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Title:

Thrombospondins: Conserved Mediators and Modulators of Metazoan Extracellular Matrix

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Running Title: Thrombospondins and extracellular matrix

Abbreviations

COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; GFP, green fluorescent protein; LRP, low-density lipoprotein receptor related protein; MMP, matrix metalloproteinase; PSACH, pseudoachrondroplasia; RFP, red fluorescent protein; siRNA, short interfering RNA; TEM, transmission electron microscopy; TGFbeta, transforming growth factor beta; TSP/TSPs, thrombospondin/thrombospondins; TSR, thrombospondin type 1 repeats; VEGF, vascular endothelial growth factor

ABSTRACT

This review relates to the BSMB Fell-Muir Lecture which I was honoured to present at the BSMB Spring Meeting in Sheffield in April 2023. It provides a personal overview of significant scientific developments in the thrombospondin field during the course of my career. Thrombospondins are multidomain, multimeric, calcium-binding extracellular glycoproteins with context-specific roles in tissue organisation, that act at cell surfaces and within ECM to regulate cell phenotype and signaling, differentiation, and assembly of collagenous ECM, along with tissue-specific roles in cartilage, angiogenesis, and synaptic function. More recently, intracellular, homeostatic roles have also been identified. Resolution of structures for the major domains of mammalian thrombospondins has facilitated major advances in understanding thrombospondin biology from molecule to tissue; for example, in illuminating molecular consequences of disease-causing coding mutations in human pseudoachrondroplasia. Although principally studied in vertebrates, thrombospondins are amongst the most ancient of animal ECM proteins, with many invertebrates encoding a single thrombospondin and the thrombospondin gene family of vertebrates originating through gene duplications. Moreover, thrombospondins form one branch of a thrombospondin superfamily that debuted at the origin of metazoans. The super-family includes additional sub-groups, present only in invertebrates, that differ in N-terminal domain organisation, share the distinctive TSP C-terminal region domain architecture and, to the limited extent studied to date, apparently contribute in tissue development and organisation. Finally, major lines of translational research are discussed, related to fibrosis; TSP1 and TSP2 and inhibition of angiogenesis, and the alleviation of chronic cartilage tissue pathologies in pseudoachrondroplasia.

Keywords: angiogenesis, cell adhesion, collagens, connective tissue, evolution, fibrosis, molecular phylogeny

1.Introduction

This review discusses advances in knowledge of the molecular, cellular and tissue-level roles of thrombospondins (TSPs). Starting with the historical context of the discovery of the first TSP, now known as thrombospondin-1 (TSP1), emphasis is then placed on the cellular interactions and tissue-specific functions of mammalian TSPs, the significance of advances in knowledge of the structural biology of TSP domains for dissecting structural /functional relationships, the complexity of extracellular interactions of TSPs at cell-surfaces and within the ECM, and intracellular activities related to cell-stress responses. These advances are placed in context of how the rapid escalation of genome sequencing projects in the 2000s facilitated studies of TSP localisation and functions in invertebrates and enabled identification of previously unappreciated TSP-related proteins, leading to a new view of the TSPs as one branch within a TSP superfamily. Major current translational research directions are briefly discussed. Given the expansion of the field in many directions over the last ~35 years, this overview has involved selection of the topics to cover in more or less depth and previous detailed reviews are cited for further reading where appropriate.

2. Identification of mammalian Thrombospondins and their structural domains

2.1 *Identification of Thrombospondins and molecular cloning*

The first TSP to be identified, now known as TSP1, was discovered in the 1970s as a thrombin-sensitive glycoprotein released from platelet alpha-granules upon platelet activation and reported to have a high content of acidic residues[1 2]. It was subsequently named thrombospondin due to its release upon thrombin activation. Biochemical analyses of purified TSP1 showed it to consist of three disulphide-bonded polypeptides of around 142,00 kDa and to bind heparin[3]. The heparin-binding property enabled a rapid and straightforward affinity purification strategy for TSP1 from lysates of expired human platelets[3,4] and, later, for other TSPs from other cell sources. Other biochemical studies showed TSP1 from platelets to undergo Ca^{2+}-dependent, disulphide-mediated inter-molecular interactions that led to formation of TSP1 aggregates or covalent linking of TSP1 with thrombin[5,6]. These properties related to the presence of free thiols within TSP1. Later, each subunit of TSP1 was shown to contain one free thiol, residing on any one of 12 Cys residues, demonstrating a propensity of TSP1 to undergo intra-molecular disulphide isomerisation[7,8]

The availability of purified TSP1 also facilitated observation of the molecule by rotary shadowing transmission electron microscopy (TEM). These studies confirmed TSP1 as a trimeric protein and identified the apparent trimerization region to be located adjacent to one of the globular ends of each polypeptide, with the rest of each polypeptide forming a flexible “arm” ending in a larger

globular region. TEM studies of TSP1 purified under different cationic conditions showed that the length of the arms was highly dependent on Ca^{2+} ion concentration, with TSP1 prepared in the presence of EDTA having longer connecting arms (~38nm compared to ~29nm in the presence of Ca^{2+} ions) and smaller C-terminal globular regions (~8nm diameter compared to ~12nm)[9,10]. Other TEM studies, that combined identification of antibody-binding sites on TSP1 with N-terminal amino acid sequencing of proteolytic fragments known to bind to specific antibodies, demonstrated the trimerization region to be adjacent to the N-terminal globular domain; the latter was also found to be the major heparin-binding region[9,11]. These studies and analyses of binding properties of major proteolytic fragments set the scene for a model of the TSP1 homotrimer (Fig. 1A), for which a number of features have proved durable[12].

The cloning and nucleotide sequencing of cDNA encoding TSP1 from a human endothelial cell cDNA library[13] considerably advanced the field with the discovery that TSP1 polypeptide subunits contain multiple types of repeated domains in their central regions, flanked by N- and C-terminal regions with unique sequences (Fig 1B). The putative oligomerisation region was positioned after the N-terminal domain and included a CXXSC motif and a series of heptad-repeat motifs. These are seven-residue motifs with a characteristic pattern of polar (P) and hydrophobic (H) residues, namely HPPHPPP, that form an alpha-helix of 3.6 residues per turn. The hydrophobic residues have an average spacing of 3.5 residues and lie on the concave face of the helix. Because a coil is imposed on the helix due to shorter hydrogen bond lengths in the vicinity of the apolar residues, two or more matching helices tend to wrap around each other to form a left-handed super-helix, also known as a coiled-coil[14,15]. The central region of the TSP1 polypeptide included a von Willebrand Factor type C domain (vWF_C) and several repeated sequence modules. The TSP type 1 domains (now referred to as thrombospondin repeated (TSR) domains) had sequence similarity with surface proteins of the circumsporozoites of the malaria parasite, *Plasmodium falciparum,* and related species. It was subsequently appreciated that TSR domains are widespread in many extracellular proteins that function in protein-protein and protein-carbohydrate interactions[16] and are modified post-translationally by unusual O-fucose-linked and C-mannose glycosylations[17]. The epidermal growth factor-like (EGF-like) domains (designated originally as TSP type 2 domains), of which there are three in TSP1, include six conserved cysteine residues that form three disulphide bonds and are also modified by O-linked glycans[18]. The EGF domains provided a recognisable commonality with the domain composition of other ECM proteins such as laminin or tenascins. In accordance with the calcium-sensitivity of TSP1 previously recognised from TEM and limited proteolysis studies, it was notable that the so-called TSP type 3 repeats were found to be rich in aspartic acid residues and thus had potential for coordination of many calcium ions. Indeed, one molecule of TSP1 binds cooperatively an average

of 35 calcium ions[19]. A single ArgGlyAsp (RGD) integrin-binding motif was identified in the last type 3 repeat of TSP1[13].

By 1995, the field had stepped on again with the realisation that TSP1 was not a singleton but instead was a member of a five gene family of TSPs in mammals (Fig. 1B). TSP2 through to TSP4 were identified by molecular cloning and numbered in order of discovery[20–22]. In separate research, a 524,000, disulphide-bonded protein designated cartilage oligomeric matrix protein (COMP), had been purified from cartilage ECM[23]. The subsequent cloning of COMP from cDNA revealed its molecular relationship with the other TSP family members[24]. Whereas TSP2 has the same domain composition as TSP1, TSP3, TSP4 and TSP5 all lack the vWF_C domain and have four EGF-like domains. Some of these EGF domains (eg, EGF domains 2 and 3 of TSP4[22]) have characteristics of calcium-binding EGF domains (InterPro IPR001881). TSP5 is distinct in lacking an N-terminal globular domain (Fig. 1B).

Electron microscopy studies of the "new" TSPs showed TSP3, TSP4 and TSP5/COMP to be pentameric rather than trimeric[25–27]. Although assumed to each form homo-pentamers, mixed pentamers of TSP4 and TSP5 have been isolated from equine tendon[28]. A terminology of two subgroups, A and B, according to whether the polypeptides form trimers (TSP1 and TSP2), or pentamers (TSP3, TSP4, TSP5/COMP) was proposed[29]. In sequence terms, the positioning of the paired cysteine residues associated with the heptad-repeats provides a 'signature' that distinguishes the two categories of oligomerisation domain: the two cysteine residues precede the heptad repeats in trimeric TSP1 and TSP2, whereas a CXXC motif is positioned after the heptad-repeats in the pentameric TSPs[25,30–32] (Fig 1C). Later analysis of the specific sequence characteristics of trimerizing or pentamerising heptad-repeat regions from a large dataset of TSP sequences identified differences of conserved residues and their chemical properties between the heptad-repeat regions of subgroup A and B TSPs. In particular, the heptad repeats of subgroup B TSPs include conserved charged residues that may be responsible for forming inter-chain hydrogen bonds and intra-helix salt bridges to stabilise the pentameric coiled-coil[33] (Fig. 1C). A timeline of research milestones for the TSP field is shown in Fig. 1D.

2.2 *Domain structures*

During the 2000s, crystal structures were solved for all the major domains of the mammalian TSPs (Fig. 1D) and these important advances led to paradigm shifts in understanding the relationships between the domains (Fig. 2). The N-terminal domain was found to belong to the laminin-G domain structural family[34]. The COMP coiled-coil was demonstrated to indeed assemble as a pentamer with a central pore (7.3nm in length and diameter 0.2-0.6nm) that, in vitro, binds and sequesters hydrophobic compounds[35,36]. Structures became available for other

examples of vWF_C domains (eg,[37]). The TSR domain was identified to form a unique structural fold with three disulphide bonds and a positively charged groove on one face[38]. Although domain diagrams such as Fig. 1 must necessarily present the domains as 'beads on a string', crystal structures of TSP C-terminal regions, (comprising either the last three type 3 repeats and the globular C-terminal domain of TSP1[39], or the entire region from the last EGF domain to the C-terminus of TSP2[40] or TSP5[41]), revealed extensive intra-molecular, calcium-dependent and disulphide-bond interactions, such that this entire highly-conserved region should be considered a single structural unit. The "C-terminal domain" itself forms a beta-sandwich 'jelly-roll" structure and belongs to the Legume-lectin (L-lectin) domain structural family[39–41]. In sequence terms, the type 3 repeats and L-lectin domain are the most highly conserved region between TSP family members[29,32]. Since both the Laminin-G domain family and the L-lectin domain families belong to the concanavalin A-like lectin/glucanase fold superfamily (InterPro 013320), the N- and C-terminal domains, although distinct in sequence terms, are in fact related in structure (Fig. 2).

3. Genetic associations and physiological roles in vertebrates

3.1 *Human disease associations*

TSP5/COMP is definitively associated with human genetic diseases: missense point mutations or small nucleotide deletions in *COMP* are causal for the autosomal dominant disorders: pseudoachrondroplasia (PSACH) and multiple epiphyseal dysplasia, type 1 (EDM1)[42,43] (OMIM 132400 and 177170). Both conditions are skeletal dysplasias characterised by post-natal short stature due to short limbs, loose joints, leg bowing in PSACH, and likelihood of early-onset osteoarthritis. The mutations most frequently involve deletion or point mutation of calcium-binding residues in the type 3 repeats, but point mutations also occur within the EGF domains or the C-terminal L-lectin domain and over 300 distinct disease-causing mutations have been identified[44,45]. Some frequent mutations have been studied in vitro for effects on the protein structure or binding interactions. For example, the mutation COMPD446N affects protein structure and reduces calcium binding by the type 3 repeats[46]. The mutation D469del affects the conformation of the type 3 repeats, reduces calcium binding and reduces binding to various collagens[47]. PSACH and EDM1 mutations have dominant negative effects, even in the heterozygous state, because the assembly of COMP molecules as homo-pentamers means that inclusion of even a single misfolded subunit promotes misfolding of the quaternary structure of the pentamer resulting, in most cases, in failure of the molecule to complete successful trafficking through the secretory pathway[48]. COMP is highly expressed in cartilage throughout life and so misfolded COMP builds up in the rough endoplasmic reticulum (rER), leading to a 'giant ER" phenotype that characterises PSACH chondrocytes. The persistence of this phenotype leads to a form of chronic ER stress, reduced chondrocyte proliferation, and cell death by apoptosis. The retention of COMP in rER also results in ER co-accumulation of ECM proteins that are binding

partners of COMP, such as matrilin-3 and collagen IX[49]. The chronic nature of this 'intracellular matrix' means that it cannot be cleared by the unfolded protein response and an ER stress state is activated[48].

Recently, heterozygous missense *COMP* mutations in two families, resulting in coding point mutations (COMPV66E in the coiled-coil domain and COMPR718W in the C-terminal L-lectin domain, respectively), were associated to carpel tunnel syndrome type 2, a common condition that results from compression of the medial nerve in the wrist. *COMP* p.R718W was also associated with EDM1 phenotypes[50]. While the V66E mutation impairs oligomerisation and thus avidity of binding of COMP to ECM binding partners[50], R718W maps to an interface between the L-lectin domain and type 3 repeat 5 and may destabilise the C-terminal region structure[39–41]. Most recently, a heterozygous variant in *THBS2*, resulting in the coding point mutation TSP2C896R, has been identified as causing Ehlers-Danlos syndrome phenotypes in humans and mice, that involve loose joints, connective tissue hypermobility, and skin fragility. The most obvious molecular model for the action of this mutation is the loss of a disulphide bond between Cys876 and Cys896, which would perturb the structure of the L-lectin domain[51].

Single nucleotide polymorphism alleles of *THBS1, THBS2* and *THBS4* have been associated in some populations with predisposition to (*THBS1, THBS4*) or protection from (*THBS2*) familial premature myocardial infarction[52] and in susceptibilities to other forms of cardiovascular disease (see reviews[53,54]). Some studies have reported a lack of association in certain populations; as one example, the *THBS1* recessive pathogenic SNP allele of rs2228262 (a2210g) was not found to associate with risk of ischemic stroke or death from stroke in a study of patients and controls from Jiangsu, China[55]. Collectively, these findings implicate important roles of TSP family members in human cardiovascular physiology. Molecular and cellular analyses of the effects of the SNP alleles on TSP structure and functions revealed that *THBS1* rs2228262 (a2210g), which results in a coding variant within the first type 3 repeat, TSP1700S, impairs a high-affinity calcium-binding site[56,57], and increases platelet aggregation relative to the common variant TSP1N700[53]. In contrast, the *Thbs4* SNP rs1866389 polymorphism g1483c is dominant (both the gc and cc alleles increase risk) and result in the coding variant TSP4387P. This variant acts as a gain-of-function relative to the predominant form, TSP4387A, by generating a new calcium-binding site in the third EGF-like domain. TSP4387P also has stronger anti-proliferative effects on endothelial cells and is a stronger activator of neutrophils that TSP4387A[53,58]. The recessive and protective *THBS2* SNP (rs8089; t3949g) is present in the 3' untranslated region and may have effects on RNA stability[53]. Since the discovery of these TSP SNP associations with susceptibilities to cardiovascular disease, several SNPs in TSP genes have been associated with other human disease conditions (Table 1). Many of these studies are as yet based on a single population

group and remain to be replicated independently, however the association in several studies of *THBS1* SNPs with glaucoma, or *THBS2* SNPs with intervertebral disc pathologies, implicate prospective physiological roles for deeper investigation.

3.2 *Targeted genetic modifications in mice*

Elucidation of the multifunctional complexity and tissue-specific roles of TSPs in mammals has depended on investigations of genetically-modified mice. Single-gene knockouts for each of *Thbs1, Thbs2, Thbs3, Thbs4* and *Comp/Thbs5* were generated during the 2000s (Fig. 1D). All are viable and fertile with apparently few overt phenotypes[59–63]. However, closer examination of individual tissues and experimental pathophysiological challenges to the mice revealed a distinct profile of non-lethal phenotypes in each case and have yielded additional insights into biological role of TSPs in mammals, including in skin wound healing and post-natal cartilage and bone development (Table 2). Remarkably, mice with gene-knockout of two, three, four or even all five TSP family members also remain viable[64–66]. Development of other compound gene-knockout mouse strains have enabled the roles of TSP1 or TSP4 to be studied in mice with susceptibility to atherosclerosis (*Thbs1$^{-/-}$/ApoE$^{-/-}$ and Thbs4$^{-/-}$/ApoE$^{-/-}$),* or muscular dystrophy (*Thbs4$^{-/-}$/mdx*), or for combined effects with gene-knockout for other ECM components, and these have revealed functional effects beyond those of the respective single gene knockouts (Table 2). In addition, targeted gene knock-in mouse strains have enabled examination of the physiological effects of point mutations in specific tissues. Because *Comp$^{-/-}$* mice have no overt aberrant phenotype[61], whereas mice with inducible transgenic expression of COMPD469del in cartilage (expressed under a collagen II promoter) recapitulate many aspects of the cartilage phenotype of human PSACH[67], particular attention has been given to characterising the phenotypic effects of knock-in of *Comp* mutations. For example, *Comp*^{D469del} mice recapitulate PSACH characteristics of slower post-natal growth with hip dysplasia and short limbs, and also show progressive disorganisation of growth plates with chondrocyte apoptosis (Table 2).

3.3 *Studies in zebrafish*

Studies in *Danio rerio* (zebrafish) are, to date, much more limited than in mice and have addressed roles of the pentameric TSPs, starting from the cloning of zebrafish homologues of TSP3 and TSP4 from cDNA[68]. A complexity for study of TSP in this species (and teleost fish in general) arises from the existence of a larger number of TSP paralogues, due to the additional whole-genome duplication considered to have taken place at the origin of the teleost lineage (see more details in section 7.2). Our phylogenomic study of an early version of the *D. rerio* genome identified encoding of 8 distinct TSP-encoding gene; three of these were unmapped or partial sequences at this time[69]. A later study based on a more complete *D. rerio* reference genome has identified encoding of seven TSPs[70]. TSP4 protein, as in mammals, is located to muscles and

tendons, including extraocular muscles, as detected with an antibody to mammalian TSP4[71]. However, zebrafish encode two *Thbs4* paralogues, designated *Tsp4a* and *Tsp4b. Tspb4* is expressed in early embryonic somites and later, as muscle differentiation proceeds, becomes restricted to tendon cells at somite boundaries and muscle attachment sites. TSP4b protein (as detected with an antibody specific for TSP4b) was similarly detected at somite boundaries and, later in development, at myotendinous junctions[72]. Silencing by antisense morpholino oligonucleotides resulted in loss of TSP4b protein from myotendinous junctions, partial basement membrane separation, decreased localisation of laminin at somite boundaries, and reduced markers of integrin signaling, such as phosphorylated focal adhesion kinase. Stimulation of muscle contraction led to a muscle detachment phenotype, which was rescued by injection of recombinant human TSP4. These results indicate organisational and signalling roles of zebrafish TSP4b within the ECM for the formation and function of myotendinous junctions[72].

A single *Comp/Thbs5* gene is present is zebrafish and, as for the mammalian COMPs, the encoded protein lacks a TSP N-terminal domain[69,70]. In common with *Tsp4b*, *Comp* is expressed in somites, myosepta and at myotendinous junctions, but, unlike mammals, not in articular cartilage. In extracts from whole fish 5 days post fertilisation, *Danio* COMP was shown to be a pentamer and to coimmunoprecipitate with TSP4b, suggesting either inter-molecular association, or intra-molecular hetero-pentamerisation, with TSP4b. Manipulation of a zebrafish line by CRISPR-Cas9 gene editing to produce an in-frame deletion of I258 and D259 in the first type 3 repeat of COMP, (the equivalents sites are PSACH mutations in human COMP) did not reveal obvious phenotypes in either heterozygous or homozygous fish. However, by immunostaining, aberrant patchy and disorganised COMP was apparent in the myosepta of homozygous mutant fish. Electron microscopy further revealed disruption of myosepta ECM without enlargement of rER[70]. In view of the importance of normal COMP expression in human cartilage, as uncovered by the PSACH mutants, it would be interesting to know when, in evolutionary terms, COMP/TSP5 began to be expressed in cartilage. The lack of COMP in zebrafish cartilage also raises the question whether other pentameric TSPs, such as TSP4, might be expressed and might serve similar functions to COMP.

4. Thrombospondins – cell-adhesive or anti-adhesive proteins?

4.1 Cell attachment and spreading

At the time of the molecular cloning of TSP1, the ‘gold-standard” assay for investigating cell-attachment activity was to coat glass or plastic surfaces with a purified ECM protein, allow cells to attach for a short time in serum-free medium, and then wash away the non-adherent cells, fix the remaining cells, and examine them by phase contrast or fluorescence microscopy. Such assays had proved very successful in the ECM field: for example, in identifying specific proteolytic fragments of fibronectin responsible for its cell-adhesive activity. These studies, and others, had

paved the way for biochemical identification of integrins as major receptors for fibronectin (and several other ECM proteins), and the ArgGlyAsp (RGD) motif as the key determinant of integrin-binding activity within the cell-binding fragment of fibronectin[73]. When molecular cloning identified the TSP1 polypeptide to include features in common with other ECM glycoproteins, including a RGD motif[13], it was a natural progression to examine if TSP1 (purified from expired human platelets through heparin-affinity chromatography) has cell-attachment activity. Whereas cell attachment experiments with fibronectin and other ECM protein typically revealed time-dependent cell spreading, equivalent cell adhesion assays with TSP1 revealed rather different functional properties: human endothelial and smooth muscle cells, that attached and spread on fibronectin, attached yet remained rounded on TSP1-coated surfaces. These assays also demonstrated strong calcium-dependence and RGD-dependence of cell attachment to TSP1. The latter implicated integrins as TSP receptors and, indeed, proteins with integrin characteristics could be eluted by RGD peptides from cell extracts subjected to thrombospondin-affinity chromatography[74]. The integrin alphav beta3 was the first TSP1-binding integrin identified from platelets, endothelial, and smooth muscle cells[75].

However, this was the beginning of an increasingly complex molecular cell biology that, overall, has led to the identification of a multiplicity of cellular receptors, including various non-integrin receptors, for different TSP domains[76–79] (Fig. 2). Cell-attachment assays with additional mammalian cell types identified cell-type specific responses to TSP1 in terms of cell spreading and the role of the RGD motif. For example, when tested systematically against the same preparations of TSP1, G361 human melanoma cells and human epidermal keratinocytes remained rounded whereas human intestinal smooth muscle cells (HISM) and MG-63 human osteosarcoma cells underwent spreading. However, only attachment of HISM and MG-63 cells was strongly inhibited by RGD-containing peptides[80]. Additional mechanistic studies with several lines of human or mouse skeletal myoblasts, identified that, although these cells spread to the same extent as sibling cells plated on fibronectin, the TSP1-attached cells showed more prominent F-actin containing lamellipodia and ruffles and the adhesion was not susceptible to inhibition by RGD-containing peptide[81]. An explanation for the variable activity of the RGD motif of TSP1 (and TSP2) relates to the context of the motif within the calcium-binding and disulphide-bonded environment of the type 3 repeats. Cell attachment and spreading was increased on intact TSP1 or a recombinant C-terminal fragment when these proteins were prepared or incubated under reduced calcium concentrations (0.1mM versus 2mM), or pre-treated with dithiothreitol to reduce disulphide bonds. The sensitivity of cell attachment to inhibition by RGD-peptide was also increased, indicating that the extent of calcium loading of the type 3 repeats and the disulphide bond status alter the availability of the RGD motif for integrin binding[39,82]

Cell-attachment activities of TSP1 were also highlighted by the identification of roles in bridging between cells. These roles are prominent in the circulatory system – for example, in platelet-platelet interactions, macrophage uptake of apoptotic cells, or in binding of sickle red blood cells to vascular endothelium[83]. Subsequently, a range of roles in cell-cell interactions in immune responses have been identified, in which the transmembrane receptors CD36 and CD47 have key roles in relation to regulation of nitric oxide signaling (reviewed by[79]). Overall, the variety of cell responses to TSP1 led to discussion over whether it could be considered an 'adhesive', 'anti-adhesive', 'adhesion-modulating' or 'de-adhesive' protein[84,85]. Subsequently, TSPs, along with other ECM proteins including tenascins and SPARC/osteonectin, gained the designation "matricellular proteins", in recognition of their multiple pericellular roles, range of adhesive properties, and apparently limited role in structural ECM fibres[86].

Evidence for anti-adhesive or adhesion-modulating properties of TSPs also came from the cell biological experimental design of pre-plating cells onto glass or other ECM proteins and then examining effects of a test protein in solution on cell spreading or focal adhesion assembly. Focal adhesions are point contacts between cells and ECM in which cell-surface integrin clustering is associated with assembly of an intracellular complex of cytoskeletal and signalling proteins. Some of the most typical components of these adhesions are the actin-associated cytoskeletal proteins, vinculin and talin[87,88]. When tested in this assay format, TSP1 in solution did not affect the extent of cell spreading, but decreased focal adhesions/cell by about 50% overall, although individual responses of cells within a population were variable[89]. This activity was mapped to a motif within the N-terminal domain of TSP1, corresponding to amino acids 17-35. The corresponding peptide, designated HepI, was sufficient to reduce focal adhesions in cell plated on fibronectin (Fig. 3A, 3B). A highly-related motif is found in TSP2 and both motifs contained clusters of basic residues (which are recognised as heparin-binding motifs[90]), suggesting that the heparin-binding activity of the amino-terminal domain could be central to the disruption of focal adhesions. Indeed, soluble heparin blocked focal adhesion disassembly by HepI[91]. The molecular pathway was later shown to depend on the formation of a tri-molecular complex between TSP1, cell-surface calreticulin and low-density lipoprotein receptor-related protein (LRP), leading to activation of phosphoinositide 3-kinase, production of phosphatidylinositol 3,4,5-trisphosphate, and release of vinculin and alpha-actinin from focal adhesions, thereby reducing cell contractility[92–94]. It was proposed that focal adhesion disassembly could be a mechanism by which TSP1 modulates cell adhesion to increase cell motility, for example in healing wounds where there are high levels of extracellular TSP1 due to platelet degranulation, and indeed the calreticulin-dependent mechanism was found to promote cell motility *in vitro*[95].

4.2 *Actin cytoskeleton and cell-ECM contact structures*

Although cell spreading on ECM surfaces was understood to depend on integrin-mediated adhesion that led to assembly of focal adhesions, analyses by immunofluorescence microscopy showed that the cell spreading of skeletal myoblasts on TSP1 under serum-free conditions occurred without focal adhesion assembly. Instead, vinculin and other focal adhesion proteins remained diffuse within the cytoplasm (Fig. 3C-F). After staining for many other cytoskeletal or signalling proteins to attempt to detect if an 'alternative' form of focal adhesion was present, convincing evidence that this was indeed cell adhesion without focal adhesions emerged with the discovery that the lamellipodia formed at cell edges contained radial F-actin bundles and microspikes in which F-actin cocalised with the actin-bundling protein, fascin (Fig. 3E). Reciprocally, the same cells plated on fibronectin under matched conditions assembled numerous focal adhesions and fascin remained diffuse within the cytoplasm[96] (Fig. 3C, 3D). Studies with recombinant proteins based on TSP1 or TSP2 showed that, although several domains independently supported cell attachment, trimeric assembly of the C-terminal region was required for spreading and fascin microspike organisation by C2C12 myoblasts or rat vascular smooth muscle cells[97].

Interestingly, the partial cell-spreading of myoblasts on the matricellular protein, tenascin-C, was also associated with fascin microspike assembly[98] and cell adhesion to laminin-1 resulted in formation of both focal adhesions and fascin microspikes[99]. Whereas RGD-containing peptides were widely known to disrupt focal adhesions (e.g.[100]), this was not the case for the fascin microspike-based adhesions. Instead, glycosaminoglycan-dependent attachment was implicated, as treatment of cells with chondroitin sulphate A or *p*-nitrophenol β-D-xylopyranoside (a competitive inhibitor of glycosaminoglycan side chain addition to protein cores) reduced cell attachment and fascin-microspike formation. These treatments had no overt effect on focal adhesion formation by cells plated on fibronectin[81,99]. In migratory cells, fascin-microspikes concentrated at the leading edge, overlapping with regions of extracellular TSP1 deposition, indicating that TSP-rich areas of ECM may provide pro-migratory cues[99].

This new direction led to over a decade of research in the laboratory, to identify cellular mechanisms linking extracellular TSP1 to the intracellular organisation of F-actin and fascin. Because this article concentrates on the TSPs, and many reviews have been written on fascin and its normal and pathological functions (e.g.[101,102]), this is discussed briefly here. In several cell types, extracellular clustering of the transmembrane proteoglycan, syndecan-1, by antibodies to its extracellular domain was found to act as a specific cue for organisation of lamellipodia and fascin-containing microspikes. Correspondingly, ectopic expression of mouse syndecan-1 in COS7 cells, (which do not spread on TSP1), led to spreading and fascin microspike formation. Stimulation of microspike formation by syndecan-1 depended on the VC2 region of the

cytoplasmic domain[103], required activity of small GTPases and was inhibited by integrin-and-protein kinase C-dependent phosphorylation of fascin[104–106]. In addition to clustering of the extracellular domain, the interaction of syndecan-1 with TSP1 involved binding of its glycosaminoglycan side chains to heparin-binding motifs of TSP1[103]. Overall, these findings identified that a distinct profile of receptor-activated intracellular pathways, in which fascin-1 is a nexus, are activated by TSP1 adhesion. The results also pointed to intracellular cell signaling and cytoskeletal regulatory mechanisms whereby responses to TSP1 are coordinated with signals activated by more abundant ECM glycoproteins such as fibronectin[101,107].

5. Thrombospondins: part of the ECM and, if so, how?

5.1 *Thrombospondins in vivo*

The reductionist experimental designs described above are valuable for identifying prominent cellular effects or adhesion receptors engaged by TSP1, yet they clearly do not recapitulate the physiological presentation of TSP1 within the complex extracellular milieus of tissues. Since TSP1 was first identified as a protein released from platelet alpha-granules that participates in the fibrin clot (a form of transient ECM), a consequential question was whether it was also a component of the ECM of cultured cells or solid tissues. In cell culture, TSP1 was found to accumulate around proliferative cells (for example[108]) or in response to inflammatory stimuli[109]. Typically, TSP1 was not detected in fibrils or a meshwork but in the form of patchy areas of fine granular structures[109,110]. An electron microscopy study resolved these as 100 to 300 nm punctate deposits at cell surfaces[111].

In vivo, TSPs were initially found to be more abundantly expressed during embryonic development, with low or absent expression in healthy post-natal tissues[77,112]. Postnatally, TSP1 was detected in several basement membranes, arterial microfibrils and wound repair ECM (for example, [113,114]). TSP5 was located with collagens XII and XIV in the dermal–epidermal junction zone of human skin[115]. Mammalian TSPs are also prominent in connective tissues, with each family member linked to a distinct expression pattern. As examples, TSP2 is located in embryonic cartilage growth plate zone, growing long bones of post-natal mice, adult cartilage and dermis[116,117], TSP3 is developmentally expressed in mouse intestine, cartilage, central nervous system and the lung[118], in adult lung and developing long bones[62] (and reviewed[119]). TSP4 is abundant in tendons and certain muscles[120], in the developing and adult nervous system and is prominent at adult neuromuscular junctions[121]. COMP/TSP5 is most highly expressed in articular cartilage and is also present in tendon, synovium and skeletal muscle[122]. In adult human or mouse tissues, expression of TSPs is often increased in pathological contexts such as healing wounds, tumour stroma, or atherosclerotic lesions (reviewed by[77]). Where examined, TSP protein distribution often appeared amorphous and not clearly related to structural ECM fibrils. However,

COMP was reported to localise to the gap region of collagen I fibrils, for example, in equine tendon[123].

Roles for TSPs in ECM organisation were further implicated by the single gene-knockout mice, due to the perturbations of collagen fibril assembly observed in certain tissues (Table 2). For example, *Thbs2*$^{-/-}$ mice have loose skin, lax tendons and disorganised, enlarged collagen fibrils in the skin[60] and altered collagen fibrillogenesis in bone[117]. In *Thbs4*$^{-/-}$ mice, total ECM and certain heparan sulphate epitopes are decreased in the soleus muscle, and, in tendons, collagen fibrils are enlarged and irregular compared to wild-type controls[120]. *Thbs1*$^{-/-}$ mice have a tendency to enlarged and irregular collagen fibrils in the dermis and tendons[124], (Fig. 4A), with the abundance of collagen I and the major collagen cross-linking enzyme, lysyl oxidase, both reduced in the skin[125].

5.2 *Binding interactions of TSPs in vitro*

In vitro assays with purified or recombinant ECM proteins showed TSP1 and the other TSPs to bind to numerous ECM glycoproteins, collagens, proteoglycans (principally via heparan sulphate sidechains), proteases including matrix metalloproteases, cytokines, and growth factors[48,76]. In many cases, cell staining has documented overlapping localisations with individual binding partners in cell cultures or tissues, and binding requirements have been mapped to specific TSP domains or proteolytic fragments (Fig. 2)[76,79,126] . TSP4 was found to bind collagen I, II, III and V in a zinc-dependent manner, with the C-terminal region in monomeric form being sufficient for binding[127]. COMP/TSP5 bound collagen IX and fibrillar collagens via the C-terminal domain[128] and was also shown to promote collagen fibrillogenesis in vitro[129]. In similar assays, TSP1 bound to pepsin-extracted collagens I, II and III, yet bound much more strongly to native collagen I fibrils and also bound specifically to the C-propeptide domain of procollagen alpha1(I), suggestive of multiple binding sites on the collagen molecule[125]. Both TSP1 and TSP2 bind latent TGFbeta1 through a WSHWSPW motif in the second TSR, but TSP1 is unique in activating latent TGFbeta1 through binding of a RFK motif in the first TSR to a LSKL motif in the latency-associated peptide[130,131]. Active TGFbeta signaling leads to downstream alterations in gene expression profiles, including upregulated expression of ECM proteins in connective tissue or epithelial cells (reviewed[132]). Thus, the effects of TSP1 and TSP2 on TGFbeta localisation and/or activation provide a separate, indirect mechanism for trimeric TSPs to alter ECM organisation and composition. Another indirect mechanism, also mediated by the TSR, is the binding of matrix metalloproteinases (MMP), MMP-2 and MMP-9, which leads to MMP uptake by low-density lipoprotein receptor-related protein (LRP) and consequent degradation. In *Thbs2*-null mice, extracellular MMP-2 and/or MMP-9 are elevated in tissue protein extracts due to the loss of TSP2 and contribute to fibroblast adhesion defects and altered ECM remodelling or angiogenesis[133,134].

5.3 *Molecular determinants for deposition of TSPs in ECM*

Despite the many identified interactions of TSPs with ECM components, the cell-based processes that lead to ECM deposition of TSPs had not been clarified. My laboratory contributed in this area by adapting a standardised ECM isolation procedure to detect ECM deposition by immunoblotting or fluorescence microscopy (details in[135]). Endogenous secretion of TSP1 or TSP5 led to deposition of the TSP proteins in arrays of insoluble ECM puncta (Fig. 4B, shown for TSP5)[136,137]. Building on the knowledge of TSP domain structures, especially with regard to the C-terminal region, expression constructs were designed for different regions of the TSP1 molecule according to structural criteria and either in monomeric or trimeric forms. Progress of these proteins through the secretory pathway and extracellular accumulation could be tracked easily by a fused monomeric red fluorescent protein (mRFP) “tag”. The initial studies identified that the C-terminal region of TSP1 (comprising the first EGF domain to the C-terminus) in trimeric form was required and sufficient for ECM deposition. In cell culture, these proteins were deposited as arrays of small puncta. The ECM puncta property was also conserved in the pentameric TSPs tested[136,137]. Both the RGD site in the last type 3 repeat and a surface exposed double calcium ion-binding motif, DDD, in the C-terminal L-type lectin domain contributed to ECM deposition. Further analysis of the molecular basis for ECM deposition of TSP1 by generating an extensive set of point mutations within the adjacent β7/β8 loop in the L-lectin domain identified two other functionally significant charged residues, such that the combination point mutant, trimeric mRFPovTSP1C/AAA/D1031R/R1036E, was blocked for ECM deposition. These residues were found to be required for inter-molecular interactions between TSP molecules and the formation of ECM-deposited puncta in vitro[124]. These results suggested that ECM deposition of TSPs depends on reaching a critical local concentration sufficient for puncta assembly. This may depend on the balance between TSP secretion and the various mechanisms at the cell surface which mediate TSP endocytosis and lysosomal degradation. These depend on binding of TSPs to heparan sulphate and low-density lipoprotein receptor-related protein[138,139].

5.4 *TSPs and collagen fibril production and cross-linking*

The above results gave insights on the entry of TSPs into ECM but did not explain the collagen fibril perturbation phenotypes observed in tissues of TSP gene-knockout mice (see section 5.1 and Fig. 4A). To gain insight into possible TSP1 binding sites on fibrillar collagens, we collaborated with Richard Farndale to work with his collagen Toolkit libraries of synthetic peptides. These triple-helical peptides span the entire triple-helical domain of the homo-trimeric collagen II[140]. The solid-phase binding assays identified a core motif for TSP1 binding, KGHR, within collagen II[125]. This motif is present near the N- and C-ends of the collagen II triple-helix domain

and both sites are very highly conserved in other collagen polypeptides. The motif is significant in collagen biology for its role in cross-linking of mature collagen molecules by lysyl oxidase[141].

In vitro, TSP1 bound to pro-lysyl oxidase and inhibited its bone morphogenetic protein-1-mediated cleavage to mature, catalytically active lysyl oxidase. In short-term (48h-96h) cultures of human dermal fibroblasts, TSP1 colocalised intracellularly (in vesicles) with both pro-lysyl oxidase and fibrillar collagens (shown for collagen I in Fig. 4C), whereas in 10-day cultures TSP1 aligned with extracellular collagen fibrils (Fig. 4D) and this colocalization was inhibitable by KGHR peptide[125]. Thus, TSP1 acts at multiple points (both intracellular and extracellular) in the process of collagen I maturation and fibril assembly, and, overall, appears to have a homeostatic role in modulating rates of collagen fibril assembly (hence the larger collagen fibril diameters in skin of *Thbs1-/-* mice). A model is shown in Fig. 4E.

The mechanisms of action of TSP1 are unique amongst the other ECM proteins known to interact with fibrillar collagens, such as FACIT collagens or fibromodulin[140,142], but appear to be shared with other mammalian TSPs. Thus, *Thbs2*$^{-/-}$ mice also show reduced abundance of dermal fibrillar collagens and lysyl oxidase and reduced collagen cross-linking relative to wild-type dermis. This study reported an indirect, miR-29 dependent mechanism for these effects[143]. In diaphyseal cortical bone, the relative distribution of pro-lysyl oxidase and mature lysyl oxidase between newly synthesised and mature, cross-linked ECM is altered in *Tbhs1*$^{-/-}$ or *Thbs2*$^{-/-}$ mice versus the wild-type[144]. Intracellular COMP/TSP5 promotes secretion of collagens I and XII by dermal fibroblasts[145]. Using an *E. coli* expressed, foldon-based peptide library based on collagen II, both TSP4 and COMP/TSP5 were shown to bind specifically to the N-terminal GVKGHR site and COMP was also shown to bind to the C-terminal KGHR[146].

Additional insights on TSP/collagen interactions continue to emerge as novel pathogenic mutations are studied. For example, a *THBS1* missense allele resulting in a TSP1R1034C point mutation has been associated with congenital glaucoma and studied in a knockin mouse model and cell culture[147]. [Footnote: residue numbering in this study includes the signal peptide; this residue corresponds to R1016 in the Kim, Christofidou et al.[124] numbering scheme]. Heterozygous and homozygous *Thbs1*^{R1034C} mice have glaucoma pathologies (Table 2) and have strongly increased deposition of TSP1 as granular patches within the trabecular meshwork of the eye. In the C-terminal domain structure, R1034 hydrogen bonds with D861 and E864 in type 3 repeat 5[39]. The loss of these bonds is predicted to affect the stability and folding of the L-lectin-like domain; instead, the cysteine residue may undergo aberrant disulphide bonding leading to protein misfolding or aggregation. Indeed, tests of TSP1R1034C in cell culture showed that overall ECM deposition, the size of TSP1 patches in ECM and colocalization with ECM fibronectin

and collagen I were all increased relative to WT-TSP1[147]. Thus, a distinct face of the L-lectin domain might participate in collagen binding.

6. Intracellular as well as extracellular mediators

Although the majority of research has focused on extracellular roles of TSPs, during the last decade functional roles within the secretory pathway started to emerge. Clearly, as proteins translated with N-terminal secretory signal peptides, TSPs are trafficked into the ER lumen and through the ER and Golgi for onward vesicular transport and secretion. During this process, the polypeptides undergo extensive post-translational modifications, including chaperone-mediated folding[148,149] leading to disulphide bond formation and isomerisation through action of protein disulphide isomerases[150,151], also the addition of N- and O-linked glycans.

Prominent intracellular pools of TSP1 were reported in early studies of protein localisation (eg, in fibroblasts, endothelial and aortic smooth muscle cells[152]). These locations were first considered representative of the outward transport of TSPs; however, later studies have revealed a functional significance of the flux of TSPs through the secretory pathway that relates to cellular homeostasis and/or the control of ER stress signaling. For example, the excessive retention of mutant COMP in the chondrocytes of persons with PSACH (discussed in 3.1) leads to accumulation of ECM binding partners of COMP within an ER “intracellular matrix”[49], indicating a role of wild-type COMP in coordinating secretion of ECM proteins needed to build the cartilage ECM.

Evidence for intracellular roles of other TSPs in cellular homeostasis and ER stress responses has arisen from experiments involving transgenic over-expression of individual TSPs in mouse heart or skeletal muscle under induced injury conditions. The over-expression conditions mimic the up-regulation of production of TSPs seen in adult mammals after tissue injury. These experiments have revealed both protective roles (e.g., TSP4 and cardioprotection[153]) and pathogenic roles under stress conditions; e.g., TSP3 exacerbates cardiac hypertrophy under induced pressure overload[66]. Mechanistically, in response to myocardial injury, TSP4 in cardiomyocytes acts by binding activating transcription factor 6α (ATF6alpha) in the ER lumen, to activate a specific, ATF6-dependent, protective ER stress response[153]. Dependence of the cardioprotective response on passage of TSP4 through the secretory pathway was demonstrated by over-expression of a secretion-defective mutant of TSP4, which resulted in cardiomyopathy rather than cardioprotection[154]. In mouse models of muscular dystrophy, (*mdx* or *sgdc*$^{-/-}$ mice) TSP4 over-expression reduced muscular dystrophy phenotypes by promoting intracellular trafficking of key muscle adhesion receptors (integrins, dystroglycan) and apparent chaperone activity, thus stabilising the sarcolemma[155].

Other TSPs examined by transgenic over-expression act through different pathways. Over-expression of TSP3 in the heart exacerbated the effects of induced pressure overload or myocardial infarction. Like TSP4, intracellular TSP3 was found to bind integrin subunits, however, TSP3-association reduced their post-Golgi trafficking, leading to reduced sarcolemma stability[66]. Cardiac over-expression of TSP1 was also pathogenic and led to cardiac atrophy and lethality, through activation of a protein kinase R-like ER kinase (PERK) and ATF4 autophagy pathway, whereas over-expression of TSP2 did not affect viability[156]. In skeletal muscle, TSP1 over-expression resulted in muscle atrophy, through a distinct autophagy pathway dependent on TGFβ-Smad2/3 and ATF4, and also decreased the lifespan[157]. Much remains to be learnt about the molecular basis for the distinctive differences in intracellular activities between TSP family members, or whether these pathways could represent opportunities for therapeutic interventions.

7.Significance of Genomics: Heady days of the 2000s onwards

7.1 *Thrombospondins of Invertebrates*

The era of eukaryotic genome sequencing began with the sequencing of the genomes of important research model organisms *C. elegans*[158] and *D. melanogaster*[159] and moved rapidly to the publication of the first draft human genome sequence in 2001[160]. Given the existence of a TSP gene family in mammals and the molecular cloning of highly related TSPs in *Xenopus* and chicken[161,162], it was of great interest to seek TSP homologues in invertebrate species. Whereas the genome sequence of *C. elegans* revealed many collagens and basement membrane components, it (and other nematode species) lacked any TSP-encoding gene. However, the existence of TSPs in invertebrates was affirmed when a TSP with domain architecture similar to mammalian TSP3 and TSP4 was identified by PCR-based cloning methods in *D. melanogaster*[163]. Systematic BLAST searches of the *Drosophila* genome and genome-predicted proteome confirmed this as the single TSP encoded in the *Drosophila* genome, leading to its designation as D-TSP or DmTSP[32,112]. Biochemical studies of DmTSP (recombinant or produced natively by *Drosophila* Clone 8 wing imaginal disc cells) showed it to oligomerise as a pentamer, and to have functions shared with the mammalian TSPs: namely, a heparin-binding activity of the amino-terminal domain and activity to incorporate into *Drosophila* cell-derived ECM. In embryos, the *Dtsp* transcript was highly enriched at muscle attachment sites and, at lower level, within wing imaginal discs[32].

Subsequently, *Dtsp* was shown to be essential for muscle attachment to tendon cells and the formation of the myotendinous junction during embryonic development; its absence led to muscle detachment and embryonic lethality[164,165]. At the molecular level, DmTSP interacts with the PS2 integrin on muscle cells[164] and its recruitment to PS2 is facilitated by the integrin co-receptor proteoglycan, Kon-tiki (a homologue of vertebrate CSPG4/NG2)[166]. Genetic interactions of

Drosophila *LamininB2* with *Dtsp* and *Kon-tiki*, and the interaction of DmTSP protein with another secreted MTJ protein, slowdown, also indicate wider roles for DmTSP in organising ECM at the myotendinous junction[167,168]. Indeed, a *Drosophila* model of muscular dystrophy can be rescued by over-expression of either DmTSP or mouse TSP4, in correlation with increased muscle cell-surface integrins[155].

There is also evidence for a role of DmTSP in another type of cell junctional structure that is bridged by ECM: the cuticle attachments of sensory chordotonal organs within the exoskeleton. In the absence of DmTSP from this ECM, cell migration is impaired and stable junctions are not formed[169].

TSPs highly related to DmTSP were identified from sequencing projects in many other arthropods. Integrating these data with a vertebrate TSP dataset produced a phylogenetic tree that estimated the evolutionary debut of pentameric TSPs at least 60 million years before that of the trimeric TSPs[32]. However, in terms of metazoans *in toto*, this was a preliminary tree, because many animal phyla were unrepresented and there was limited representation of invertebrates versus vertebrates. Exponential growth of the NCBI DNA databases continued throughout the 2000s[170] and the continuing growth in sequenced metazoan genomes allowed us to keep referring back to this issue - in the process providing a seemingly never-ending source of student projects - and to continue to identify more TSPs in different phyla. New data flowed so fast that ideas, data charts and figures had to be continually modified, but this also offered scope to rapidly ask and answer questions *in silico*. By 2010, we demonstrated that TSPs were encoded in most metazoan phyla, although limited or absent data or partial sequences precluded firm conclusions on the early-diverging phyla of Porifera (sponges), Placozoa, or Ctenophora. From the available full-length TSP sequences, it was apparent that a B-type, (pentameric) form of TSP predominated in protostomes (annelids, arthropods and molluscs) as the single form of TSP. The A-type domain architecture appeared to originate in early-diverging deuterostomes (echinoderms and hemichordates) as a single gene product and was also encoded as a single gene in early-diverging chordates (the urochordate *Ciona intestinalis* and the cephalochordate *Branchiostoma floridae*)[171]. These findings supported a model for the origin of TSP1 and TSP2 from a gene duplication of "*TSP-A*" early in the vertebrate lineage.

In view that TSPs were highly conserved in metazoans, it was of great interest to also examine if TSP ligands for the major domains of the canonical TSPs and their cognate binding motifs on TSPs, where known, are also conserved. Of the ligands examined, the most highly-conserved interactions were with heparan sulphate glycosaminoglycans (ligands of both the N-terminal domain and lower affinity sites within the TSRs of TSP1 and TSP2), and fibrillar collagens, at the

time thought to be specific ligands of the C-terminal, L-lectin-like domain. The presence of RGD, integrin-binding motifs within the type 3 repeats proved a much more variable feature of the TSPs[171].

7.2. *Phylogenomics and the relationships of TSP gene family members*

Whilst this research was in progress, exciting developments in understanding of the structure of the human genome opened up a new route to assess the relationships between the five mammalian TSPs. This had become something of a vexed issue in the field for the pentameric TSPs. TSP3 and TSP4 clearly have highly related domain architectures and COMP/TSP5 was distinct due to the lack of an N-terminal globular domain (see Figure 1), yet the pairwise sequence identities of TSP3, TSP4 and TSP5 are all very similar in a given tetrapod species, as are the intron-exon gene structures. An initial phylogenetic tree placed TSP3 and TSP4 as more closely related to each other than to COMP/TSP5[172]; however, a tree based on the type 3 repeats and C-terminal domain (the most conserved regions in all TSPs) placed TSP4 and TSP5 as the closest sequences with TSP3 on a distinct branch[32].

The appreciation that the genomes of early chordates enlarged through large-scale, *en bloc* genomic duplications (two rounds of genome duplication associated with the common vertebrate ancestor[173,174] and a further round of genome duplication unique to the teleost fish lineage[175], with partial conservation of the order of genes along a chromosome throughout subsequent genome evolution), provided a new framework to assess the evolutionary relationships between members of a vertebrate gene family. The criterion for conservation of synteny is that orthologous gene loci are found to be linked in different species, albeit with alterations to the exact gene order or the existence of non-conserved intervening genes[176,177]. By examining conservation of gene neighbours on the chromosomes, rather than the individual TSP protein sequences, we reported that the *Tbhs3*, *Thbs4* and *Comp/Thbs5* genes each had gene neighbours conserved between the human, mouse, chicken and fish genomes[69]. With the great benefit of the breakthrough analysis that identified largescale paralogous chromosomal regions within the human genome[176,177], we were able to establish that *THBS4* and *COMP/THBS5* genes are located in related paralogous regions. The location of *THBS3* also has paralogy with that of *THBS4*. These findings led to a new evolutionary model in which *Thbs3* and *Thbs4* of vertebrates originated and diverged from the early first round of genome duplication whereas *Thbs4* and *Comp* diverged more recently from the secondary round of genome duplication. Interestingly, the *THBS1* locus also showed weak paralogy, indicative of an earlier duplication event (potentially within the early chordate lineage and in line with the molecular evidence on the emergence of TSP1 and TSP2), whereas *THBS2* was located at a non-paralogous locus, suggesting that *THBS2* underwent replicative transposition after the gene duplication event that gave rise to *THBS1* and *THBS2*[69].

The skills acquired during this study were rapidly transposed to a study of the syndecan gene family, where paralogy and conservation of synteny again proved critical for establishing relationships of the four syndecan (*Sdc*) genes of tetrapods (land animals) and specific loss of the syndecan-1 gene in bony fish[178]. Propelled by prior collaborations and conversations at conferences, fruitful collaborations analysed relationships within the tenascin gene family[179] and the MACIT gene family[180].

7.3 *Identification of the Thrombospondin super-family*

From these studies, and database searches that continued over time, we began to appreciate that invertebrates encode proteins identifiable as TSP-like by the conserved domain architecture of the C-terminal region, yet with distinct domain compositions in the N-terminal region. TSPs with repeated chitin-binding domains at their N-terminal ends had been independently identified in shrimps and prawns[181–183]. Our *in silico* "digging" also identified apparent TSPs encoded in basal deuterostomes and basal chordates that included a discoidin-like domain[171]. These distinctive forms of TSP lacked a heptad-repeat domain and so would appear to be secreted as monomers. However, the chitin-binding domain and discoidin domain are protein-protein interaction domains, raising the question of whether TSP "molecular aggregation" could occur by non-covalent interactions. Indeed, a group studying prawn TSP in the economically significant species, *P. monodon*, has proposed a conceptual model in which PmTSP may form self-associating, extracellular networks through protein-carbohydrate interactions of the chitin-binding domains with O-β-GlcNAc saccharide substitutions on the EGF domains[184].

Efforts to examine the TSPs of the cnidarian *Nematostella vectensis* provided evidence for the existence of four TSPs in this species[185], all of which are transcribed in adult polyps[186]. NvTSP168100, the first of these to be studied in detail by my collaborator Richard Tucker, has a typical B-type TSP domain architecture with the exception of an unusual, approximately 100 amino acid-long region rich in proline, threonine and arginine, that precedes a short heptad-repeat region. According to molecular phylogenetic analysis, NvTSP168100 and the closely related Nv30790 are most related to B-type TSPs, yet appear distinct as an evolutionary clade, and may represent the products of a *N. vectensis*-specific gene duplication. Immunohistochemistry for NvTSP168100 identified protein localisation in neuron-like cells within the mesoglea (the ECM layer of cnidarians) of the retractor muscles and pharynx of unchallenged adult polyps, and upregulation of the protein in the epidermal glycocalyx during regeneration[186].

A breakthrough on the TSP-like sequences that contain discoidin-like domains came with the molecular cloning of full-length cDNA sequences from two cnidarians, *Hydra magnipapillata* and

Nematostella vectensis. Striking, these proteins are of very large size, over 2700 amino acids long, and, apart from the conserved C-terminal region, have a different domain organisation from the canonical TSPs (Fig. 5A). In view of the large size, we named these proteins 'mega-TSPs'. In the course of identifying these proteins, we also identified the existence of additional types of TSP-related proteins: the 'sushi-TSPs" (so-named for the presence of sushi domains near the N-terminus), that were identified in certain classes of poriferans and cnidarians, also the 'poriferan-TSPs' (identified only in poriferans)[187] (Fig. 5A). All the newly identified TSP super-family members lack coiled-coil domains and so are predicted to be secreted as monomers. All, however, contain known protein-protein interaction domains (leucine-rich repeat domains in the mega-TSPs; sushi domains in the sushi-TSPs) or, in the case of poriferan-TSPs, potential for protein-carbohydrate interactions in the form of the TSRs, which may provide capacity for extracellular self-assembly into networks. Particular features of the C-terminal regions of the newly identified super-family members include variable numbers of EGF domains (a single domain in sushi-TSPs and four or more in mega-TSPs), and the presence of two tandem L-Lectin/Con A-like domains at the C-terminus of mega-TSPs and sushi-TSPs (Fig. 5A). These could be modelled based on the known structure of the TSP1 L-lectin/Con A domain with good correspondence and with the two domains placed contiguously with known functional motifs well-exposed Thus, the intensively studied canonical TSPs form just one branch of a much larger super-family of proteins that appears to have been present from the origin of animals. Phylogenetic analyses showed that the TSP C-terminal regions of sushi-TSPs are most closely related to those of the mega-TSPs[187] (Fig. 5B). Mega-TSPs are conserved in many animal phyla, from ctenophores to basal chordates, but are not present in arthropods, nematodes or the craniates (the latter comprise hagfish, lamprey and all jawed vertebrates). Strikingly, only mega-TSPs were identified in ctenophores and placazoans, whereas poriferans encoded mega-TSPs, sushi-TSPs and poriferan TSPs[187,188] (Fig. 5C).

8. Expression and Functions of TSP super-family members in cnidarians

The appreciation of a TSP super-family set up a host of new questions on the expression patterns of the newly identified super-family members, their functions, and how these might overlap or be distinct from functions of canonical TSPs. These questions have been addressed first with regard to mega-TSPs, as the newly-identified family member with the widest phylogenetic distribution. The focus for these studies has been cnidarians, because mega-TSPs are present in this phylum and it is the earliest-diverging phylum in which canonical TSPs have also been identified. With regard to laboratory experiments, Phylum Cnidaria includes some very well-studied species[189,190]. For ECM biologists, additional interesting aspects of cnidarians are 1) that these are diploblastic animals in which the majority of the ECM is found as a defined mesoglea layer between the outer

ectoderm and inner endoderm, and 2), with regard to studies of ECM proteins conserved between invertebrates and vertebrates, cnidarians, unlike *Drosophila*[159], encode several fibrillar collagens.

8.1 *Hydra Thrombospondin*

In *Hydra*, the mesoglea is acellular and can be separated from the ectodermal and endodermal cell layers by a detergent extraction and freeze-thaw procedure. An unbiased proteomic study of the composition of the isolated *Hydra* mesoglea identified various collagens, laminin and Hydra thrombospondin (HmTSP) as the most abundant components[191]. In molecular cell biology experiments, HmTSP was found to oligomerise as a pentamer. Despite the abundance of the protein in mesoglea, *HmTSP* transcript is tightly localised in adult polyps to ectodermal cells around the hypostome (oral cavity), with weaker expression in endodermal cells at the base of the tentacles. An additional site of high expression is in the ectoderm at the tips of growing buds (these structures form on and ultimately detach from the body column of adult polyps in the process of cnidarian asexual reproduction) (Fig. 6A). These body sites are known zones of Wnt signaling activity, and Wnt signaling has a central role in the setup of the body plan of *Hydra* polyps (reviewed by[190]). Perturbation of Wnt signaling by either upregulating or decreasing beta-catenin abundance by appropriate pharmacological inhibitors, along with chromatin immunoprecipitation experiments to investigate beta-catenin occupancy of T-cell factor (TCF)-binding motifs within the *HmTSP* promoter region, identified that *HmTSP* is transcriptionally regulated by beta-catenin signaling activity. Although siRNA knockdown of *HmTSP* (by electroporation of polyps) did not alter the body pattern or number of tenacles in animals at steady state, *HmTSP* knockdown in conjunction with Alsterpaullone treatment to increase beta-catenin signaling led to a massive increase in tentacle formation[191] (Fig. 6C-E). These results identified a native role of HmTSP in a negative feedback regulation of the Wnt source region, i.e., in ensuring tight regulation of Wnt specification of the ‘head’ region along the length of the body axis. This was the first study to link the function of a thrombospondin to Wnt signaling. Subsequently, an investigation of the role of TSP1 in *Trypanosoma cruzi* infection of heart endothelial cells in mice found TSP1 to have a role in dampening down the Wnt5a / beta-catenin signaling (with regard to timecourse and abundance of beta-catenin) that is linked to early stages of parasite infection and which contributes to successful infectivity[192].

8.2 *Hydra and Nematostella Mega-Thrombospondin*

Further to these studies, expression of *mega-TSP* was examined by in situ hybridisation in the cnidarians *Hydra magnipapillata* and the sea anemone, *Nematostella vectensis*. In *N. vectensis*, *NvMT* was found to be expressed in planular larvae and in the endoderm of primary polyps, specifically in the endoderm of mesenteries and within tentacles. In adult polyps, expression was widespread in the body wall and mesenteries and predominantly associated with the ectodermal

layer. In adult *H. magnipapillata* polyps, *HmMT* is expressed within the ectoderm of the body wall, being highest in the apical and budding regions of the body column, lower in the distal foot region and weak/absent within the tentacles[187] (Fig. 6B). In Hydra, as in other cnidarians, regeneration capacity can be assessed by transecting the adult polyp in the central region of the body and monitoring the regrowth of foot or hypostome regions, respectively, by the two halves[193]. By comparing regeneration activity of control or siRNA-*HmMT* knockdown Hydra, we identified that head regeneration was blocked after *HmMT*-silencing (Fig. 6F-H), whereas foot regeneration was unaffected. The mechanism for this functional effect remains to be established, but could relate directly to disruption to the mesoglea or to indirect effects of altered mesoglea properties on cell migration into the re-forming hypostome and tentacles[187].

9. Translational Prospects

Although the invertebrate-specific family members of the TSP super-family are present in a vast range of species, to date research into these family members remains limited. The most active research areas relate to species that are deemed economically significant and farmed for human consumption: predominantly various prawn and shrimp species, where the lineage-specific chitin-binding TSPs are under investigation for possible roles in innate immunity against viral infections, or roles in the oocyte extracellular matrix that support fertilisation (reviewed in[194]; [184]). Overall, the main directions of translational research in the TSP field focus on the intensively studied mammalian TSPs. The possibility of targeting inter-molecular connections of TSPs to reduce or inhibit fibrosis has been of long-standing interest, due initially to the role of TSP1 as a major activator of latent TGFbeta1 in some tissues. Here, the focus has been on peptide LSKL and derivatives, that compete for TSP1 binding to the latency-associated peptide and have shown efficacy in mouse models: for example, in reducing renal injury and active TGFbeta in a model of diabetic nephropathy[195]. Efforts to lengthen the half-life of the peptides in the circulation and develop a peptide targeted to inhibit an extracellular interaction (ie, with reduced cellular uptake) have led to identification of a tripeptide with promising properties[196]. Since excessive deposition of collagen fibrils is a defining hallmark of fibrosis, and the TSRs of TSP1 and TSP2 bind and promote clearance/reduce activation of MMP-2 and MMP-9, strategies to manipulate these interactions of TSP1 or TSP2 could also offer entry points for new targeted therapies. Inhibition of the lysyl oxidase collagen cross-linking enzymes is already under investigation as a potential anti-fibrotic treatment (eg[197]). The recent identification of the TSP-binding KGHR motif that is also involved in collagen cross-linking[125,146], offers a potential new direction in this area. However, the current major directions for translational TSP research relate to the roles of the TSR domain in inhibiting angiogenesis, or to ameliorating the pathological effects of COMP mutations in PSACH and EDM1; these are discussed below.

9.1 *The TSR domains and inhibition of angiogenesis*

The importance of tumour vascularisation in driving aggressive tumour progression is well-recognised (reviewed[198]). The identification of anti-angiogenic activity by the TSRS of TSP1 and TSP2[199,200] has resulted in extensive investigations of the TSRs and TSR-derived peptides as potential angiogenesis-inhibitory therapeutics[78,201]. Mapping of anti-angiogenic activity within the TSRs identified related active, 19-mer peptides, SPWSSCSVTCGDGVITRIR and SPWDICSVTCGGGVKQRSK, from the second and third TSR domains, respectively[200]. Overlapping peptides KRFKQDGGWSHWSPWSSC and SPWSSCSVTCGDGVITRIR were identified as angiogenesis inhibitors in the chicken chorioallantoic membrane assay of angiogenesis[202]. Other studies on TSP1 had previously identified the CSVTCG motif (which is also conserved in TSP2) as critical for binding CD36, a cell-surface receptor prominent on microvascular endothelial cells[203]. Indeed, CD36 was later identified to mediate inhibition of endothelial cell proliferation and migration[204], to activate pro-apoptotic signalling in endothelial cells[205,206], and to be required for angiogenesis inhibition by TSP1 in vivo[205].

Further research, to improve the stability and activity profile of the anti-angiogenic short peptides, led to the identification of a D-isoleucyl enantiomer based on the GVITRIR motif. This peptide had greatly improved specific activity to inhibit endothelial cell migration and proliferation in vivo and block tumour growth in mouse models[207,208]. Further optimisations resulted in development of the peptide mimetic ABT-510[209]. Although showing promising anti-tumour activity in pre-clinical models and reaching phase II clinical trials, ABT510 did not show clear clinical efficacy against several forms of human cancer as a single agent[210–212]. Nevertheless, possible utility of ABT-510, or its later derivative ABT-898, as an adjuvant that increases the efficacy of chemotherapy has been indicated in mouse cancer models[213]. ABT-898 has also shown promise in models of neovascular age-related macular degeneration, where choroidal neovascularisation is a key driver of vision loss, yet its utility is limited by low binding affinity to CD36 and rapid turnover in vivo (0.77h half-life in mice after intravitreal injection[214]). Development of ABT-898 ‘protein-like polymers’ in which peptides are polymerised synthetically at high density to form globular structures appears a promising new direction. These proteomimetic polymers have increased affinity for CD36, are non-toxic in cell culture or the retinas of mice, and are much more stable in vivo (circulation half-life of 13.1h). The polymers are also more effective than ABT-898 in blocking angiogenesis in a laser-induced model of choroidal neovascularization in mice[214].

The anti-angiogenic activity of TSP1 or TSP2 via the TSRs is now appreciated to involve multiple additional physiological pathways, that include inhibition of vascular endothelial growth factor (VEGF) signaling by several mechanisms (reviewed by[201]), inhibition of nitric oxide signaling in endothelial cells[215], or blockade of beta1 integrin-dependent endothelial cell migration[216]. Agents

including or mimicking the intact TSR domains, such as recombinant proteins that include all three TSR of TSP1 or TSP2 (designated “3TSR”) are therefore under investigation, with the goal of engaging multiple interactions within the native structural context. These proteins also have advantages of increased binding affinity/avidity and better stability in vivo over the short peptides. 3TSR protein inhibited tumour growth in mouse models by several mechanisms[217]. The half-life in circulation of this protein is around 14h. A dimerising version of 3TSR was developed as a fusion protein in which the Fc domain of human IgG1 was fused in-frame N-terminal to 3TSR. Protein “Fc3TSR” is more stable (half-life in mice of ~ 5 days) and more potent than 3TSR in blocking tumour growth in a mouse model of ovarian cancer[218]. A further improvement has been to develop adeno-associated viral expression vectors for 3TSR and Fc3TSR. In a mouse model of ovarian cancer, a single injection of AAV-3TSR or AAV-Fc3TSR led to sustained expression of the respective proteins, and Fc3TSR extended survival relative to control, tumour-bearing mice from a median of 96 days to a median of 121.5 days[219].

A separate tissue mechanism that alters the angiogenic ‘balance” between angiogenesis stimulators and inhibitors in favour of pathological angiogenesis is a reduction in the abundance of TSP1 and TSP2 within the tumour microenvironment, for example as detected in a subset of human breast cancers[220,221]. A distinct translational approach has therefore been to explore how TSP1 production might be stimulated within the TME, with the goal of restricting tumour angiogenesis through native TSP1-dependent mechanisms. A tumour cell-secreted inhibitor of metastasis, prosaposin, was identified in vitro to act via p53 to increase TSP1 production by fibroblasts and to promote TSP1-dependent inhibition of experimental metastasis in mice[222]. These findings led to the development of a small cyclic peptide, VT1021, that mimics a functional motif of prosaposin, resulting in induction of TSP1 and anti-metastatic activity in mouse cancer models[223]. In humans, VT1021 has proved safe and tolerable in a phase I clinical trial, with all the treated patients showing increased TSP1 in plasma and on the surface of peripheral blood mononuclear cells. VT1021 is currently under evaluation in the GBM AGILE phase II/III clinical trial in glioblastoma patients[224] (ClinicalTrials.gov Identifier: NCT03970447).

9.2 *COMP/TSP5, PSACH and Endoplasmic Reticulum Stress*

The other major direction of TSP translational research relates to the roles of COMP/TSP5 in PSACH and EDM1. Whereas the feasibility of gene editing to correct the causal mutations appeared an initial prospect, the large number of small deletions or point mutations in COMP that have been identified to be disease-causing *(*over 300 at present) presents a major challenge for the implementation of such an approach[44,45]. Instead, the discovery that endoplasmic reticulum stress acts as a major central mechanism for the deleterious phenotypic changes of cartilage and joints in PSACH has opened more accessible new directions for potential therapies. As discussed

in section 3.1, in PSACH, the mutant forms of COMP are misfolded and retained within the endoplasmic reticulum (ER), which results in co-accumulation of ECM binding partners of COMP in the ER, leading to the ER swelling that characterises chondrocytes of PSACH individuals[49]. The chronic accumulation of misfolded COMP leads to ER stress responses, inflammation, and apoptotic death of chondrocytes with consequent abnormalities of the growth plate and impaired growth of long bones[225]. These phenotypes of human PSACH are recapitulated by expression of a disease-causing COMP mutant in mice, but not in COMP-knockout mice, showing that it is the properties of mutant COMP, rather than the loss of normal COMP functions, that are key drivers of PSACH pathologies[61,67,226,227]. Proof that induction of ER stress is a major cause of disease pathology came from experiments in which transgenic over-expression of a 'model', non-ECM protein (thyroglobulin) in chondrocytes of mice was shown to be sufficient to recapitulate effects of mutant COMP on chondrocyte proliferation and long bone growth[148].

Thus, more recent research on PSACH has focused on the targeting of ER stress and on related pathways of oxidative stress or inflammation. For example, treatment of mice expressing mutant COMP with the anti-inflammatory agent resveratrol led to improved chondrocyte viability and growth of the long bones[228]. It was necessary for the treatment to begin within 4 weeks of birth to effectively prevent joint degeneration[229]. The plant-derived polyphenol, curcumin, in particular in the highly absorbable form, CurQ+, is another anti-inflammatory agent that shows promise for increasing chondrocyte viability and long bone growth in mouse models[230]. While these directions appear highly promising for development of safe treatments with potential to mitigate major, chronic pathologies of PSACH, the status of resveratrol and curcumin as non-regulated dietary supplements poses certain obstacles to progression into clinical trials (reviewed[231]).

10. Perspective

This field has developed from the study of a single molecule, thrombospondin-1, to multi-faceted and multi-disciplinary research that embraces molecular, cellular and tissue biology processes from simple animals such as cnidarians to organ-specific human diseases, and from basic 'blue skies' research to tissue engineering and translation. This article emphasises fundamental attributes of TSPs and their evolution, yet much current research takes place within discipline areas, for example vascular biology, cartilage biology, or immunology. Inter-disciplinary approaches will continue to be valuable for coherent views on the biological functions, similarities and differences of the TSPs and the wider TSP super-family. Several promising translational directions are underway and it can be hoped that TSP-based agents may reach therapeutic use in future years.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1. Evolving views of the thrombospondins.
A, Model of TSP1 molecule from biochemical and biophysical studies (reproduced with permission from[12] under Elsevier License 5813221395195).
B, Domain architectures of mammalian TSPs from molecular cloning. Reproduced with re-labelling from[187] under Creative Commons CC-BY. Key: Key: SP, signal peptide; N (LNG), N-terminal laminin G-like domain; cc, coiled coil oligomerisation regions (short vertical lines indicate positions of the conserved paired cysteines that support oligomerisation); vWF_C, von Willebrand

Factor type C domain; TSR, thrombospondin type 1 domains; EGF, EGF-like domains (bar across domain indicates predicted calcium-binding EGF domain); L-lectin, L-type lectin-like domain.
C, Characteristics of the oligomerisation domain in subgroup A and B TSPs.
Sequence Logos were prepared from MUSCLE[232] multiple sequence alignments in WebLogo 3[233] and are based on the oligomerisation domains of TSPs from human, chicken and amphibian (*Xenopus tropicalis*). Colours indicate amino acid chemical group (polar, green; neutral, purple; basic, blue; acidic, red; hydrophobic, black) and the height of a letter indicates the conservation at that position. Cysteine residues that form disulphide bonds between TSP polypeptides (asterisks) are conserved before the heptad-repeats in subgroup A and after the repeats in subgroup B. Hydrophobic and neutral residues are well-conserved within the heptad repeat pattern.
D, A research timeline for the TSPs, from identification of TSP (now known as TSP1) to the TSP super-family.

Figure 2. Structures and functions of major domains of TSP1 and TSP2.
Structures of the N-terminal domain of TSP1[34], second and third TSR of TSP1[234], and the C-terminal region of TSP2[40] are shown with the associated PDB ID numbers and in relation to a domain diagram of the TSP1 polypeptide. The structures are viewed in iCn3D[235] and beta-sheets are shown in green, helices in red, loops in blue, disulphide bonds in yellow and bound calcium ions as grey balls. Glycan post-translational modifications identified in the structures are also shown. Major functions and binding partners of these regions of TSP1 are listed below.

Figure 3. Cell adhesion and de-adhesion activities of TSP1.
A, B, Bovine aortic endothelial cells plated on fibronectin for 24 hr and then treated for 2h with either BSA or 0.4μM Hep1 peptide. Focal adhesion remained abundant under control conditions (A) but are mostly lost after treatment with HepI peptide (B). Reproduced from[91] under Creative Commons CC-BY.
C-F, Cytoskeletal organisation in H9c2 rat myoblasts plated on fibronectin- (C, D) or TSP1- (E, F) coated surfaces. Cells were plated for 2h under serum-free conditions, fixed and stained for fascin (C, E), or vinculin (D, F). In cells on fibronectin, fascin is unorganised (C) and focal adhesions are assembled (D), whereas spreading on TSP1 results in lamellipodia with organised fascin/F-actin bundles (E) and a lack of focal adhesions (F).
Scale = 10 microns. Panel E re-used from Journal of Cell Science (1995)[96] with permission of Company of Biologists, Licence 1498388-1.

Figure 4. TSPs and ECM.
A, Cross-sectional TEM views of collagen fibrils from mouse skin and tendon, showing perturbation of fibril organisation in *Thbs1*$^{-/-}$ mice compared to age and sex-matched wild-type C57Bl/6 mice. Reproduced from[124] under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).
B, Cell-derived isolated ECM produced by rat chondrosarcoma cells, immuno-stained for TSP5 to show its deposition as arrays of small puncta in ECM. Reproduced from[137] under Creative Commons Attribution License 3.0 (https://creativecommons.org/licenses/by/3.0/)
C, D, Intracellular and extracellular colocalisations of TSP1 and collagen I in human dermal fibroblasts. Merged view of cells co-stained for TSP1 (in green), collagen I (in red) and DAPI (blue) after 72h of culture, when co-localisation is mostly in intracellular vesicles, (C), or after 10 days, when co-localisation is predominantly on extracellular collagen fibrils (D).
Reproduced with permission from[125] under License Number 5815560139809 from The American Association for The Advancement of Science.
E, Model for mechanisms of TSP1 modulation of collagen fibril assembly, through intracellular interactions with proLox and procollagen, and extracellular, KGHR-dependent interactions with collagen, that collectively contribute to homeostatic regulation of fibril cross-linking and assembly.

Figure 5. The thrombospondin superfamily.
A, Examples of domain architectures of canonical TSPs and other superfamily members from the indicated species. The poriferan-, sushi- and mega-TSPs are predicted to be secreted as

monomers. Key: SP, signal peptide; LNG, laminin G domain; o, the oligomerisation domain; vWF_C, von Willebrand Factor type C domain; TSR, thrombospondin type 1 domain; EGF, EGF-like domain (bar across domain indicates predicted calcium-binding EGF domain); Con A, Concanavalin A-type domain; SCR, short consensus repeat; LRR, Leucine-rich repeat; PA14, PA14 domain; CAD, cadherin domain.
B, Schematic evolutionary tree of the relationships of the superfamily clades, based on phylogenetic trees prepared from the universally conserved C-terminal region[187] (region used is shown by dotted line in A).
C, Representation of the different TSP superfamily members across major metazoan phyla. Diagrams in A and C reproduced with relabelling from[187] under Creative Commons CC-BY.

Figure 6. Functional studies of TSP superfamily members in the cnidarian, *Hydra magnipapillata.*
A, B: In situ hybridisation on adult Hydra polyps for *TSP* transcript (A), *Mega-TSP* transcript (B). In A, h = hypostome, t= tentacle, f= foot region. Arrow indicates polyp budding by asexual reproduction.
C-E, role of Hydra TSP in restriction of the head region, as identified by siRNA silencing in transgenic Hydra expressing ectodermal GFP. siGFP was included in all experiments as a control for efficiency of silencing. C, no overt effect of siHMTSP in control adult polyps. D, treatment of control polyps with Alsterpaullone, a Wnt pathway activator, disrupts the longitudinal body axis, with tentacle formation along the body. E, Alsterpaullone treatment of polyps pre-treated with siHmTSP leads to an elevated 'hyper-tentacle' phenotype.
Brightfield images. Scale = 0.5 mm. See[191] for details.
F-H, role of Hydra mega-TSP in head region regeneration. F, control polyp treated with siGFP immediately after transection of the body column below the head region. G, polyps treated with siGFP regenerate the hypostome region and tentacles within 96h; G' inset shows the same polyp at time 0. H, polyps treated with siGFP and siHmMegaTSP (siMT1&2) fail to regenerate the head region by 96h; H' inset shows the same polyp at time 0. Brightfield images. Scale in F- H = 0.5 mm. Scale in G' and H' = 0.25 mm. See[187] for details. A and C-E reused with relabelling from[191] under Creative Commons Attribution 4.0 International Licence (http://creativecommons.org/licenses/by/4.0/). B, F-H, reused with relabelling from[187] under Creative Commons CC-BY.

TABLES

Table 1. Thrombospondin single nucleotide polymorphisms (SNPs) identified from population studies and missense coding alleles identified in single families that have been associated with disease states unrelated to cardiovascular disease. For discussions of TSP SNPs related to cardiovascular conditions see [53,54].

TSP gene	SNP or mutation	Disease correlation	Reference
Thbs1	rs1478604	Increased risk of pterygium	236
	rs1478604 rs7170682 rs2664141 rs12912082 rs3743125	Risk factors for *P. falciparum* malaria	237
	s2228261 rs2292305	Susceptibility to schizophrenia	238
	rs2292305	Autism risk	239
	rs2725797	Protective effect against loss of ambulation in Duchenne muscular dystrophy	240
	rs1478604	Corneal allograft rejection	241
	rs1478604 rs2228262 rs2292305	Susceptibility to chronic keratoconjunctivitis	242
	rs1478604 rs2228262	Development of autoimmune dry eye syndrome	243
	rs2228262	Over-represented in patients responsive to anti-VEGF therapy for age-related macular degeneration	244
	rs2228262	Association with hereditary primary open-angle glaucoma and increased intraocular pressure	245
	Missense alleles *THBS1*$^{R1034C/+}$ *THBS1*$^{R1034S/+}$	Correlation with congenital glaucoma	147
Thbs2	rs9406328	Lumbar disc herniation risk	246
	rs6422747 rs6422748	Susceptibility to, but not severity of, intervertebral disc degeneration	247
	rs9406328	Intervertebral disc degeneration	248
	Missense allele *THBS2*$^{C896R/+}$	Novel Ehlers-Danlos syndrome with vascular features	51

Table 2. Phenotypes of mouse gene knock-out or knock-in models for thrombospondin gene family members.

Mouse Model	Recognised Phenotypes	References
Single gene-knockout		
Thbs1$^{-/-}$	Spinal lordosis; increased circulating monocytes; post-natal pneumonia	59
	Reduced TGF-beta activation (lung, pancreas)	249
	Increased vascular density (retina)	250
	Delayed skin wound healing	64
	Increased inflammation and granulation tissue (healing myocardial infarcts)	251
	Reduced platelet attachment, thrombus formation in endothelial injury	252
	Reduced smooth muscle cell activation, neointima formation on carotid artery ligation	253 254
	Reduced glomerular active TGFbeta (experimental diabetic nephropathy)	255 256
	Increased tissue survival after ischemia	257
	Decreased age-related susceptibility to ischemic injury	258
	Increased soft tissue resistance to radiation	259
	Increased angiogenesis (experimental inflammatory bowel disease)	124
	Increased cardiac and skeletal muscle capillarity and exercise capacity	144
	Irregular collagen fibrils, enlarged diameters (skin, tendon)	260
	Reduced cross-linked collagen in female bone; altered LOX distribution in bone of both sexes	261 262
	Increased vascular density (iris)	
	Reduced intra-ocular pressure	263
	Reduced muscle and adipose tissue fibrosis and insulin resistance response to high-fat diet (male mice)	157 60
	Reduced adiposity and inflammation response to high-fat diet	264
	Reduced hindlimb muscle atrophy after denervation or fasting	260 265
Thbs2$^{-/-}$	Fragile skin, lax tendons; increased bone density; prolonged bleeding time; accelerated skin wound healing; enlarged, irregular collagen fibrils (dermis)	266 267 268
	Increased vascularisation (foreign body reaction)	269
	Increased vascular density (iris)	270
	Increased age-related dilated cardiomyopathy and mortality	271
	Inhibition of adipogenesis	117
	Increased tumor angiogenesis (skin cancer progression)	144
	Increased inflammation and MMP-2 activity (experimental glomerulonephritis)	261 62
	Altered lamellar morphology (lumbar discs)	
	Increased endosteal bone formation	
	Altered bone formation upon mechanical loading	
	Brittle bone phenotype	65
	Reduced cross-linked collagen; altered LOX distribution	66
	Reduced intra-ocular pressure	272
Thbs3$^{-/-}$	Transient abnormalities of post-natal skeleton: altered biomechanical properties of long bones transient increase in bone cortical area accelerated maturation of femoral head growth plate	273 274 153
	Altered growth plate organisation (age 1 and 2 months)	275
	Reduced cardiac hypertrophy and ventricular dilation responses to induced pressure overload; reduced cardiac fibrosis; increase of specific integrins in sarcolemma	276 277
Thbs4$^{-/-}$	Lack of cardiac enhanced contractility response to acute pressure overload	120
	Reduced neuropathic pain (spinal nerve ligation model)	155
	Increased fibrosis and altered myocardial remodelling response to induced pressure overload	278
	Increased lethality of induced pressure overload or myocardial infarction	279

Thbs4$^{-/-}$ *continued*	Increased cardiac hypertrophy and aortic aneurysms in response to angiotensin-2 Increased aortic wall thickness, inflammation and adventitial collagen fibril diameters in response to angiotensin-2 Reduced neointima formation and macrophage infiltrates in response to arterial wire injury Enlarged tendon collagen fibrils; reduced soleus muscle size and altered ECM composition; reduced grip strength Age-dependent (by 1yr) loss in running capacity and muscular dystrophy phenotypes Transient reduction in articular cartilage thickness (26 wks) Impaired developmental migration of neurons in the rostral migratory stream to the olfactory bulb	61 65
Comp$^{-/-}$*(aka Thbs5*$^{-/-}$*)*	No overt skeletal phenotypes Altered growth plate organisation (age 1 and 2 months)	
Compound gene-knockouts		
Thbs1$^{-/-}$*/Thbs2*$^{-/-}$	Delayed skin wound healing with impaired inflammatory response; pulmonary inflammation; prolonged bleeding time Decreased synaptic puncta in post-natal cerebral cortex (P8, P21) Reduced functional recovery after stroke with synaptic plasticity deficits Aberrant barrel cortex plasticity phenotype Altered proteome of diaphyseal cortical bone; brittle bone phenotype and decreased trabecular bone mass in males	64 280 281 282 283
Thbs1$^{-/-}$*/ApoE*$^{-/-}$	Increased atherosclerotic plaque maturation Protection against leptin-induced atherosclerosis	284 285
Thbs2$^{-/-}$*/Sparc*$^{-/-}$	Accelerated skin wound healing; reduced collagen fibril diameters and increased fibrovascular invasion (wound tissue); early cataracts	286
Thbs4$^{-/-}$*/ApoE*$^{-/-}$	Reduced atherosclerotic lesions and vascular inflammation than *ApoE*$^{-/-}$ mice Reduced macrophage infiltrates in visceral adipose tissue; reduced injury-induced neointima and cytokines than in *ApoE*$^{-/-}$	63 277
Thbs4$^{-/-}$*mdx, Thbs4*$^{-/-}$*/Sgcd*$^{-/-}$	Increased severity of muscular dystrophy phenotypes at 3 months of age and reduced running performance relative to *Sgcd-/-* or *mdx* mice	155
Thbs5$^{-/-}$*/ColIX*$^{-/-}$	Altered epiphyseal cartilage architecture	287
Thbs1$^{-/-}$*/Thbs3*$^{-/-}$ */Thbs5*$^{-/-}$*/ColIX*$^{-/-}$	Altered growth plate architecture (age 1 and 2 months; 10-20% shortening of long bones (hind limbs)	65
Thbs1$^{-/-}$*/Thbs2*$^{-/-}$ */Thbs4*$^{-/-}$*/Thbs5*$^{-/-}$	Early neonatal growth delay; death by d14 of induced pressure overload; impaired sarcolemma stability with reduced residency of β1D integrins	66
Thbs1$^{-/-}$*/Thbs2*$^{-/-}$ */Thbs3*$^{-/-}$*/Thbs4*$^{-/-}$ */Thbs5*$^{-/-}$	Early neonatal growth delay; survival of induced pressure overload	66
Knock-in models		
Thbs1$^{R1034C/+}$*, Thbs1*$^{R1034C/\ R1034C}$	Increased intra-ocular pressure; decreased aqueous humor outflow; decreased retinal ganglion cell density by 14 months	147
Thbs2$^{C896R/+}$	Mimics novel human Ehlers-Danlos syndrome with vascular features; excessive skin and tail tendon flexibility; increased bleeding time; disorganised collagen fibrils (dermis)	51
Thbs4$^{387P/387P}$	Increased macrophage infiltration of atherosclerotic lesions	289
Thbs4$^{387P/387P}$	Increased activity to promote pro-inflammatory differentiation and apoptosis in macrophages in LPS-induced peritonitis, relative to TSP4387A	290

Comp$^{T583M/T583M}$	Slower post-natal growth; shorter limbs and hip dysplasia; disorganised cartilage growth plate; increased chondrocyte ER stress and apoptosis; [288]age-dependent degeneration of articular cartilage	288
Comp$^{T585M/T585M}$	Progressive muscle weakness; mild myopathy of perimysium and MTJ; increased collagen fibril diameters (tendon) and disorganisation; altered tendon biomechanics	122
Comp$^{D469del/D469del}$	Slower post-natal growth; short limbs and hip dysplasia; disorganised growth plate and collagen ECM; increased chondrocyte apoptosis; reduced *Prdx2* expression	291
Comp$^{V65E/+}$, *Comp*$^{V65E/V65E}$	Normal long bones; enlarged ER in tenocytes; decreased *Scx*-positive tenocytes and reduced Achilles tendon healing; fibrosis of carpel tunnel and Achilles tendon (by 20 months)	50