Thrombospondins and Novel TSR-containing Proteins, R-spondins, Regulate Bone Formation and Remodeling

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Abstract Thrombospondins (TSPs) are a family of five secreted multimeric matricellular proteins that share homology in the type II and III repeats and carboxy-terminal region. Type I repeats, also known as properdin or thrombospondin repeats (TSRs), are found in TSP1/2, but not TSP3-5. A variety of other secreted proteins contain TSRs, including the novel extracellular molecules, R-spondins. TSP family and many TSR-containing proteins, including R-spondins, are highly expressed in skeletal tissues during development and postnatal. TSP2 regulates the osteoblast lineage, influencing bone mass and geometry, as well as response to fracture healing, ovariectomy, and mechanical loading. Compound knockout mice of TSPs have revealed important mechanistic insights. TSP1/2 knockout mice have craniofacial dysmorphism, and TSP1/3/5 compound knockout mice display growth plate abnormalities. R-spondins promote osteoblast differentiation and R-spondin-2 deficiency results in skeletal developmental defects. Overall, TSP and other TSR molecules influence multiple aspects of bone development and remodeling.

Keywords Thrombospondins · R-spondins · Osteoblast · Bone regeneration

Introduction

Bone and cartilage are relatively acellular and characterized by extensive extracellular matrix (ECM). Although ECM provides structural integrity for tissue function, some ECM molecules play a nonstructural, modulatory role that is more pleiotropic, affecting cell behavior, growth factor activity, and progenitor cell differentiation. This group of proteins is referred to as matricellular proteins [1]. A functional, rather than structural, classification defines matricellular proteins, and prototypical representatives are the thrombospondin (TSP) gene family [1, 2].

The TSP gene family has five members. The first TSP protein (TSP1) was identified in the 1980s as a component of platelet α granules and then later four additional family members were cloned [2–5]. TSPs are secreted ECM proteins that do not have a primary structural role. Characteristic of other matricellular proteins, knockout mice of TSP do not result in embryonic lethality or severe structural abnormalities in the skeleton, but rather show relatively mild skeletal phenotypes and a variety of nonskeletal phenotypes [6, 7].

Structurally, TSPs are large multimeric, modular proteins (Fig. 1). TSP1/2 are trimers and are more similar to one another than to TSP3-5. TSPs show increasing homology as the molecules are compared from the amino to the carboxy-terminus. Named domains include the globular N-terminus, von Willebrand factor/procollagen domain, type I repeats (also known as properdin or thrombospondin repeats [TSRs]), type II repeats (epidermal growth factor [EGF]-like), type III repeats (calcium binding), and a globular C-terminus (Fig. 1). Whereas TSP1/2 are trimeric proteins and contain three TSRs, TSP3-5 are pentameric and do not contain a TSR.

Despite having significant structural homology, TSP1 and TSP2 have divergent promoter regions and show differential gene regulation. TSP1, and to a lesser degree TSP2, have been studied most extensively as inhibitors of angiogenesis. The TSP1/2 group binds to cells through integrin receptors, heparin sulfated proteoglycans, lipoprotein-related receptor

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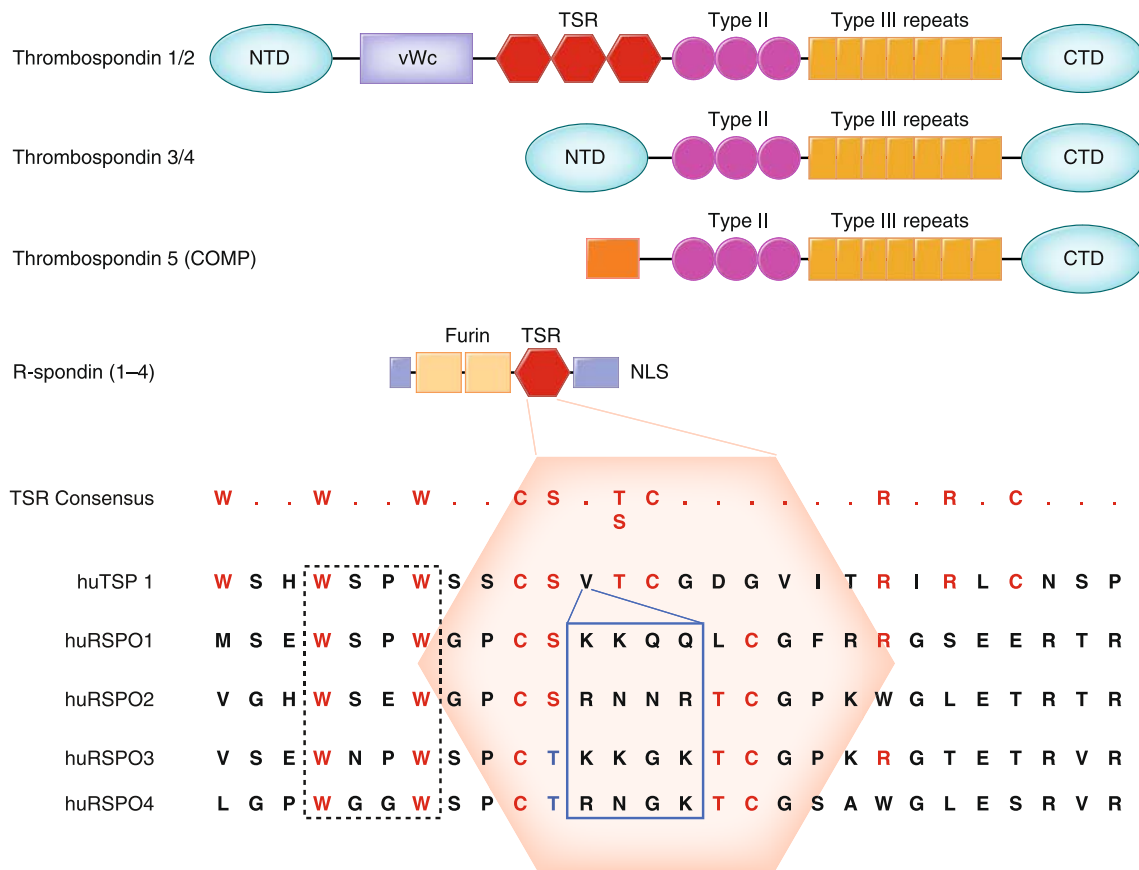
Fig. 1 Alignment of thrombospondins and R-spondins. Thrombospondins (TSPs) are depicted as monomers of the homopentameric TSP3-5 or the homotrimeric TSP1/2. TSP1/2 trimerize through the von Willebrand factor/procollagen domain (vWc). All five TSPs share homology in the type II repeats, type III repeats, and the C-terminal globular domain (CTD). TSP1/2 also contains three thrombospondin type I repeats (TSRs), a domain that is also found conserved in many non-TSP family proteins, including R-spondins. The *hexagonal inset* shows the most conserved portion of the amino acid sequence and alignment of the TSR domains. The TSR consensus sequence is shown in *red at the top* with conserved amino acids also shown in *red below*. The *blue text* indicates a conserved amino acid change. The WSxW sequence shown (*dashed box*) has been shown to bind to cells, heparin, and the latent complex of transforming growth factor-β. The *blue box* indicates the basic amino acid–rich sequence that is found inserted in all R-spondins into an otherwise conserved CSxTC sequence. COMP—cartilage oligomeric matrix protein; huRSP—human R-spondin; huTSP—human thrombospondin; NLS—nuclear localization; NTD—N-terminal domain

protein (LRP) receptors, CD36 (fatty acid translocase), and the integrin-associated protein CD47. Also, the molecules have been shown to bind to a variety of growth factors, including transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and fibroblast growth factor, and other matrix proteins such as collagens and fibronectin.

There are at least 41 different human extracellular proteins containing one to seven TSRs. Proteins containing TSRs include F-spondins, ADAMTS (a disintegrin and metalloproteinase with a TSP type 1 motif), connective tissue growth factor, and a novel family of ECM proteins, the R-spondins [8]. X-ray crystallographic data show that a TSR contains three strands, A–C, arranged in a β sheet [9]. Although there is variation between TSR sequences they are commonly defined by the presence of six cysteines and three functional regions: 1) tryptophan-rich–containing

WXXW repeats, 2) a cysteine-rich region, and 3) basic amino acid–containing region (Fig. 1). The three TSRs from TSP1 are perhaps the best characterized and bind to growth factors, heparin, and the cell surface receptor CD36 [5]. The second TSR of TSP1 contains a TGF-β activation domain [10]. TSR appears to be the portion of the molecule that has functional activity regulating proliferation and apoptosis.

The other three pentameric TSP family members, TSP3-5, do not contain TSRs, but show relatively strong homology in the type II, type III, and globular C-terminus domains. Whereas TSP3-5 are all expressed in cartilage and bone, the roles of TSP3/4 are perhaps the least well-characterized of the five TSPs. Conversely, TSP5, also known as cartilage oligomeric matrix protein (COMP), has been extensively studied [11]. TSP5 is highly expressed in cartilage and joints, and increased serum levels are associated with joint erosion.

Most importantly, mutations in TSP5 are responsible for pseudoachondroplasia (PSACH) [11, 12].

R-spondins are a recently identified TSR-containing protein family that play a role in skeletal biology [13]. R-spondins are secreted proteins that contain a single TSR and contain furin repeats (Fig. 1). There are four R-spondin family members and all are expressed in the skeleton. R-spondins have not been widely studied, but initial work suggests that R-spondins may function to increase Wnt signaling through interactions with LRP5/6 and frizzled receptors [13].

Thrombospondin-2

TSP2 expression in the developing skeleton has been studied using in situ hybridization and immunohistochemistry [14, 15]. TSP2 is detected starting at E14 in the mouse and localizes to the proliferating and hypertrophic chondrocytes of growth plate, perichondrium, and intramembranous ossification centers of skull bones. TSP2 is also detected in the femoral periosteum of 8-week-old mice. In a tibial fracture model, TSP2 expression is highest in undifferentiated mesenchyme 5 days post fracture and decreases in mature fracture calluses [16•]. TSP2 expression increases when marrow stromal cells (MSCs) are induced to undergo osteogenic differentiation and decreases as committed osteoblasts undergo terminal differentiation and mineralization (Shitaye and Hankenson, unpublished observations) [17•, 18]. The promoter structure of TSP2 contains multiple activator protein-1 (AP-1) elements. Transgenic mice overexpressing the AP-1 family member, Fos-related antigen-1 (Fra-1), have decreased TSP2 expression in calvarial osteoblasts and long bones, suggesting that AP-1 family members play an important role in regulating TSP2 expression [18].

Analysis of the TSP2-null skeleton revealed that TSP2 plays an important role in bone homeostasis (Table 1) [18]. TSP2-null mice have increased cortical bone thickness due

Table 1 Overview of skeletal phenotypes of TSP family and Rspo family mutant mice

	Appendicular skeleton phenotype	Axial skeleton phenotype	Other phenotypes of interest
TSP1	No reported defects	Spinal lordosis. Craniofacial dysmorphism that is more pronounced in compound TSP1/2-null mice	Altered rates of wound healing/tissue remodeling in various injury models
TSP2	Excess cortical bone mass attributed to increased bone formation	Moderate spinal lordosis	Increased skin and tail flexibility due to improper fibrillar collagen organization. Increased vascularization. Altered wound healing.
TSP3	Early ossification of femoral head and growth plate chondrocyte disorganization in TSP1/3/5 compound knockout mice	None reported	None reported
TSP4	No mutant mouse has been reported		
TSP5/COMP	Mutations: shortened long bones, disproportion short stature, early OA, joint abnormalities. Null: growth plate chondrocyte disorganization and mild exercise-induced articular cartilage flattening	Mutations: lumbar lordosis, scoliosis, vertebral abnormalities that resolve in adolescence	Mutations cause skeletal dysplasias (PSACH and MED/EDM1)
Rspo1	No reported skeletal phenotypes		Required for appropriate ovarian and mammary gland development. Mutation in mice and humans causes XX sex reversal
Rspo2	Required for maintenance of the apical ectodermal ridge. Extensive skeletal defects	Cleft palate; craniofacial malformation	Lethal E18.5, day 1 post-natal. Pulmonary defect, cleft palate, kidney defect
Rspo3	No reported skeletal phenotypes		Knockouts lethal E10. Attributed to placental hemorrhage and inappropriate vasculature. Promotes angiogenesis through regulation of VEGF
Rspo4	No reported skeletal phenotypes		Mutation in the furin region of Rspo4 is attributed to loss or malformation of nails in human familial anonychia [54]

COMP cartilage oligomeric matrix protein, *MED* multiple epiphyseal dysplasia, *OA* osteoarthritis, *PSACH* pseudoachondroplasia, *Rspo* R-spondin, *TSP* thrombospondin, *VEGF* vascular endothelial growth factor

to enhanced endocortical bone formation. The increase in bone formation in TSP2-null mice is most likely due to higher numbers of osteoprogenitors (MSCs) obtained from the marrow of TSP2-null long bones. Furthermore, these MSCs show increased proliferation in vitro. Paradoxically, mineralized matrix formation is delayed in MSCs from TSP2-null mice and RNA interference (RNAi)–mediated knockdown of TSP2 in preosteoblasts (MC3T3-E1) decreases mineralized matrix formation [17•]. Therefore, the function of TSP2 in mesenchymal lineage cells appears to be highly contextual, and the overall effect of the presence or absence of TSP2 on bone formation is likely dependent on the cell type present and stage of differentiation.

Findings from studies in which bone homeostasis is perturbed in TSP2-null mice further supports the hypothesis that the TSP2 role in bone formation is highly contextual (Table 2) [1, 18]. For instance, mechanical loading in TSP2-null mice results in an atypical pattern of bone formation. [1, 18] In wild-type mice, the normal response to bending load is deposition of bone at the periosteum with relatively little endocortical new bone formation; however, in TSP2-null mice, this envelope-specific pattern of bone formation is reversed [1, 18]. In the absence of TSP2, periosteal osteoblasts produce less bone matrix in response to mechanical loading, whereas at the endocortical surface, increased osteoprogenitor proliferation results in increased osteoblast numbers and thus increased bone formation. Similarly, TSP2-null mice are protected from ovariectomy-induced bone loss due to an increase in bone formation at the endocortical surface with a concomitant reduction in new periosteal bone [1, 18]. Also, ovariectomized TSP2-null mice show decreased bone resorption. A potential explanation for the altered phenotype in ovariectomized TSP2-null mice is that endocortical MSC numbers, as determined using a colony-forming unit—fibroblastoid (CFU-F) assay, increase four-fold in TSP2-null mice after ovariectomy.

TSP2-null mice also show altered fracture healing following semi-stabilized closed fractures of the tibia [16•]. TSP2-null fracture calluses contain more bone and less cartilage 10 days post fracture. Analysis of the fracture callus at 5 days post fracture shows an increase in vascular density and a decrease in VEGF and Glut-1 (two markers of hypoxia-inducible factor signaling). Because oxygen tension influences chondrogenesis and osteogenesis [19], the alteration in the proportion of cartilage and bone in TSP2-null mice relative to wild-type mice may arise from differences in oxemic status between TSP2-null and wild-type fractures.

The molecular mechanism through which TSP2 influences MSC proliferation and differentiation is not currently known. A recent study by Meng et al. [20•] found that TSP2 physically interacts with Notch receptors and ligands and modulates activation of the signaling pathway in lung cancer cell lines and primary smooth muscle cells. Inactivating Notch signaling in MSCs results in premature differentiation and depletion of the osteoprogenitor pool [21]. We have recently shown that Jagged-1 (a ligand for Notch)–mediated activation of Notch signaling is increased in TSP2-null MSCs relative to wild-type controls (Shitaye and Hankenson, unpublished results). Interestingly, Jagged-1 increases MSC proliferation and inhibits their osteogenic differentiation. Therefore, in the absence of TSP2, Notch signaling could be increased, resulting in an expansion of the osteoprogenitor pool while decreasing osteogenic differentiation.

Thrombospondin-1

The role of TSP1 in bone has not been examined as extensively as TSP2. In the developing skeleton, TSP1 has a broader pattern of expression than TSP2 [5]. TSP1 is detected in mesenchymal condensations that give rise to rib bones at E13 and in the ECM of chondrocytes at E14 in mice. TSP1 expression can also be detected in cartilage proper and in resting and proliferating chondrocytes of growth plate cartilage. TSP1 expression colocalizes with TSP2 in ossification centers of skull bones formed by intramembranous ossification.

TSP1-null mice have no apparent defect in bone mass, as determined by radiographic studies, but show a mild

Table 2 Remodeling phenotype of TSP2-null mice

Pathophysiologic challenge	Phenotype
Ovariectomy	TSP2-null mice show resistance to ovariectomy-induced bone loss. There is an increase in endocortical bone formation and MSC number, and reduction in periosteal bone formation
Mechanical loading	TSP2-null mice show differential surface responsiveness to mechanical loading
Fracture healing	TSP2-null mice show increased bone formation and reduced chondrogenesis in response to fracture
Aging	TSP2-null mice show alterations in bone mass and geometry associated with aging. At advanced age, an absence of TSP2 appears to protect against age-associated bone loss[a]

[a] (Terkhorn and Hankenson, unpublished results.)

MSC marrow stromal cell, TSP2 thrombospondin-2

lordotic curvature of the spine (Table 1) [10]. Similar to TSP2, TSP1 expression is decreased in calvarial osteoblasts and long bones of Fra-1–overexpressing mice [18]. Interestingly, TSP1-null mice have craniofacial dysmorphism similar to Fra-1 transgenic mice (Table 1). The craniofacial phenotype is more pronounced in TSP1/TSP2 double-null mice, indicating that the two proteins may have overlapping roles in cranial bone development.

The role of TSP1 in osteogenic differentiation in vitro has been examined in MC3T3-E1 preosteoblast cell lines and, rather surprisingly, shows the opposite effect of TSP2. TSP1 knockdown in MC3T3-E1 cells results in increased alkaline phosphatase activity, increased osteocalcin expression, and accelerated mineralization, whereas stable overexpression, or treatment with purified TSP1 protein, results in a decrease in mineralized matrix formation. Subcutaneous implantation of collagen sponges loaded with cells stably overexpressing TSP1 (E1 cells) in immunocompromised mice results in less bone formation than control cells. Therefore, in MC3T3-E1 cells, TSP1 appears to inhibit osteogenic differentiation [22].

Thrombopsondin-3

TSP3 expression has been detected in the developing skeleton of several species (chick, frog, and mice) and in a variety of nonskeletal tissues [5]. Specifically, in situ hybridization in the developing chick shows TSP3 expression in proliferative and hypertrophic chondrocytes in cartilage. During mouse development, TSP3 is expressed early in the outer layer of extra-embryonic mesoderm and later in developing cartilage, calcified cartilage (developing bone), and neuronal tissue. Although there are no published studies that detail the regulation of TSP3 expression level in bone tissue, a microarray study to identify genes related to osteosarcoma tumor progression showed that high levels of TSP3 after chemotherapy treatment are associated with a poor prognosis and metastasis [23].

To characterize the role of TSP3 in vivo, a knockout mouse was generated and skeletal and growth parameters were measured [24]. Skeletal development was not perturbed before birth; however, by 9 weeks of age, trabecular bone is present in the secondary ossification center (femoral head) in the TSP3-null but not in the wild-type mice [24]. By 15 weeks of age, the femoral head in TSP3-null male mice is completely ossified, whereas the wild-type mice have no trabecular bone in the secondary ossification center at this stage [24]. Additionally, the TSP3-null mice have an increase in femoral cortical bone periosteal circumference and parameters of mechanical strength at 9 and 15 weeks of age. The phenotype of the TSP3-null mouse shows that TSP3 is involved in ossification of cartilage anlagen and

perhaps influences the timing of bone maturation; however, the molecular mechanisms involving TSP3 regulation of ossification are unknown [24]

The high degree of homology and structural similarity between TSP3 and TSP5 proteins led the authors to generate a TSP3-TSP5 double-null mouse line [24]. The mechanical and geometric properties of the double-null femur were similar to that of the TSP3-null, suggesting that these two TSPs are functionally distinct in modulation of bone geometry [24]. Further studies of the double-null growth plate demonstrated that both TSP3 and TSP5 contribute to columnar organization of chondrocytes in the growth plate and mild shortening (8%) of the hind limbs at 1 month of age [25•]. These growth plate studies of the TSP3-TSP5 double-null are consistent with TSP3 being involved in endochondral ossification and bone growth.

Thrombospondin-4

TSP4 is expressed in a variety of tissues during development and adulthood [5]. In chick, TSP4 is expressed in the periosteum of developing bone at the beginning of osteoblast differentiation. In the developing *Xenopus* embryo, TSP4 is present in the skeletal muscle and somitic mesoderm, which gives rise to many tissues rich in ECM, including bone, muscle, connective tissue, reproductive system, and urinary tract.

Currently, there are no genetic models for TSP4, and TSP4 has received very little attention in studies of skeletal biology. In vitro, TSP4 has been shown to support myoblast attachment and promote adhesion and neurite outgrowth indirectly through interaction with laminins. Cellular adhesion is mediated through the interaction with ECM components. The C-terminal domain of TSP4 binds to collagens I, II, III, and V, as well as laminin, fibronectin, and matrilin-2 [5].

Thrombospondin-5/COMP

TSP5/COMP, once thought to be cartilage-specific, has been detected in other tissues, including tendon, ligament, smooth muscle, synovium, and osteoblasts [5]. TSP5 is expressed during early limb development, and expression is particularly high in growth plate cartilage [26]. The proximal 375 bp of the human TSP5 promoter is sufficient to drive cartilage expression in vivo, and sequence elements between 1.0 and 1.7 kb upregulate TSP5 expression [27].

During skeletal growth, cartilage turnover is rapid and, therefore, higher levels of serum TSP5 are detected in children under 16 years of age compared with adults [28]. Lower serum levels of TSP5 are associated with growth deficiencies observed in juvenile arthritis [28]. High levels

of TSP5 in serum/synovial fluid of adults indicate cartilage erosion and are associated with adverse long-term outcome for injured or arthritic joints [11]. Chondrocytes close to the cartilage defect express high levels of TSP5, suggesting that TSP5 may contribute to joint resurfacing [29]. Tendon studies show that weight-bearing tendons have more TSP5 than tendons not under strain, indicating that TSP5 may be expressed in response to loading [30].

Mutations in TSP5 cause PSACH, a dwarfing condition associated with early-onset osteoarthritis [11, 31]. Irregular epiphyses and metaphyses are observed in radiographs [31]. PSACH-associated abnormalities are limited to the skeletal system and include disproportionate short stature, shortened fingers, extremely lax joints, scoliosis, lumbar lordosis, and osteoarthritis beginning in the second and third decades of life [11, 32].

TSP5 mutations disrupt the three-dimensional structure of the protein and inhibit protein export, leading to pronounced accumulation of TSP5 in rough endoplasmic reticulum (rER) cisternae of chondrocytes [33]. This accumulation is cytotoxic and causes premature chondrocyte death [11]. Decreasing the number of growth plate chondrocytes decreases linear growth of long bones [34]. Additionally, mutant TSP5 impedes the export of type IX collagen and matrilin-3 and together these ECM proteins form an intracellular matrix network that may decrease degradation of the intracellular material exacerbating rER accumulation [35]. The decrease of these proteins in the ECM causes disorganization and results in joints that are easily eroded translating into early onset osteoarthritis [11].

Initial studies of TSP5-null mice reported no phenotypic abnormalities [36]. However, a more detailed study of the growth plate of these mice showed that loss of TSP5 decreases chondrocyte columnar organization (Table 1) [25•]. Mild exercise-induced flattening of the articular cartilage superficial cap was observed in the TSP5 knockout mice [25•]. Complex crosses of knockouts for TSP5 in combination with TSP1-null and TSP3-null result in significantly altered growth plate organization and limb length, suggesting that TSP may be redundant in growth plate function [25•]. Recent work describing a mouse line that expresses mutant TSP5 (D469del) shows a dramatic phenotype mimicking the PSACH growth plate pathology [37••]. Mice expressing mutant TSP5 had intracellular retention of TSP5, types II and IX collagens, and matrilin-3, and premature intracellular ECM assembly [37••]. Additionally, less type IX collagen and matrilin-3 are observed in the ECM reminiscent of the PSACH ECM and, most importantly, apoptosis is upregulated by expression of mutant TSP5 in the growth plate [37••].

TSP5 interacts with collagenous and noncollagenous components of the ECM. TSP5 binds to integrins, aggrecan, heparin, heparin sulfate, and chondroitin sulfates, and calcium depletion of TSP5 decreases binding [38]. TSP5 expression enhances chondrogenesis perhaps by regulating proliferation and attachment [39]. The EGF domain of TSP5 binds to an autocrine growth factor, granulin-epithelin precursor, stimulating chondrocyte proliferation [40]. The RGD motif of TSP5 interacts with integrins and supports chondrocyte attachment [41]. Mutant COMP (D469del) supports less chondrocyte attachment most likely due to changes in three-dimensional calcium dependent structure [41].

Type I, II, and IX collagens bind to the C-terminal globular domain of TSP5 [42, 43] and both types II and IX collagens are co-retained in the rER of PSACH chondrocytes [35]. Pentameric TSP5 promotes collagen fibril assembly, whereas the monomeric form inhibits collagen fibrillization [44]. The TSP5 pentamer may catalyze fibril formation by increasing the effective concentration of free collagen molecules [44]. TSP5 only interacts with immature collagen fibrils, which is consistent with the interaction of TSP5 and other ECM protein in the endoplasmic reticulum of PSACH chondrocytes [44]. Additionally, the ECM that surrounds PSACH chondrocytes is disorganized and fibril diameter is altered, suggesting that TSP5's role in collagen fibril formation contributes to the PSACH ECM pathology [45].

R-spondins

Among the newest identified members of the TSR superfamily are the R-spondins (Rspo). In mammals, the R-spondin family includes four proteins, which are encoded by separate genes. Rspo homologues have also been identified in non-mammalian vertebrates and invertebrate species [13]. The first Rspo gene (Rspo3) was identified in a fetal brain cDNA library, although it was named only after the discovery of Rspo1. Rspo1 was isolated from a screen of genes expressed in a cell line derived from spinal cord/neuroblastoma cells (NSC-19) [46]. In situ hybridization demonstrated expression of Rspo1 in the roof plate; thus, the protein was named for both the roof plate localization (R) and the TSR (spondin) [46].

All four R-spondins are comprised of a signal sequence, two sequential cysteine-rich furin domains, a single TSR, and a nuclear localization signal [13]. The signal sequence and the nuclear localization signal have been functionally verified as R-spondins are detected both extracellularly in 293 cells and within the nucleus of COS7 cells [46]. The furin domains are required for activation of the Wnt signaling pathway [47, 48]. The Rspo TSR binds to heparin and heparin sulfate proteoglycans [48] and loss of that domain significantly reduces Wnt signaling. Both the furin

domains and the TSR are required for cell surface binding of Rspo2 [48].

In a thorough assessment of expression of all four mouse R-spondins, Nam et al. [49] demonstrated that R-spondins are expressed throughout development but each R-spondin expression pattern is unique. All R-spondins are detected in the developing limb bud of wildtype mice during embryonic stages E9–10.5. *Rspo2* is tightly associated with the apical ectodermal ridge (AER), whereas *Rspo4* is seen in the zone of polarizing activity and along the anterior region of the limb bud. *Rspo3* occupies a region of the limb bud framed by the dual expression patterns of *Rspo2* and *Rspo4*. Expression of *Rsop1* is restricted to a small zone in the most proximal region of the limb bud [49]. Only in the spinal cord and developing skeleton (including long bones and calvaria) are all four R-spondins detected across embryonic stages 12.5–17.5. In addition, *Rspo2* and *Rspo4* are expressed at the most distal tip of the digits, whereas only *Rspo2* is expressed in presumptive dentin.

Because of similarities between Wnt and *Rspo1* expression in the limb bud, *Rspo1* expression was evaluated in Wnt-1, Wnt-3a, and Wnt-1/3a knockout mice [46]. In all three mice, *Rspo1* expression was greatly diminished, suggesting a link between Rspo proteins and Wnts. More recent studies have demonstrated that all four Rspo proteins appear to regulate Wnt signaling through blocking of Dkk interactions with LRP or promoting Wnt-LRP interactions [47]. Wnt family members are expressed throughout skeletal development and, more recently, mechanistic studies have implicated Wnts as critical regulators of bone formation, maintenance, and healing [50].

Knockout studies of all four Rspos have been reported, but only *Rspo2*-deficient mice appear to have a skeletal defect. Footless mice, generated by random insertional transgene mutagenesis, have a disruption in Rspo2 [48]. Rspo2 nulls were also generated by three additional groups [51–53]. Both the Rspo2-null mice and the footless mutant mice (which are also Rspo2-null) demonstrate that Rspo2 is required for maintenance of the AER and that the loss or disruption of the *Rspo2* gene results in severe distal hindlimb defects, loss of some digits and all fingernails of the forelimbs, cleft palate, lung hypoplasia, craniofacial malformation, and death at birth. Interestingly, a mutation in the human *Rspo4* gene is responsible for familial anonychia, characterized by the loss or malformation of finger and toenails, a phenotype similar to that seen in the digits of the Rspo2-null mice [54].

As with the Rspo1 knockout mouse, Rspo2-null mice have a loss of *Wnt3* gene expression within the AER [49]. In addition, Rspo2 regulation of skeletal development through Wnt signaling was further demonstrated by investigating the canonical Wnt signaling coreceptor, LRP6. The phenotype of the footless mutant mice closely resembled that of the LRP6-null mice, and a cross of the LRP6-null and Rspo2-null mice resulted in exacerbated limb phenotypes with forelimb skeletons completely ablated in the LRP6-null/Rspo2-null mouse [48].

Although skeletal defects seen in the Rspo2-null mice are attributed to a disrupted AER and resultant loss of distal skeletal structures, we recently published evidence that Rspo2 can also directly affect the maturation and mineralization of osteoblasts through the regulation of bone morphogenetic protein (BMP) signaling [55]. In vitro, overexpression of Wnt11 in BMP-2–treated MC3T3-E1 cells upregulates expression of Rspo2 as well as increasing expression of osteoblast-associated genes and matrix mineralization. The regulation of Wnt-11 and Rspo2 expression appears to be somewhat reciprocal because knockdown of Rspo2 expression via microRNAi abrogates the ability of Wnt-11 to increase osteoblast differentiation and mineralization in response to BMP-2. Similar to results with Rspo2, Rspo1 also appears to regulate osteoblast differentiation. Rspo1 synergizes with Wnt-3a to promote expression of osteocalcin and alkaline phosphatase in C2C12 cells and primary murine osteoblasts [56•].

Conclusions

TSP family members and TSR-containing proteins are widely expressed in skeletal tissues during development, growth, modeling, remodeling, and aging. Although the physiologic significance of TSP, other than TSP5, in human bone biology is still being pursued, studies with knockout mice, and other mutant mice, suggest that these extracellular, matricellular proteins play important modulatory roles in skeletal development and function. Importantly, compound deficiencies in these proteins likely exacerbate functional deficits, which suggest that in some cases these proteins may be functionally redundant in the skeleton. Studies of the TSR-containing R-spondins are more recent and show that R-spondins likely modulate bone development, but similar to TSP2, also increase osteoblast activity. Because Rspo2 mice die at birth, functional in vivo studies of the role of Rspo2 in bone maintenance and healing have not yet been published; however, given the role of Rspo2 in the regulation of bone formation in vitro, it seems likely that conditional or localized knockdown of in vivo Rspo2 expression will demonstrate a role for Rspo regulation of postnatal bone remodeling. More work is required to fully understand the significance of the TSR domain in the pro-osteoblastic effects of TSP2 and R-spondins, and to determine whether there are molecular parallels in the function of TSR-containing molecules in osteoblastogenesis.

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