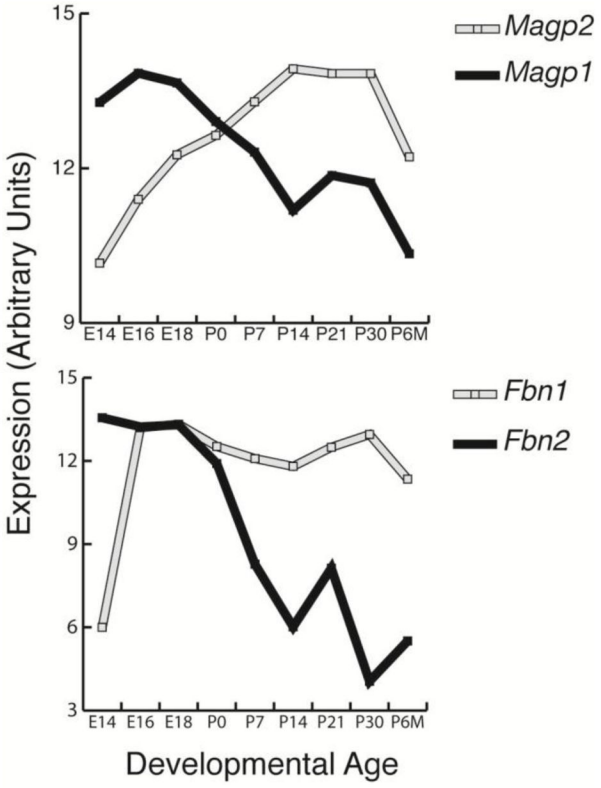

Figure 3.

MAGP and Fibrillin expression in developing mouse aorta as determined by gene array analysis. Expression values in arbitrary units are plotted against developmental age, which begins at embryonic day 14 (E14) through 6 months (P6M).

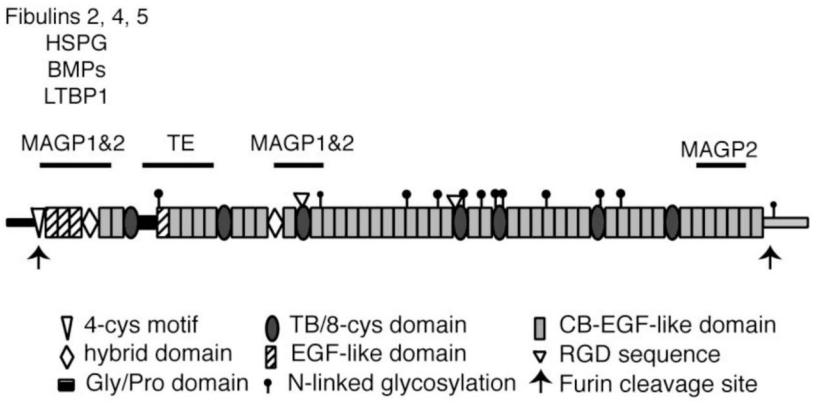

Figure 4.

MAGP-1 and MAGP-2 binding sites in fibrillin. Two binding sites for MAGP-1 and MAGP-2 have been identified in fibrillin-1 and fibrillin-2. The N-terminal binding site overlaps with binding sites for the large latent TGFβ complex, pro-BMPs, and several other proteins. The second MAGP binding site is located in or near the third TB domain, which lies in the region of fibrillin where mutations produce the most severe phenotypes associated with neonatal Marfan syndrome (fibrillin-1) and congenital contractural arachnodactyly (fibrillin-2). The last seven calcium-binding EGF-like domains in fibrillin-2 define a third binding site for MAGP-2. TE, tropoelastin; HSPG, heparan sulfate proteoglycan.

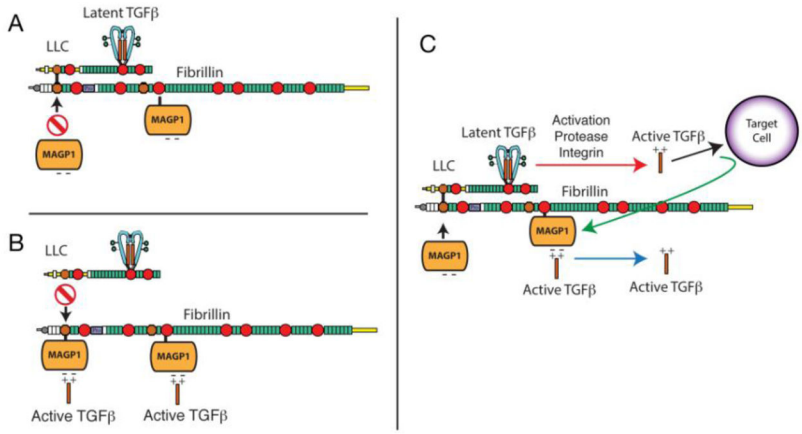

Figure 5.

Models for TGFβ regulation by MAGP. A) Competition between MAGP-1 and TGFβ-large latent complex (LLC) for the same binding site in fibrillin could influence TGFβ loading. Both LLC and MAGP-1 bind to the N-terminal end of fibrillin-1. *In vitro* evidence suggests that each protein blocks the binding of the other so that when the LLC is bound to fibrillin, MAGP-1 does not bind and the microfibril is load with latent TGFβ. B) Conversely, if MAGP-1 is bound to fibrillin, the LLC cannot bind and the microfibril is loaded with active growth factor bound to MAGP-1. C) In this model, the microfibril can bind both LLC and MAGP-1. Once latent TGFβ is activated, it can either bind receptors on its target cell or be sequestered back into the ECM by binding to MAGP-1. In this way, MAGP-1 could serve to suppress TGFβ signaling by removing excess TGFβ from the immediate membrane microenvironment.

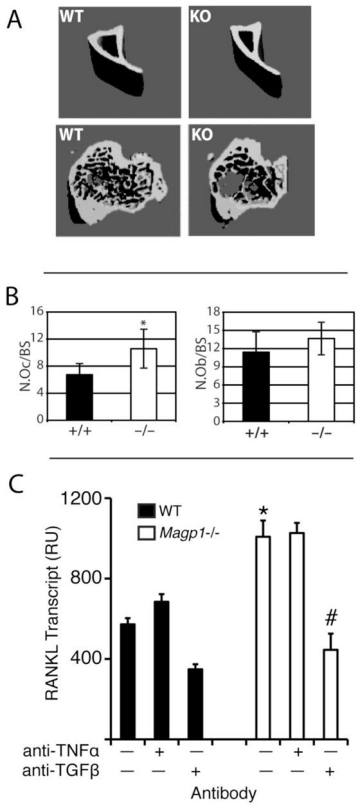

Figure 6.

Osteopenia in MAGP-1-deficient mice. A) μCT images of cortical (top) and trabecular (bottom) bone in WT and *Magp1*$^{-/-}$ mice demonstrate a smaller marrow cavity and significantly less trabecular bone mass in the MAGP-1-deficient animals. B) A major reason for the osteopenia is that *Magp1*$^{-/-}$ mice have more osteoclasts than WT animals with no change in osteoblast number. C) Osteoblasts from *Magp1*$^{-/-}$ mice express more RANKL than WT animals, which is a driver for the elevated osteoclast number in MAGP-1-deficient bone. Treatment with a neutralizing antibody to TGFβ reduced RANKL expression in MAGP-1-deficient osteoblasts to WT levels. These findings indicate that MAGP-1 is a regulator of bone remodeling through a mechanism involving TGFβ. A & B from [55] and C from [56]. Used with permission.

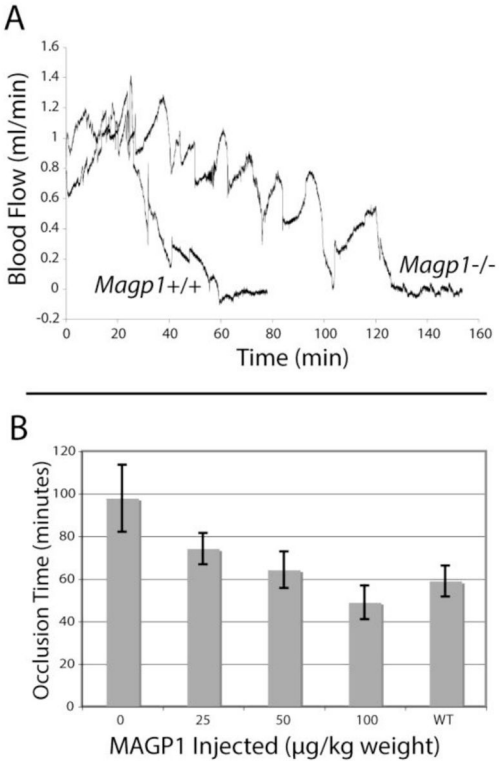

Figure 7.

Extended thrombotic occlusion time in *Magp1*$^{-/-}$ mice. A) Blood flow recordings showing a delayed thrombotic occlusion time and periodic flow pattern in a carotid injury model in *Magp1*$^{-/-}$ animals. B) Infusion of recombinant MAGP-1 reestablishes normal occlusion time in *Magp1*$^{-/-}$ mice when injected into the tail vein 5 minutes before vascular injury. From [82]. Used with permission.

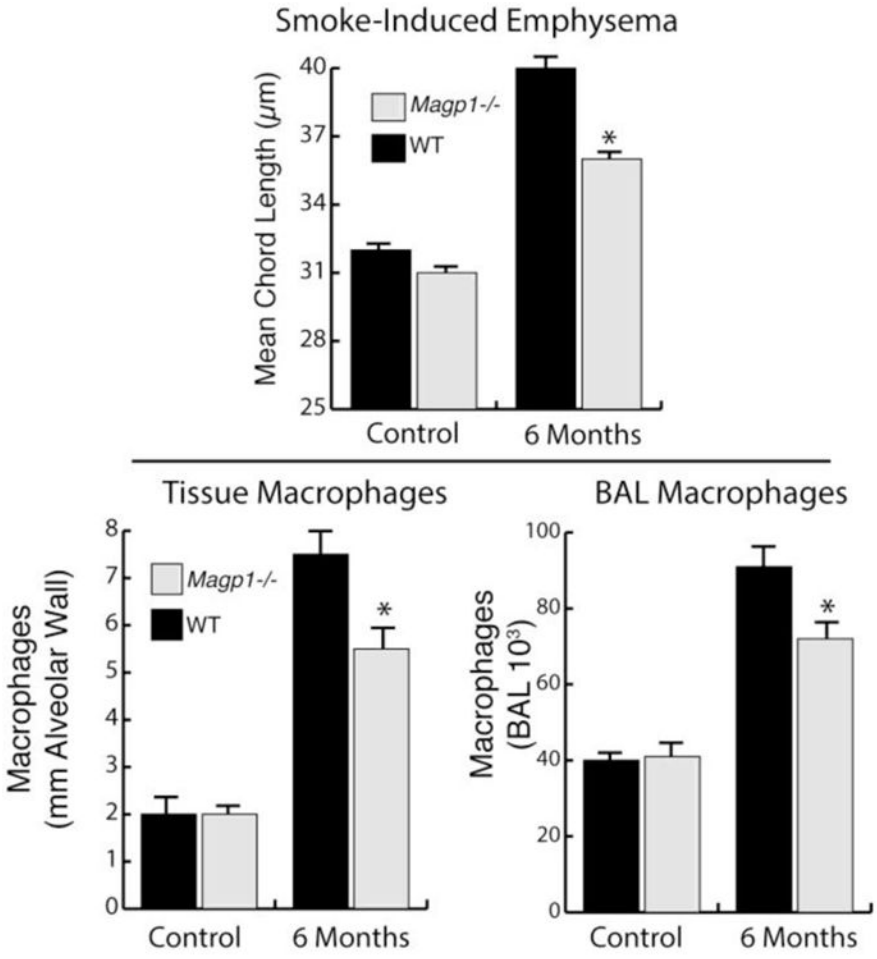

Figure 8.

Attenuated lung damage in *Magp1*$^{-/-}$ mice exposed to cigarette smoke. MAGP-1-deficient mice show attenuated lung damage (top) and have fewer tissue and bronchoalveolar lavage (BAL) macrophages (bottom) than WT animals after 6 months of cigarette smoke.

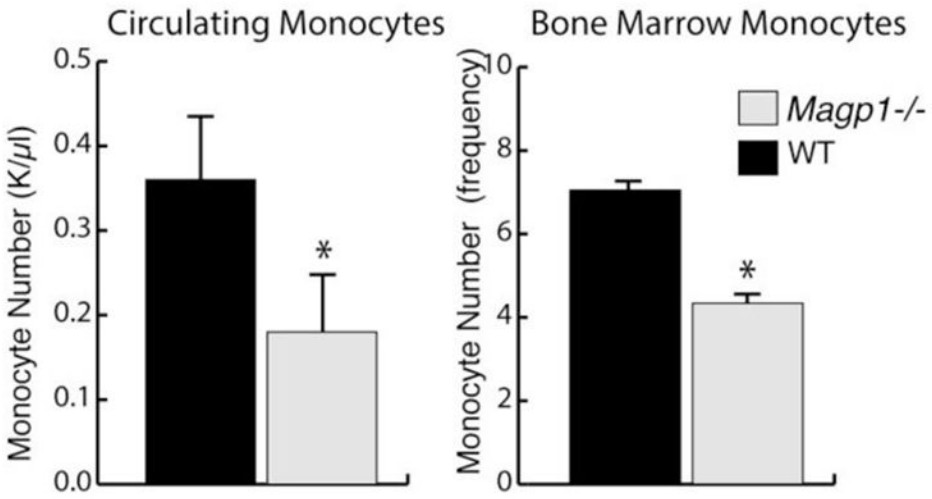

Figure 9.

Monocytopenia associated with MAGP-1-deficiency. A) *Magp1*$^{-/-}$ mice have fewer circulating monocytes and fewer monocytes in the bone marrow.

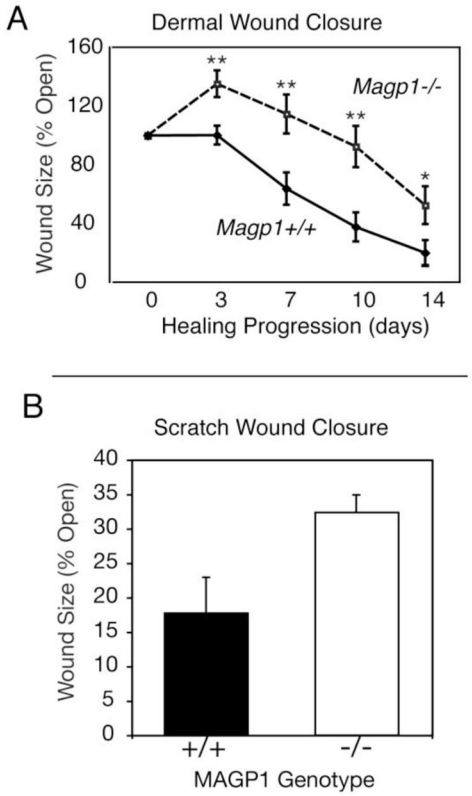

Figure 10.

Wound healing defects. A) *Magp1*$^{-/-}$ mice show delayed closure of excisional dermal wounds compared to WT animals. B) Scratch-wound assays using dermal fibroblasts from MAGP-1-deficient mice shows a ~2-fold delay in wound closure, indicative of slower *Magp1*$^{-/-}$ fibroblast migration. Panel A is from [20]. Used with permission.

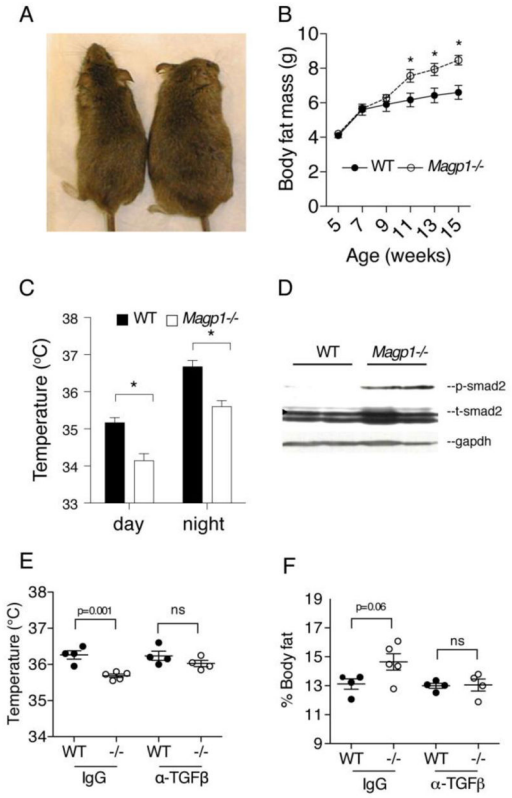

Fig 11.

Obesity and metabolic disease associated with elevated TGFβ levels in *Magp1*$^{-/-}$ mice. A) MAGP-1-deficient mice (right) are larger than age-matched wild-type animals (left) due to increased body fat. B) Longitudinal EchoMRI study of whole-body adiposity from 5 to 15 weeks of age shows that Increased adiposity due to MAGP-1 deletion is apparent by 9–10 weeks and progresses with age. C) Core body temperature in *Magp1*$^{-/-}$ mice is lower than in WT animals due to changes in brown fat that impair the ability of MAGP-1-deficient mice to regulate thermogenesis. D) An underlying mechanism for the obesity phenotype is elevated TGFβ signaling in adipose tissue as shown by elevated p-Smad2 levels in white adipose tissue lysate from *Magp1*$^{-/-}$ compared to WT mice (2 animals per group). The immunoblot was developed using antibodies to phosphorylated Smad2 (p-Smad2), total Smad2 (t-Smad2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). D & E) Treatment of *Magp1*$^{-/-}$ mice for 5 weeks with a neutralizing antibody to TGFβ improves body temperature and prevents excess fat accumulation. Panel A is from [20] and panels B-F from [92]. Used with permission.

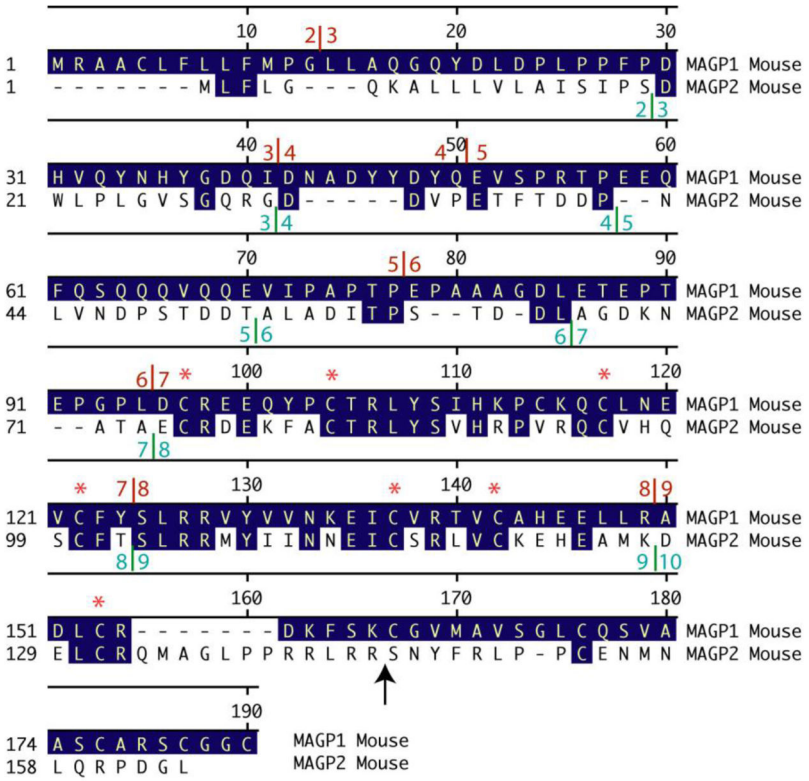

Fig 12.

Sequence alignment of mouse MAGP-1 and MAGP-2. Sequence alignment diagram showing conservation of the 7 cysteines in the matrix-binding domain (red stars). While there is relatively high sequence homology within this domain, regions outside of the matrix-binding domain share little homology. Red lines and green lines are exon boundaries for *Magp1* and *Magp2*, respectively. The arrow indicates the furin cleavage site in MAGP-2.