Published in final edited form as:

Matrix Biol. 2019 January ; 75-76: 300–313. doi:10.1016/j.matbio.2017.11.006.

Thrombospondin-4 in tissue remodeling

Olga Stenina-Adognravi and **Edward F. Plow**

Department of Molecular Cardiology, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH, 44195, U.S.A.

Abstract

Thrombospondin-4 (TSP-4) belongs to the thrombospondin protein family that consists of five highly homologous members. A number of novel functions have been recently assigned to TSP-4 in cardiovascular and nervous systems, inflammation, cancer, and the motor unit, which have attracted attention to this extracellular matrix (ECM) protein. These newly discovered functions set TSP-4 apart from other thrombospondins. For example, TSP-4 promotes angiogenesis while other TSPs either prevent it or have no effect on new blood vessel growth; TSP-4 reduces fibrosis and collagen production while TSP-1 and TSP-2 promote fibrosis in several organs; unlike other TSPs, TSP-4 appears to have some structural functions in ECM. The current information about TSP-4 functions in different organs and physiological systems suggests that this evolutionary conserved protein is a major regulator of the extracellular matrix (ECM) organization and production and tissue remodeling during the embryonic development and response to injury. In this review article, we summarize the properties and functions of TSP-4 and discuss its role in tissue remodeling.

Keywords

extracellular matrix (ECM); angiogenesis; nociception; fibrosis; skeletal muscle; myocardium

In a growing tissue, the composition of the extracellular matrix (ECM) and its organization are critical for supporting and guiding the assembly of cellular structures and for regulating cellular processes and responses. The growth and re-organization of tissues are essential for development of the embryo; but, in the adult organism, the fetal growth programs can become re-activated in response to injury (1) or in carcinogenesis (2, 3). The activation of fetal programs requires intracellular signaling in response to low oxygen and/or low nutrients in the environment, activation of transcription of specific genes regulating tissue remodeling, and synthesis and secretion of ECM proteins. One of the ECM proteins actively produced in the developing embryo and in remodeling adult tissues is thrombospondin-4 (TSP-4), a member of thrombospondin (TSP) protein family consisting of 5 distinct

Corresponding author: Olga Stenina-Adognravi, Ph.D., Staff (Professor), Department of Molecular Cardiology, Cleveland Clinic, Lerner College of Medicine/CWRU, Taussig Cancer Center, 9500 Euclid Ave, Cleveland, OH 44195, tel: (216) 444-9057, fax: (216) 445-8204, stenino@ccf.org.

homologous proteins that are products of distinct genes on different chromosomes. Despite the high homology in amino acid sequences of the C-terminal halves of all TSPs (4, 5), distinct functions have been ascribed to each member of the family. In particular, TSP-4 has assigned a number of remarkable functions that distinguishes it from other TSPs, and, in several cases, these functions counterbalance contributions of other family members. As a striking example, TSP-1 and TSP-2, which were discovered first and were the focus of many early studies (5–14), are renown for their potent anti-angiogenic effects (6, 9–11, 15), whereas TSP-4 promotes angiogenesis, and its expression is associated with enhanced vascularization of tissue (16–18). As another example, TSP-1 promotes fibrosis by activating TGF-beta (19–23), and higher levels of TSP-1 have been associated with accelerated fibrosis in several organs (22, 24–29), whereas TSP-4 suppresses production of ECM by fibroblasts in cell culture (18, 30) and mouse models (18, 31). Currently, it is unclear what cellular and molecular processes activated by TSP-4 result in its distinction from other TSPs.

The high expression of TSP-4 in injured tissues and the phenotype of TSP-4 knockout mice suggest that this matricellular protein is a key regulator of tissue growth and remodeling. With the emergence of evidence distinguishing TSP-4 from other TSP family members, we felt that a review focused on TSP-4 was timely and appropriate.

Structure of TSP-4

TSP-4 (THBS4) is a member of subgroup B of TSPs, which encompasses TSP3, TSP-4 and TSP-5. Each of the three group member is a pentameter; generally, they are homopentamers comprised of a single TSP-3, TSP-4 TSP-5 subunit although heteropentamers involving TSP-4 and TSP-5 have been reported (32). Subgroup A THBSs (THBS-1, TSP-1 and THBS-2, TSP-2) form trimers. The subunits of subgroup A are larger than those of subgroup B members, a difference arising from the presence of a distinct N-terminal domain followed by a von Willebrand factor Type C domain. A schematic model depicting the domain organization of the TSP-4 is shown in Fig.1A. Based on its mRNA sequence, the TSP-4 subunit is predicted to have a molecular weight of ~103K; however, glycosylation raises its molecular weight to ~120K, still considerably smaller that the subunit of TSP-1, ~145K. TSP-4 lacks the distinctive features of the N-terminal domain found in the two subgroup A members. The N-terminal domain of TSP-4 and other subgroup B members is followed by a coiled coil region that harbors the oligimerization site, in which the 5 subunits become disulfide linked (Fig.1B). In subgroup B, multiple Type 2 EGF-like domains (TSP-4 has four of these EGF-like repeats), are followed by a series of Type 3 calcium-binding repeats, also referred to a “calcium-binding wire module” by Mosher and colleagues (33–38). These are then followed by the C-terminal “signature” domain, which defines the TSP family. The C-terminal signature domain are highly conserved (53-82%) across both the subgroup A and group B members.

The oligimerization of the subunits to form the TSP-4 pentamer is shown in Fig. 1B. Calcium plays an important role in maintaining the tertiary structures of all the TSPs, including TSP-4; removal of calcium affects the overall conformation of TSP-4 (39, 40). Even though the signature domains are highly homologous, differences in the effects of calcium binding to the signature domains of TSP-4 and TSP-2 were noted (39). A single

nucleotide polymorphism (SNP) in TSP-4, a proline at position 387 compared to alanine in the predominant form of TSP-4, has been associated with cardiovascular diseases (41–48). This SNP resides in the third EGF-like repeat and enhances its calcium binding affinity (40).

TSP-4 receptors

Multiple TSP-4 receptors have been identified, primarily using antibody blocking or small molecule blocking approaches. Among the TSP-4 receptors identified using these strategies are several integrins that support cell adhesion and/or migration on TSP-4 containing substratum. The involvement of specific integrins in recognition of TSP-4 depends on cellular context. On leukocytes, both monocytes and neutrophils, integrins αMβ2 (Mac-1, CD11b/18) and αvβ3, have been implicated as TSP-4 receptors (49, 50). The involvement of αMβ2 in TSP-4 recognition was corroborated using cells derived from mice deficient in this leukocyte integrin. Recognition of TSP-4 by αMβ2 depends upon engagement of the I domain of the integrin with the EGF-like repeats of TSP-4, and this interaction activates intracellular signaling events in leukocytes. Vascular cells, endothelial cells and smooth muscles cells, express αvβ3 although the participation of this integrin in endothelial cell adhesion to and migration on TSP-4 may be different (17). Another integrin with a ligand specificity very similar to αvβ3, αvβ5, has not been directly implicated in TSP-4 recognition. Also, integrin α2β1 was implicated in endothelial cell migration on TSP-4 using an α2-specific antibody (17). A non-integrin channels (17, 51–54). Gabapentin is a ligand for $\alpha_2\delta$-1 and blocks the binding of TSP-4 to $\alpha_2\delta$-1. $\alpha_2\delta$-1 also reacts with subgroup A TSPs in immunoprecipitation assays from cerebral cortex tissues. These interactions involve the EGF-like repeats of the TSPs.

TSP-4 expression in embryonic development and adult organisms

TSP-4 or its orthologs are present in lower organisms starting with *Basal metazoa* (55), unlike TSP-1 and TSP-2 that appear later in evolution during the development of a vascular system (55). In *Xenopus*, TSP-4 is expressed in the developing embryo starting with neurulation stage (56). In adult human tissues, the highest expression of TSP-4 is detected in hearts and skeletal muscles (57). It was noted by Tucker et al (34) that the pattern of expression of the TSP-4 transcript was clearly different from the patterns of expression of other TSPs.

Expression of TSP-4 in the osteogenic tissues

In chick embryo, TSP-4 was found in osteogenic tissues in the mesenchyme surrounding the bone anlage during the initial stages of osteogenesis, but the TSP-4 mRNA disappeared at later stages (58). In the same study, TSP-4 mRNA was detected in the eye of chick embryos, where it was associated with later ossified structures, and in corneal fibroblasts beneath the corneal epithelium (58). TSP-4 was also detected in myotendinous junctions of the extraocular muscle of zebrafish (59) and in the basement membrane zone of human ocular surface epithelia, a specialized microenvironment in which maintenance, self-renewal, activation, and proliferation of stem cells by external signals occurs (60).

Expression of TSP-4 in the tendon

TSP-4 is abundant in the tendon (61–64) and is accepted as one of the markers of mature tenocytes (65). In tendon, TSP-4 is associated with fibrillar structures that also contain TSP-5 and have a different direction than TSP-3-containing fibers (30). TSP-4 in tendon does not co-localize with TSP-3, but the staining for TSP-4 and TSP-5 largely overlap (30). Analysis of TSP-4 from equine tendon revealed that TSP-4 and TSP-5 monomers assemble into heteropentamers that cannot be separated under non-reducing conditions (32). The significance of heteropentamers remains unclear. There were no obvious signs of compensation in the tendon of TSP-4 KO mouse: the levels of TSP-3 and TSP-5 did not change with the knockout of TSP-4 suggesting a distinct function and regulation for TSP-4 in tendon.

In *Drosophila*, there is only one TSP that has the structural features of group B thrombospondins (TSP-3, TSP-4, and TSP-5 in humans). *Drosophila*'s TSP-4 ortholog was found to be highly expressed in tendon progenitors (66).

Expression of TSP-4 in the skeletal muscle

In *Drosophila* and zebrafish, the TSP-4 ortholog is necessary for the formation of myotendinous junctions, and without it, the muscles detach upon contraction (67). Injections of human TSP-4 into zebrafish restored the function of muscles and revealed that TSP-4 provides a scaffold for a proper ECM assembly in myotendinous junctions and supports intracellular signaling.

In axolotl, the ortholog of TSP-4 is highly expressed during limb regeneration, and the patterns of expression overlap with the expression pattern during larval limb development (68).

Expression of TSP-4 in the nervous system

The role of TSP-4 in skeletal muscle is not limited to maintaining the structure and function of myotendinous junctions; it is influential everywhere where the muscle connects to another tissue. TSP-4 is expressed in neuromuscular junctions, likely by the muscle interstitial cells, and its expression increases dramatically after denervation of a muscle (69). In this location, TSP-4 incorporates into ECM and promotes the outgrowth of neurites after denervation. These functions of TSP-4 are strikingly distinct from the functions of other TSPs. High levels of TSP-4 but not other TSPs, were found in a wide range of neurons and were associated with synapse-rich layers in the adult nervous system (69).

Expression of TSP-4 in the eye

Expression of TSP-4 was detected in embryonic mouse retina at day 15 (70). In the adult mouse eye, TSP-4 was detected in ganglion cell layer, the inner nuclear layer, the plexiform layers, and in the sclera (70). In the adult bovine eye, TSP-4 was expressed in multiple eye structures: cornea, conjunctiva, aqueous ducts, sclera, iris, ciliary processes and muscle, trabecular meshwork, Bruch's membrane, retina, lamina cribrosa, optic nerve, and blood vessel wall (71). Cultured retinal pigment epithelial cells (RPE) express TSP-4 together with three other TSP family members, TSP1, 2, and 3 (72).

Expression of TSP-4 in the vasculature and the heart

TSP-4 is produced in the vascular wall by endothelial cells (EC) and smooth muscle cells (SMC) (73) and normally localizes either on the outer side of capillaries or in the adventitia of larger blood vessel (30, 49).

In myocardium, the highest expression was seen in the fibrous skeleton of the heart, which provides structure, tensile strength, and stiffness to the myocardium (29). The protein was also present in perimysium between bundles of cardiomyocytes and in the endomysium, the extracellular space between cardiomyocytes. TSP-4 was also consistently present in blood vessels of various sizes in the heart, in the adventitia of larger vessels and in the sub-endothelial matrix of capillaries.

The expression of TSP-4 is tightly regulated, similarly to the expression of other TSPs, but the unique spatial and temporal patterns suggest that it performs functions distinct from other TSPs (74).

TSP-4 expression in pathology

During the development of an atherosclerotic lesion, TSP-4 was found in the lesion in the *Tunica Intima* where it contributed to the inflammatory atherosclerotic process (49).

Consistent with the production of TSP-4 in the blood vessel wall, a high TSP-4 expression is detected in cancers, where the growth of the tumor relies critically on the vasculature. TSP-4 was in top 1% of most upregulated genes in several types of cancer: e.g., gastric cancer (75–77), and especially in breast cancer (78–80).

Perhaps the most striking increase in TSP-4 expression levels was detected in remodeling hypertrophic and failing hearts, both human and murine (18, 31, 81), suggesting its important role in the remodeling of the myocardium in response to the pressure overload.

Role of TSP-4 in the connective tissue

The expression patterns of TSP-4 in axolotl during the limb regeneration and larval limb development suggests the involvement of TSP-4 in ECM remodeling and formation of “transitional matrix” (68). In higher organisms that have lost the ability to regenerate limbs, TSP-4 participates in wound healing and scar formation.

Similar to other TSPs, TSP-4 regulates the production and assembly of collagen and organization, repair, and remodeling of ECM. J. Adams proposed that the conserved L-lectin domain of all TSPs serves to flexibly associate TSPs with ECM to regulate the interstitial ECM of various tissues (82). TSP-1, TSP-2, or TSP-4 deficiency all result in the formation of disorganized collagen fibrils and increased number of fibrils of larger diameter (30, 82–84). In addition to this organizational role, TSP-1 and TSP-2 knockouts altered the shape of the fibrils. While TSP-4 deficiency did not result in shape change, it caused increased spacing between fibrils. Although the original concept of matricellular proteins suggested that they are not structural proteins and may only affect the fibrillogenesis by regulating the functions of fibroblasts, TSP-4 may also function as a structural protein: it is very abundant

in the tendon (32, 61, 62) and presents as a well-organized fibrillar network (30). TSP-4 binds to collagens I, II, III, and V, and non-collagenous proteins are found in close proximity to TSP-4 in ECM, e.g., laminin-1, fibronectin, matrilin-2, but not to collagen IV (85). TSP-4 binds most ECM protein ligands independently of the N-terminal and coil-coiled domain, and the interactions are regulated by divalent cations, with binding to collagens induced by Zn^{2+}(85). The complex multiple strong interactions of TSP-4 with ECM proteins suggest a role as an adaptor protein in extracellular matrix assembly (85). The N-terminal heparin-binding domain is thought to bind the cell surface, and this binding may account for the high TSP-4 immunoreactivity in the pericellular compartment with close association to the cell surface in the tendon (86) (Fig.1C).

The production of TSP-4 is regulated by the properties of the ECM. Mesenchymal stem cells (MSC) produced 2.5-fold more TSP-4 when grown on highly anisotropic matrices than the cells on low anisotropy matrices (87), reflecting the tenogenic differentiation of MSC that also produced high levels of collagen I and III. TSP-4 upregulation seems to be always associated with the formation of tendon tissue: treatment of the murine mesenchymal stem cell line with BMP12 and BMP13 that induce formation of the tendon-like tissue (versus osteogenic differentiation in response to BMP2) resulted in a dose-dependent TSP-4 upregulation (88).

TSP-4 appears to play a role in wound healing and hypertrophic scar formation. It was one of the 6 genes selectively upregulated in hypertrophic scars, along with collagen I (89). Excisional wound healing was significantly delayed in *Thbs4*$^{-/-}$ mice, with decreased angiogenesis (17).

Regulation of ECM by TSP-4

The effects of TSP-4 on the production of ECM are tissue-specific. For example, in the heart, TSP-4 deficiency augmented deposition of ECM (18), while in the skeletal muscle TSP-4 knockout was associated with the loss of ECM (30). Consistent with these observations in *Thbs4*$^{-/-}$ mice, rTSP-4 administration increased production of collagens by cultured fibroblasts (18), but treatment of cultured EC with rTSP-4 resulted in a loss of specific ECM components (30).

TSP-4 controls modifications of heparan sulfates (HS) chains the levels of HS core proteins the levels of HS core proteins in skeletal muscle tissue and (30). Glypican and beta-glycan, a receptor for TGF-beta, were remarkably downregulated in the skeletal muscle tissue of *Thbs4*$^{-/-}$ mice (30). In cultured endothelial cells (EC), TSP-4 induced the production of beta-glycan and increased TGF-beta signaling. TSP-4 appears to regulate the production of HS core proteins by influencing their expression rather than their retention in tissues: the levels of two enzymes responsible for the key steps in synthesis of HS, heparan sulfate 2-O-sulfotransferase 1 and N-heparan sulfate sulfotransferase 3, were reduced in *Thbs4*$^{-/-}$ mice, and cultured EC had increased levels of these enzymes in response to rTSP-4 (30).

In the skeletal myofibers, TSP-4 enhances vesicular trafficking of dystrophin-glycoprotein and integrin attachment complexes to stabilize the sarcolemma (90). Interestingly, in a

Drosophila model of muscular dystrophy, muscle-specific overexpression of either *Drosophila* TSP-4 ortholog or mouse TSP-4 rescued the phenotype, stressing the structural and functional evolutionary conservation of TSP-4 (90).

Expression of TSP-4 appears to be closely associated with TGF-beta signaling (16, 30, 91). However, the associations are complex and point to a multi-stage reciprocal regulation between TGF-beta and TSP-4. In addition to the loss of beta-glycan in skeletal muscle of *Thbs4*$^{-/-}$ mice and EC from these mice, the effect of TSP-4 on TGF-beta signaling was augmented in *Thbs4*$^{-/-}$ EC, where beta-glycan was absent, suggesting that beta-glycan played an inhibitory role and that TSP-4 regulates both the initiation of TGF-beta signaling and its feedback inhibition. TSP-4 was upregulated in response to TGF-beta in cultured EC, *in vivo* (16), and other cell types (unpublished data), but higher levels of TSP-4 were detected in the ventricular cardiomyocytes of TGF-deficient mice (91). Although the signaling pathways connecting TSP-4 and TGF-beta are not completely understood, it is clear that TSP-4 is regulated by these pathways and is in turn affecting TGF-beta signaling.

TSP-4 in skeletal muscle

High levels of TSP-4 in myotendinous and neuromuscular junctions suggest that it is important for the correct assembly and function of the motor unit. Indeed, without TSP-4 skeletal muscle does not function properly. Both the *Drosophila* and the zebrafish TSP-4 orthologs are required for the assembly of the myotendinous junctions and their repair after injury (67, 92). The protein interacted with the integrins and organized a proper laminin localization to promote attachment, and both muscle and tendon TSP-4 was required for attachment. The pentameric structure of TSP-4 was essential for these scaffolding functions, confirming its scaffolding function and simultaneous binding to multiple ligands and receptors as is schematically demonstrated in Figure 1C. In zebrafish, TSP-4 was a component of extraocular tendon at muscle origins (59).

Although the phenotype in mice is less striking, TSP-4 knockout in mice still results in lower muscle mass and poorer performance of limbs. Compared to WT mice, *Thbs4*$^{-/-}$ mice are less capable of maintaining grip strength with age, thereby implying possible impairment of muscle function (30). By stabilizing the membrane and cellular attachment in skeletal muscle, TSP-4 regulates the skeletal muscle integrity (90). Loss of TSP-4 gene resulted in spontaneous dystrophic changes in skeletal muscle with aging and accelerated muscular dystrophy in mouse models, and overexpression of TSP-4 mitigated the dystrophic disease (90).

Although TSP-4 seems to have some structural role in the tendon, in skeletal muscle it appears to function as a classical matricellular protein, regulating the composition of ECM and affecting the cellular functions of myocytes. There, TSP-4 is associated with myotendinous junctions, perimysium, endomysium, and endothelial cells (EC) (30). In the cell culture, the C-terminal fragment of TSP-4 promoted myoblast adhesion although it was not as potent as a similar fragment of TSP-1, despite very high homology between TSPs in this region (93). *In vivo*, TSP-4 regulates the production and composition of ECM in skeletal muscle and the cellular metabolic functions. TSP-4 is especially abundant in red muscles

that primarily utilize oxidative metabolism. TSP-4 deficiency results in impaired metabolism in red muscles: the uptake of very low-density lipoproteins (VLDL) was decreased in red muscles of *Thbs4*$^{-/-}$ mice and in cultured EC from these mice (30). While the activity of lipoprotein lipase, the enzyme regulating the uptake of VLDL, was decreased both *in vivo* and *in vitro*, TSP-4 did not affect the levels of lipoprotein lipase. It appears that the regulation of HS proteoglycans influenced the activity of lipoprotein lipase that has to bind the surface of a cell to become active. Thus, TSP-4 maintains the LPL activity and the metabolism of red muscle by supporting production and modification of specific HS. *Thbs4*$^{-/-}$ mice on the high fat/high carbohydrate content diet developed higher blood levels of total cholesterol and VLDL, consistent with the inability to process VLDL due to the impaired function of endothelial LPL (30).

Functions of TSP-4 in myocardium

Healthy and especially remodeling hearts express high levels of TSP-4 (18, 31, 81, 94, 95). Studies in *Thbs4*$^{-/-}$ mice revealed the importance of TSP-4 in heart remodeling and maintenance of a healthy heart ECM (18, 31). In response to pressure overload, *Thbs4*$^{-/-}$ mice developed a significantly higher heart weight to body weight ratio than WT mice, and function of the heart was impaired. Control of fibrosis caused by pressure overload is the main function of TSP-4 in remodeling heart. TSP-4 does not affect the size or apoptosis of cardiomyocytes, but prevents deposition of interstitial ECM and heart hypertrophy and maintains the adaptive mechanisms that augment contractility and activate stretch-response upon pressure overload. TSP-4 prevents depositions of collagen I, II, III, and V, without affecting the levels of collagen IV (30). The increased levels of TSP-4 mRNA in remodeling hearts were associated with interstitial fibroblasts (30). Interestingly, ECM deposition increases in aging mice without pressure overload (18), suggesting that TSP-4 becomes more influential with age, consistent with the observation of age-related changes in skeletal muscle of aged mice (30). One of the signals upregulating TSP-4 in angiotensin II infusion model of cardiac hypertrophy is Kruppel-like factor 6 (Klf6) (96): in *Klf6*$^{+/-}$ mice (*Klf6*$^{-/-}$ is embryonically lethal), diminished cardiac fibrosis was associated with increased expression of TSP-4. Chromatin precipitation showed recruitment of Klf6 to TSP-4 promoter and a dose-dependent repression of TSP-4 promoter activity by Klf6. The loss of the contractility and stress-response adaptation was associated with changes in the composition of the matrix and direct regulation by TSP-4 in response to pressure overload: incubation of the muscle with recombinant TSP-4 restored the contractility, suggesting that TSP4 is a myocyte-interstitial mechano-signaling protein regulating adaptive cardiac contractile responses to acute stress (31).

Similar to other tissues, in the myocardium, TSP-4 is upregulated in many situations involving injury and tissue remodeling. Increased expression of TSP-4 was detected in the chronic ischemic myocardium of non-failing human left ventricle and was associated with chronic myocardium remodeling and expression of a number of other ECM genes involved in matrix remodeling (97).

A surprising intracellular function for TSP-4 in cardiomyocytes was associated with its functions in secretory pathways. The type-3 repeat domain of TSP-4 bound the ER luminal

domain of activating transcription factor 6α (Atf6α) to promote its nuclear shuttling and to activate the protective ER stress response (95). This function is shared by other TSPs and is not limited to cardiomyocytes: TSP-4 activated of the ER stress response in skeletal myocytes as well (95). In addition to activation of the ER stress response, TSPs, including TSP-4, were found to affect cellular calcium signaling through an interaction with STIM1, a transmembrane protein that functions in the endoplasmic reticulum (ER) to detect calcium depletion, in the ER and plasma membrane (98). These novel functions for TSP-4 in ER open a whole new direction of study of TSP-4 and other TSPs – their intracellular regulatory roles associated with secretory pathways that have not been appreciated earlier. Not only protein interactions can occur in the ER environment before proteins are secreted, but important signaling events can be initiated by TSP-4 and other secreted proteins while progressing through the secretory pathways.

Interestingly, the binding site that mediated the interaction of TSP-4 to Atf6α was localized to Type 3 repeats of TSP-4, the same region where SNP associated with increased risk of the myocardial infarction (MI) and the coronary artery disease (CAD) was found (44) and confirmed in several human population studies (41–48). This region of TSP-4 appears to be important in most of TSP-4 functions, and the mutation corresponding to the disease-associated SNP results in a change of TSP-4 properties and its interactions with the cells (4, 17, 40, 50, 73, 99).

TSP-4 in remodeling of vasculature

TSP-4 may affect the development of MI/CAD in several complementary ways: by regulating the adaptive responses of the myocardium, by influencing the growth and remodeling of the blood vessels, and by affecting the inflammatory responses. Atherosclerotic lesions in *Thbs4*$^{-/-}$/*ApoE*$^{-/-}$ mice were smaller and less cellular, with decreased levels of inflammatory markers and EC activation in blood vessels (49). TSP-4 was abundant in atherosclerotic lesions and areas of blood vessels prone to development of lesions, and TSP-4 deficiency prevented accumulation of macrophages in lesions. rTSP-4 supported migration and adhesion of macrophages and neutrophils and activation of pro-inflammatory signaling in leukocytes (49, 50). These observations assigned another important role for TSP-4 in tissue remodeling – regulation of local inflammation through the effects on leukocytes.

Inflammation and tissue remodeling are intimately associated with angiogenesis and remodeling of the vasculature. The first indication that TSP-4 may regulate these processes came from a study of remodeling myocardium in response to the pressure overload: in *Thbs4*$^{-/-}$ mice, the area occupied by microvessels was dramatically decreased after transverse aortic constriction (TAC) as compared to WT mice (30). When the ability of TSP-4 to support angiogenesis was tested directly *in vivo* in mouse models of angiogenesis and *in vitro*, TSP-4 was found to promote the growth of new blood vessels and to support the pro-angiogenic functions of EC (17). In developing retina, in cancer and Matrigel plug models, and in wound healing, TSP-4 knockout mice had delayed angiogenic response, while the mutant MI/CAD-promoting TSP-4 was even more pro-angiogenic than WT TSP-4.

The pro-angiogenic properties of TSP-4 set this TSP apart from other TSPs: TSP-1 and TSP-2 are two well-known, potent inhibitors of angiogenesis while TSP-3 did not affect angiogenesis (100), and no angiogenesis-related effects were reported for TSP-5. TSP-3, TSP-4, and TSP-5 do not harbor the protein domains that mediate the anti-angiogenic activities of TSP-1 and TSP-2 (5, 7). More intriguing is the difference between TSP-4 and TSP-3/TSP-5 that have a very high homology to each other.

Based on the increased pro-angiogenic properties of TSP-4 with the mutation in type 3 repeat region and the dependence of pro-angiogenic effects on α2δ-1 receptor for gabapentin that was shown to interact with this region of TSPs (17, 52), the third EGF-like repeat is critical for the pro-angiogenic functions of TSP-4.

TSP-4 and TGF-beta

TSP-1 activates TGF-beta (20–23, 101, 102) and is regulated by TGF-beta (16, 103, 104). It is not surprising that TSP-4, a regulator of ECM remodeling and synthesis, is also involved in responses mediated by TGF-beta, the main regulator of ECM production.

TSP-4 was required for angiogenesis in response to TGF-beta: in *Thbs4*$^{-/-}$ mice or in models where TSP-4 production was suppressed using shRNA, the angiogenic response to TGF-beta was dramatically reduced, and the growth of a tumor caused by TGF-beta was completely lost (16). TGF-beta induced production of TSP-4 but not other TSPs in EC. More than one report showed that this response to TGF-beta is cell-type-specific. TSP-4 was not detected in response to TGF-beta in vascular smooth muscle cells *in vitro* (16), and the myocyte-selective TGFβ inhibition augmented the synthesis of TSP-4 in myocytes.

The cell-specificity of the production of TSP-4 in response to TGF-beta suggests that TSP-4 may mediate stage-specific effects of TGF-beta, i.e., acceleration of tumor growth at the later stages when a tumor is well vascularized, and ECs provide sufficient amounts of TSP-4 to support further angiogenesis. Although the details of the regulation of TSP-4 production in response to TGF-beta are not fully known, the involvement of SMAD3 was reported (16).

TSP-4 in cancer

The pro-angiogenic properties of TSP-4 may explain its high expression in several cancers. Increased expression of TSP-4 in cancer tissues is associated with cancer progression. Remarkably, TSP-4 has been identified among the top 1% of most upregulated genes in several types of cancer (75–80). In breast cancer, increased TSP-4 expression was associated with the stromal response to invasive cancer, suggesting that TSP-4 in ECM contributes to the tumor progression and facilitates the invasion of tumor cells (105). Differential expression of TSP-4 was detected in lobular versus ductal breast carcinomas (106), suggesting that it could be a marker of a specific breast cancer type, and studying its effect could be important to understand the basis of phenotyping differences in breast cancers. TSP-4 also showed the strongest correlation to a histological type of gastric adenocarcinoma, being overexpressed in diffuse type versus intestinal type (107). These associations with specific forms of cancers versus other forms could shed light on TSP-4 functions in cancer growth if further investigated. TSP-4 was upregulated in Wilms tumor

and mesoblastic nephroma (108) and prostate cancers (109). In hepatocellular carcinoma, TSP-4 was overexpressed and correlated with prognosis (110), and its knockdown inhibited hepatocellular-carcinoma-induced angiogenesis, migration, and invasion of the cancer cells. TSP-4 was identified as one of the members of a gene network, which regulates liver bud expansion by controlling hepatoblast migration and adhesion during the liver development and was proposed as a candidate gene for investigation of liver carcinogenesis (111).

The roles of TSP-4 in different cancers are as complex as the roles of another TSP, TSP-1, widely associated with cancer: both have been reported as promoting certain types of cancers and inhibiting others. TSP-4 gene was methylated and silenced in colon cancer (112, 113) and in primary cutaneous T-cell lymphoma (114). Based on these observations, a protective role for TSP-4 was proposed. However, further examination of the TSP-4 role in cancer is warranted to distinguish between the silencing of TSP-4 as a tumor suppressor or its suppression as a feed-back protective anti-cancer mechanism. A direct effect of TSP-4 on cancer cells is also possible, although opposite roles for TSP-4 were found in different cancer cells: forced expression of TSP-4 in colonies of colorectal cancers caused dramatic repression of tumor growth (113), but knockdown of TSP-4 in prostate cancer cells significantly reduced their migratory and invasive abilities and decreased the expression levels of p38 and matrix metalloproteinase (MMP)-9 (109). As with TSP-1, effects of TSP-4 in cancerare complex and suggest tissue specific and cell specific roles.

Role of TSP-4 in the nervous system

Important and unexpected functions have been recently ascribed to TSP-4 in the nervous system.

Over twenty years ago, Arber and Caroni reported that TSP-4 is expressed by neurons, promotes neurite outgrowth, and is especially abundant in synapse-rich structures in the cerebellum and retina of adults (69). These unanticipated observations were made during the search for muscle genes that may be involved in neuromuscular signaling. TSP-4 was tested for effects on cultured motor, sensory, and retina neurons, and the results suggested that TSP-4 was a preferred substrate and promoted neurite outgrowth. The effect of TSP-4 is dependent on laminin, and TSP-4 acts as an organizer of adhesive and axon outgrowth-promoting molecule in the ECM (70). TSPs were identified as a necessary and sufficient synaptogenic signal secreted by astrocytes that increases synapse number (115, 116).

The CNS synaptogenesis was dependent on interaction of α2δ-1 gabapentin receptor with Type 3 (EGF-like repeats) domains of TSPs (52). TSP-4 promoted neuronal differentiation of neural progenitors that may be a potential source of therapy for neurological disorders (117). The idea that TSP-4 may be involved in signaling during development and repair processes in nervous system was further tested in other models. A localized photothrombotic/ischaemic cortical injury initiated a marked increase in astrocyte production of TSP-4 from the postnatal subventricular zone, where the neural stem cells express a remarkably high level of TSP-4 (118). In *Thbs4*$^{-/-}$ mice, migration of newly formed neurons along the rostral migratory stream (RMS) to eventually integrate the olfactory bulb neuronal circuitry was impaired: some neurons migrated out of RMS (119),

supporting the role for astrocyte-produced TSP-4 in the migration of newly formed neurons to the olfactory bulb. The robust post-injury astrogenic response was modulated by TSP-4 via direct Notch1 receptor binding and activated downstream signals for glia production. *Thbs4*$^{-/-}$ mice had severe defects in cortical-injury-induced SVZ astrogenesis and abnormal glial scar formation in response to injury, as well as increased hemorrhage. These results implicate TSP-4 into the post-injury recruitment of neural stem cells (118). After a subtle trauma of the brain, increased expression of TSP-4 was detected in the hippocampal tissue at the injured side and the contralesional side, implicating TSP-4 in reparative response involving activation of astrocytes and triggering molecular and structural changes in the uninjured hemisphere (120).

Binding of TSP-4 to a receptor for an anti-epileptic and analgesic drug gabapentin (52) and its high expression upon injury suggested a role for TSP-4 in pain development after injury. In a neuropathic pain model of spinal nerve ligation injury, increased expression of TSP-4 was demonstrated at the injury side of dorsal spinal cord that correlates with the development of neuropathic pain states. Blockade of TSP-4 or inactivation of Thbs4 gene prevented hypersensitivity, while injections of TSP-4 caused hypersensitivity and increased the frequency of excitatory postsynaptic potential (121). The effect of TSP-4 was due to decreasing high-voltage-activated calcium current and increasing low-voltage-activated calcium current in dorsal root ganglia (DRG) (53) by activating its receptor α2δ-1 calcium channel subunit (54). Further investigation of this new function of TSP-4 in various models (51, 122, 123) suggested that TSP-4 could become a potential target for development of antagonists with therapeutic potential for target-specific neuropathic pain management.

Concluding Remarks

TSP-4 was first identified in 1993 (57) but interest in the molecule was modest for the remainder of the twentieth century. Now there are over 140 publications related to TPS-4 in PubMed. These reports identify numerous unanticipated functions of TSP-4, often found in gene expression profiles, in many different tissues and in various physiological and pathological responses. We can anticipate that the exponential increase in interest in TSP-4 will continue and may even explore TSP-4 as a therapeutic target to encourage angiogenesis under ischemic conditions, to suppress tumor growth in various tissues, and to alleviate pain.

Acknowledgments

Funding

This work was supported by the National Institutes of Health [grant numbers HL117216 and CA177771]

Abbreviations

Atf6a	activating transcription factor 6a
ApoE	apolipoprotein E
BMP	bone morphogenic protein
CAD	coronary artery disease

CNS	central nervous system
DRG	dorsal root ganglia
EC	endothelial cells
EGF	epithelial growth factor
ECM	extracellular matrix
ER	endoplasmic reticulum
HS	heparin sulfate
Klf6	Kruppel-like factor 6
KO	knockout
MI	myocardial infarction
MMP	matrix metalloproteinase
MSC	mesenchymal stem cells
RMS	rostral migratory stream
shRNA	small hairpin RNA
SMAD3	Mothers against decapentaplegic homolog 3
SMC	smooth muscle cells
SNP	single nucleotide polymorphism
STIM1	Stromal interaction molecule 1
SVZ	subventricular zone
TAC	transverse aortic constriction
Thbs4	mouse TSP-4 gene
TGF	transforming growth factor
TSP	thrombospondin
VLDL	very low density lipoprotein

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Highlights

- Thrombospondin-4 (TSP-4) regulates ECM synthesis, composition, and organization
- TSP-4 regulates tissue and ECM remodeling in embryonic development and in adults after injury
- TSP-4 has intracellular functions in endoplasmic reticulum where it initiates stress-response signaling
- TSP-4 has functions distinct form other members of thrombospondin family members
- TSP-4 regulates remodeling of multiple tissues and organs including the heart, the nervous system, the skeletal muscle, the tendon, and cancers.

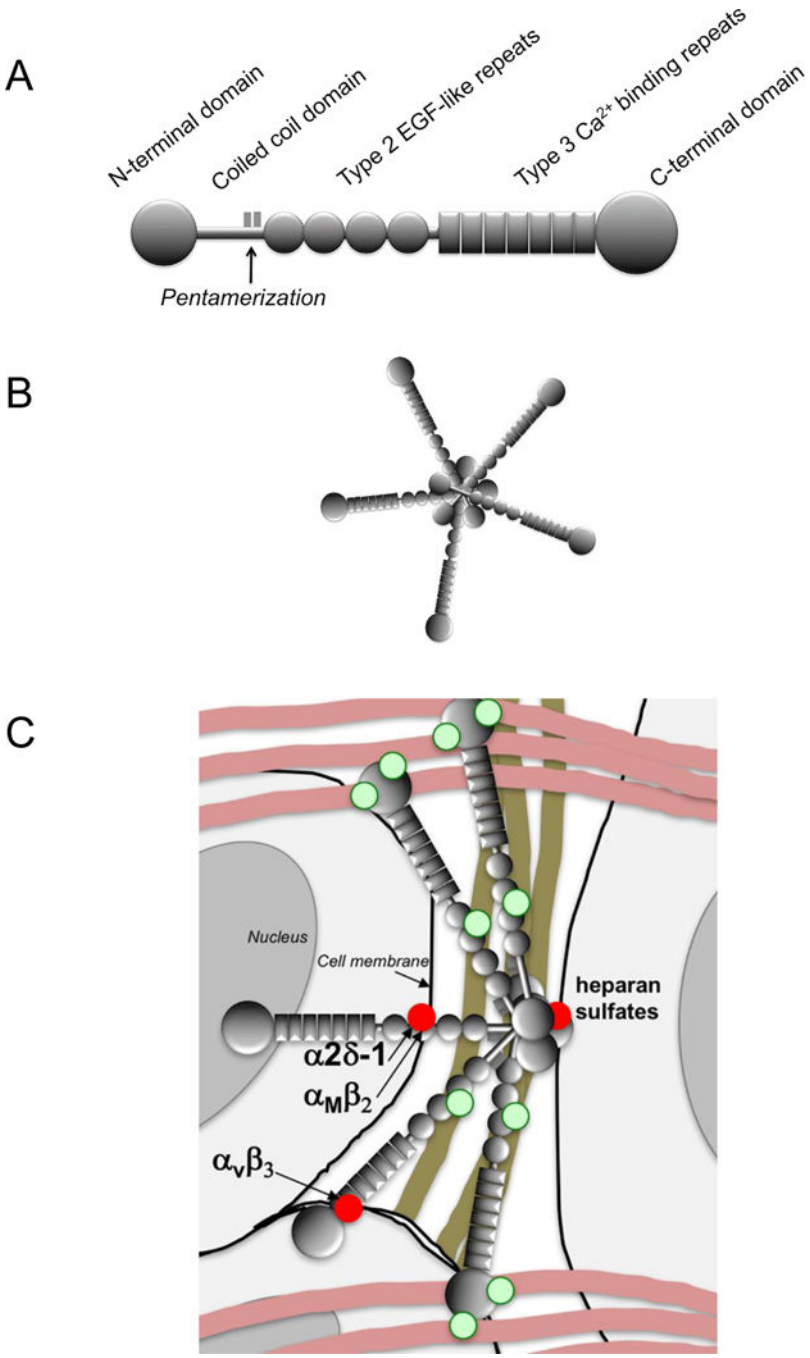

Figure 1. Domain structure of TSP-4 and its interaction with cells and ECM

A: Domains of a TSP-4 monomer; B: TSP-4 is a pentamer; C: The pentameric structure allows TSP-4 to engage in multiple interactions with cells and proteins in ECM through binding sites in heparin-binding N-terminal domain, EGF-like domains, and conserved L-lectin C-terminal domain. Binding sites for the cellular receptors are marked with red dots (e.g., heparan sulfates, integrins $\alpha_v\beta_3$, and $\alpha_M\beta_2$, gabapentin receptor $\alpha_2\delta$-1); binding sites for ECM proteins are marked with green dots. There are both the ECM ligands binding the L-lectin domain (shown in pink), and ligands with binding sites in other domains of TSP-4,

e.g., in EGF-like domains (shown in brown). Thus, a single molecule of TSP-4 can simultaneously engage multiple cellular receptors and ECM ligands and organize ECM and the interaction of the cells with ECM.

Table 1

Functions of TSP-4

Function	Location	Receptor	Signaling	References
Promotes angiogenesis	Tumor, Heart, Skin wound Endothelial cells	α2δ-1 α_2-integrin		(16–18)
Mediates effects of TGF-β	Tumor, Endothelial cells		TSP-4 is upregulated by TGF-β via SMAD3; TSP-4 increases levels of TGF-β receptor β-glycan	(16, 30)
Suppresses production of ECM	Heart, Cultured fibrobalsts			(18, 30, 31)
Stimulates production of ECM	Skeletal muscle		TGF-β	(30)
Organizes ECM	Muscle Tendon			(67, 85, 90, 92)
Controls modifications of Heparan Sulfates chains	Skeletal muscle Endothelial cells		Heparan sulfate 2-O-sulfotransferase 1 and N-heparan sulfate sulfotransferase 3	(30)
Regulates remodeling of myocardium	Heart		Kruppel-like factor 6 (Klf6) upregulates TSP-4 in angiotensin II infusion model of cardiac hypertrophy	(18, 30, 31, 96)
Regulation of adaptive cardiac contractile response	Heart		ERK1/2; Akt	(31)
Limb regeneration	Transitional matrix, axolotl			(68)
Organization of collagen fibers	Tendon			(30)
Regulates skeletal muscle integrity and metabolism				(30, 59, 67, 69, 90, 92)
Organization of myotendinous and neuromuscular junction	Skeletal muscle			(59, 67, 69, 92)
Wound healing and scar formation				(17, 89)
Regulation of functions of stem cells	Eye (ocular surface epithelia)			(60)
Promotes local inflammation	Blood vessels Endothelial cells			(18)
Promotes atherogenesis and CAD				(18, 41–48)
Promotes adhesion, migration, recruitment of leukocytes into tissues, and pro-inflammatory responses		$\alpha_M\beta_2$-integrin, $\alpha_V\beta_3$-integrin,	P38 MAPK	(49, 50)
Regulates cancer growth	Gastric cancer; Breast cancer; Liver cancer; Colorectal cancer, Prostate cancer			(16, 17, 75–80, 105–111, 113)
Promotes neurite outgrowth, neuronal differentiation, and	Nervous system	Notch1		(69, 70, 117–120)

Function	Location	Receptor	Signaling	References
post-injury recruitment of neural stem cells				
Promotes formation of synapses	Nervous system	α2δ-1		(115, 116)
Nociception	Nervous system	α2δ1	Decreasing high-voltage- activated calcium current and increasing low-voltage-activated calcium current	(51, 53, 54, 121–123)
Activates ER stress response	ER of cardiomyocytes	Activating transcription factor 6α (Atf6α)	Activating transcription factor 6α (Atf6α)	(95)
Regulates Ca^{2+} signaling	ER, variety of cells	STIM1	Calcium release activated calcium (CRAC) channel and arachidonic acid calcium (ARC) channel	(98)