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Dynamic Expression of Two Thrombospondins During Axolotl Limb Regeneration

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Abstract

The molecular processes underlying regeneration remain largely unknown. Several potential factors have been elucidated by focusing on the regenerative function of genes originally identified in a developmental context. A complementary approach is to consider the roles of factors involved in wound healing. Here we focus on the Thrombospondins, a family of secreted extracellular matrix proteins that have been implicated in skin wound healing in mammals. We show that a subset of Thrombospondins are expressed at distinct times and in particular cell types during axolotl limb regeneration. Our studies have revealed the axolotl orthologs of *thrombospondin-1* (*tsp-1*) and *thrombospondin-4* (*tsp-4*) are highly upregulated during limb regeneration in patterns both distinct and similar to larval limb development. Our data suggests that *thrombospondins* may be key regulators of limb regeneration in axolotl, while their activation appears to be relegated solely to wound healing in vertebrates that have lost the ability to regenerate limbs.

Keywords

thrombospondins; limb regeneration; axolotl; wound epidermis; blastema

INTRODUCTION

Urodele amphibians, such as the axolotl, harbor remarkable regenerative abilities. Following amputation, adult axolotls can regenerate an entire limb, a process which is still poorly understood. In contrast, mammals have largely lost the ability to regenerate limbs, although digit tips can be regrown under certain conditions (Illingworth, 1974), reviewed in (Han et al., 2005).

One hypothesis regarding this loss of regenerative ability over evolutionary time is that it could be coupled to the differences in wound-healing between mammals and amphibians. Mammals have evolved a wound-healing scheme that leads to protective scar formation, whereas salamanders do not heal wounds with overt scarring, which may be critical for a regenerative response. As a consequence, they may have evolved to keep active pathways necessary for regeneration that are shut down in higher vertebrates. Indeed, the earliest morphological step leading to a regenerated limb is the migration of epithelial cells across the freshly amputated stump, a process akin to mammalian wound healing, to form a “wound epithelium.” While mammalian wounds heal with scabbing and then scarring, the salamander wound epithelium never scabs or scars and histological data suggests the underlying extracellular matrix is very different from a mammalian wound (Levesque et al.,

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2010; reviewed in Harty et al., 2003). Cells in the salamander wound epithelium are thought to signal to the underlying stump tissue to instruct the formation of a regenerative blastema, a collection of relatively de-differentiated cells beneath the wound epidermis. However, little is known about this potential interaction between the wound epithelium and the underlying mesenchymal blastema.

An increased understanding of the molecular factors supporting regeneration in axolotls might inform efforts to restore regeneration in mammals or at least to enhance healing. In this report, we analyze the distribution during various stages of axolotl limb regeneration of a family of mRNA transcripts encoding proteins implicated in mammalian wound healing. We initiated this screen by focusing on molecules whose activity is downstream of Thrombin, a serine protease in the coagulation cascade that also activates platelets, and ultimately focused on the TSP family. There are numerous reports in the literature implicating TSP family members as having critical functions during wound healing. For example, Thrombospondin-1 (TSP-1), the founding member of the Thrombospondin family, is released by human platelets in response to Thrombin (Lawler et al., 1978). Subsequent research has demonstrated TSPs play a vital role in diverse biological processes in mammals including cell proliferation, cell migration, and angiogenesis, all necessary processes for wound healing (reviewed in (Adams and Lawler, 2004)). In both mice and humans, high levels of TSP-1 protein are detectable in tissues beneath skin wounds (Raugi et al., 1987). Loss-of-function mouse mutants for *tsp-1* or *tsp-2* result in altered wound healing kinetics (Kyriakides et al., 1999; Agah et al., 2002). Despite the vast amount of research focused on the role of TSPs in mammalian wounding, TSPs have never been implicated in regeneration. In this paper, we show two members of the TSP family are expressed in discrete tissues and stages of regeneration, and provide suggestive evidence that molecules necessary for mammalian wound-healing could be key factors in urodele regeneration.

RESULTS

Cloning axolotl thrombospondins

Degenerate PCR was used to recover portions of *thrombospondin* transcripts from mixed-stage axolotl blastema cDNA preparations. We were able to isolate transcript fragments from axolotl *tsp-1*, *tsp-3*, and *tsp-4*. The 5' and 3' ends of the *tsp-1* and *tsp-4* ORFs were cloned by 5' RACE, cDNA library screening, and modified degenerate PCR (see methods). Full-length *tsp1* and *tsp4* ORFs were cloned by long-range PCR from cDNA prepared from blastemas from a single axolotl. The cDNAs and translated protein sequences have been submitted to Genbank under accessions HQ380179 (*tsp-1*) and HQ380181 (*tsp-4*). We also cloned a fragment of axolotl *tsp-3* (Genbank accession number HQ380180), for which we have not obtained a full transcript to date. The recovered portion of axolotl TSP-3 shares 93% amino acid identity with *X. tropicalis* TSP-3 over amino acids 539–785.

Tsp-1 is predicted to encode an 1176-aa protein, while *tsp-4* is predicted to encode a 956-aa protein. Alignment to other vertebrate proteins indicates both TSP-1 and TSP-4 are highly conserved across vertebrate species, and all identified molecular interaction motifs are present in the axolotl proteins (Fig. 1B and 1D, full protein alignments in Sup. Fig. 1). Within both the known CD36 and CD47 binding domains (CSVTCG and RFYVVMWK, respectively), axolotl TSP-1 is 100% identical to the homologous domains of TSP-1 of other vertebrates. The Fibronectin binding domain (GGWSHW) is also entirely conserved between other vertebrates and axolotl TSP-1, and the Integrin binding domain is nearly identical, with only a valine to leucine substitution at position 221 in axolotl TSP-1. One notable difference between axolotl TSP-1 and all other vertebrates aligned, except *Xenopus*, is a five-amino-acid insertion immediately following the heparin binding site in the N-terminal globular domain (Fig. 1B, inset). As this amino acid insertion is also not seen in

out-group zebrafish, it appears to represent a synapomorphy or shared-derived characteristic in modern amphibians.

TSP-4 is less-well characterized at the molecular level than other Thrombospondins. Hence, we have more limited data about the specific protein-protein interaction motifs to anticipate within the newly-cloned axolotl TSP-4. Within the four EGF-like domains and seven type III thrombospondin repeats, all cysteines predicted to participate in disulfide bonding are conserved between axolotl and other vertebrate TSP-4 proteins. Two potentially interesting differences between the axolotl TSP-4 and other vertebrate TSP-4 proteins are highlighted in Fig. 1D insets. The first area of interest lies between the N-terminal domain and the COMP family sequence. The second divergent area spans the amino acids between COMP and beginning of the first EGF-like domain.

Isolating *tsp-1*, *tsp-3*, and *tsp-4* from mixed-stage blastema cDNA shows that all three of these molecules are present during regeneration, but reveals no detailed information on the timing or specificity of the expression. To begin to understand the potential roles of Thrombospondins in vertebrate limb regeneration, we analyzed their expression in regenerating axolotl limbs. *Tsp-3* expression at two weeks post-amputation was not detectable in the blastema or epidermis, and was weakly detected in muscle tissue on the stump (data not shown). In contrast, striking expression patterns during regeneration were observed for *tsp-1* and *tsp-4* transcripts, hence subsequent work was focused on these two genes.

Thrombospondin-1 and thrombospondin-4 are expressed in discrete tissues in intact limbs

We analyzed the expression of *tsp-1* and *tsp-4* in intact, unamputated axolotl limbs to provide a reference point for where to expect stump expression in freshly-amputated limbs, in regenerating limbs proximal to the dedifferentiation zone, and in limbs that have completed regeneration entirely. *Tsp-1* is expressed in the chondrocytes of long bones, such as the metacarpels, carpels, radius, ulna, and humerus (Fig. 2A, 2D, 2G, 2I). Expression is diminished in the areas of skeletal elements undergoing ossification (Fig. 2A, 2C). Importantly, in the context of its expression during regeneration (see below), expression of *tsp-1* was never detected in the epidermis by in situ hybridization. Unlike the expression found for *tsp-1*, we found robust expression of *tsp-4* in several tissues of the musculoskeletal system of intact limbs, including tendons, myotendonous junctions, and perichondrium (Fig. 2B, 2E–F, 2H, 2K–L). Within this context of their expression in unamputated limbs, we next turned to studying their expression during the process of limb regeneration, starting with TSP-1.

Thrombospondin-1 is enriched in wound epidermis

Thrombospondin-1 expression was detected in the primary cells of the wound epidermis at 24 hours post amputation (Fig. 3A, 3E). These cells are distinct in their position within the wound epidermis as well as in their morphology. Within the wound epidermis, the primary epidermal cells lie at the basal layer of this tissue, have a squat shape and are connected in a brick-like pattern to one another, while epidermal cells that appear later atop these basal cells are packed in a cobblestone manner. *Tsp-1* expression persists well into regeneration, where it is clearly detected in the basal layer of the wound epidermis at one and two weeks post-amputation (Fig. 3B, 3C, 3F, 3G). This can most dramatically be seen in regenerates derived from proximal amputations, where nearly all of the epidermis expresses *tsp-1* in the basal layer, presumably because much of it is participating directly in the regeneration process. To determine if the basal layer expression was specific to the regenerating tissue, amputations were performed more distally, at the mid-radius/ulna level, which allowed for a

longer stump to be included in the analysis. Interestingly, relatively little *tsp-1* expression is evident in the basal layer of the proximal epidermis, indicating *tsp-1* is specific to the regenerative wound epidermis (Fig. 3G). Furthermore, as noted above, *tsp-1* does not appear to be appreciably expressed in non-regenerating axolotl limb epidermis (for example, Fig. 2A, 2J). Quantitative RT-PCR reveals that *tsp-1* transcript is upregulated approximately fivefold in wound epidermis (60 hours post amputation) versus intact skin (data not shown).

Tsp-1 is also expressed during regeneration in tissues other than the epidermis. Within 24 hours post-amputation, *tsp-1* becomes highly expressed in scattered cells within the distal end of the stump (Fig. 3A). At one week post-amputation, scattered cells within the forming blastema express *tsp-1* (Fig. 3B), and larger blastemas also exhibit *tsp-1* expression. By three weeks post-amputation, *tsp-1* expression in the blastema begins to resolve into chondrocyte expression (Fig. 3D, 3H, 3K), and is robustly expressed the chondrocytes in the central portion of the skeletal element (Fig. 3L). In the less mature, posterior digits, *tsp-1* expression remains as scattered mesenchymal cells (Fig. 3I), and along the posterior epidermis, *tsp-1* expression is maintained within the basal layer (Fig. 3J).

Thrombospondin-4 is expressed in blastema cells

In situ hybridization for *tsp-4* was performed on serial sections of the same limbs used in examining *tsp-1* expression. At 24 hours post-amputation, *tsp-4* transcripts are not detectable in the wound epidermis, and only faint expression of *tsp-4* in the mesenchyme immediately beneath it is detectable (Fig. 4A, 4E). In contrast, at one week post-amputation, within the early blastema, many mesenchymal cells accumulating directly beneath the wound epidermis express *thrombospondin-4* robustly (Fig. 4B, 4F). *tsp-4* expression intensifies as the blastema cells proliferate and the blastema matures (two and three weeks post amputation, Fig. 4C–D, 4G–L). A very large proportion of the blastema cells express the *tsp-4* transcript. In contrast, none of the epidermal cells express detectable levels of *tsp-4*, including those within the wound epidermis. Within the stump, intense *tsp-4* expression is evident in tendons (Fig. 4H), and connective tissues surrounding skeletal elements (Fig. 4L) throughout all regenerative stages analyzed (tendons were identified by their position connecting muscle and skeletal elements as well as by comparing the expression pattern to tendon patterns in the axolotl limb described in Holder, 1989). The blastema-cell expression begins to display a restricted pattern by three weeks post-amputation. Notably, in the earliest-regenerating digits, *tsp-4* expression appears confined to the cells surrounding the condensing chondrocytes, the developing perichondrium (Fig. 4D, 4I–K). In dorsal and ventral sections above and below the chondrocytes, *tsp-4* expression in the developing digit perichondrium is seen as a sheath (Fig. 4I), while sections taken directly through the condensing chondrocytes show *tsp-4* expression outlining the chondrocytes (Fig. 4J). Interestingly, the chondrocytes themselves, but not the perichondrium, express *tsp-1* very robustly (compare to Fig. 3H, the serial section immediately preceding section in 4J), thus providing another example of a reciprocal expression pattern between *tsp-1* and *tsp-4*. In the less-differentiated posterior digits, *tsp-4* expression is maintained at levels similar to late-bud blastemas (Fig. 4K). *Tsp-4* is also expressed in the central portion of developing skeletal elements such as the humerus (Fig. 4L).

Mesenchymal expression of *tsp-4* is regeneration-specific

A central issue in regenerative biology is whether regeneration of a body part is simply a recapitulation of development (albeit on a larger scale) or whether it is a novel process (or some combination of both). In this context, we compared *tsp-1* and *tsp-4* expression patterns during regeneration with their respective expression during the larval limb development. In axolotls, the forelimb grows from a limb bud after the animal has hatched from the egg. Larvae were harvested at various stages of development, fixed, sectioned, and subjected to

in situ hybridization for *tsp-1* and *tsp-4* (limbs were staged according to Nye et al., 2003). *Tsp-1* expression was apparent in epidermis overlying the larval limb bud similar to its expression in the regenerative wound epidermis (Fig. 5A, 5B). This indicates that *tsp-1* is not regeneration-specific, and might play a role in regeneration analogous to its function during very early limb bud development. However, *tsp-1* expression was not highly enriched specifically in the basal layer of the epidermis in later stages when the epidermis is several-cell layers thick, and it is not enriched distally, so it may also be playing a distinct role in regeneration that it does not in development in this respect. From stage 46.5 onward, condensing chondrocytes express *tsp-1* robustly (Fig 5C, 5D), consistent with its expression in regenerating limbs. In contrast to the high expression seen in the mesenchymal cells of the regenerating blastema, virtually no *tsp-4* expression is detectable in the mesenchymal cells of the early undifferentiated limb bud (Fig. 5E). Other areas of the larva included in the same section, such as the dorsal aorta, express *tsp-4*, providing an internal positive control (data not shown). The discrepancy between blastema and limb bud *tsp-4* expression represents an unusual difference between development and regeneration, and indicates that in the context of *tsp-4* the molecular programs underlying the two are not equivalent. In slightly more mature buds (stage 45), *tsp-4* expression is largely undetectable (Fig. 5F), but in more dorsal sections (Fig. 5G), a faint but distinct area of expression is seen at the posterior proximal margin of the mesenchyme. By stage 48, *tsp-4* is expressed throughout much of the distal mesenchyme (Fig. 5H), similar to mid- and late-stage blastemas. Apparent developing tendons express *tsp-4* (Fig. 5I–K) similarly to tendon progenitors seen in developing chick limb buds (Schweitzer et al., 2001). Posterior digits that develop later than anterior digits in the forelimb continue to express *tsp-4* before they are fully developed much the same way posterior areas of the regenerating autopod express *tsp-4* (Fig. 5L).

***Tsp-1* and *tsp-4* expression in blastemas does not require innervation**

Limb regeneration has been shown to be critically dependent upon innervation (reviewed in (Brookes, 1987)). A limb that has been denervated prior to amputation can form a wound epidermis and a blastema, but the blastema cannot mature and subsequent regenerative events fail to occur. Innervated left forelimbs harvested at three weeks post amputation show the predicted *tsp-1* expression pattern (Fig. 6A, 6C). Consistent with previous studies, contralateral limbs that had been denervated before amputation formed a blastema that did not elongate and did not initiate digit formation. We find that denervated limbs do express *tsp-1* appropriately in the basal layer of the wound epidermis, and that this expression persists to three weeks post-amputation when the experiment was ended (Fig. 6B, 6D). Since *tsp-4* is highly upregulated in blastema cells, and its expression persists into the regeneration stages dependent upon innervation, we sought to determine whether its expression is maintained by innervation. In contralateral, innervated limbs, *tsp-4* shows the expected mesenchymal expression (Fig. 6E, 6G). In denervated limbs, *tsp-4* is still robustly expressed throughout much of the mesenchyme, demonstrating that its expression in blastema cells is independent of innervation (Fig. 6F, 6H), and a decrease in *tsp-4* activity is not a factor in the block in regeneration effected by denervation. Interestingly, we noticed that in some of these stunted, denervated regenerates, *tsp-4* is ectopically expressed by the basal wound epidermal cells which normally do not express *tsp-4* but do express *tsp-1* (data not shown, n=1/5).

DISCUSSION

We have analyzed the expression of *thrombospondin-1* and *thrombospondin-4* during axolotl limb regeneration, and we find that both genes are dynamically expressed in a manner consistent with potential roles for supporting the regeneration program. *Tsp-1* marks a subset of the epithelium, specifically the basal layer of the wound epithelium, cells long-

speculated to play a special role in signaling to the underlying mesenchyme during regeneration. Interestingly, we find *tsp-1* to be expressed in the epithelium overlying the developing limb bud in larval hatchlings. These data suggest that *tsp-1* may be involved in instructing the formation of the limb bud and blastema, and it may play a role in maintaining these structures once they form. The juxtaposition of the basal cells of the wound epidermis with the underlying mesenchymal cells of the blastema suggests these cells may signal to the blastema cells, perhaps providing a signal that sustains the population or instructs it in some way. Consistent with this hypothesis, *tsp-1* is not expressed in the basal epidermis of mature limb tissue or in basal epidermal cells proximal to the dedifferentiation front during limb regeneration.

Robust expression of *tsp-1* transcripts in the basal layer of the early wound epidermis suggests several possible functions in limb regeneration. *Tsp* genes play important roles in mammalian wound-healing, thus an obvious candidate function for TSP-1 is the regulation of clotting and the immediate wound-healing response to amputation. It may indeed initially play such a role, however *tsp-1* expression persists in the basal epidermis and might therefore be performing additional functions. One candidate function for persistent TSP-1 in the matured wound epidermis is the negative regulation of angiogenesis. Following amputation, thrombosis-induced pathways, left unopposed, might tend to promote the sprouting of new vessels from those left on the stump. However, previous work has shown that the blastema is relatively avascular at early stages, and substantial ingrowth of large vessels occurs only after the blastema matures (Smith and Wolpert, 1975). Hence, a reasonable hypothesis is that TSP-1 in the wound epidermis might perform an anti-angiogenic function as it is known to do in other biological instances (reviewed in (Lawler, 2002). Recent work has shown that Nitric Oxide Synthetase (NOS) is enriched in the basal layer of the wound epidermis (Rao et al., 2009). While NOS catalyzes the formation of nitric oxide, which in turn promotes angiogenesis, this signaling cascade can be antagonized by TSP-1 (Isenberg et al., 2005; Ridnour et al., 2005).

Another possible function of TSP-1 secreted from the basal layer of the wound epidermis might be activation of matrix metalloproteinases. *Mmp-9* transcripts are also upregulated in the basal layer of wound epidermis in axolotl (Yang et al., 1999) and newt limbs (Vinarsky et al., 2005), and MMPs have been postulated to facilitate the breakdown of tissues required for normal regeneration while antagonizing pathways that lead to scars. TSP-1 has been previously shown to stimulate the collagenase activity of MMP-9 (Qian et al., 1997). A thick layer of collagen underlies the limb epidermis in both intact and stump samples, while there is essentially no collagen beneath the wound epidermis (JLW, data not shown). Thus, this work might intimately connect two processes required for salamander limb regeneration: thrombosis and matrix degradation.

The *tsp-4* expression patterns suggest TSP-4 is likely involved in some aspect of extracellular matrix remodeling during limb regeneration. Recently, a “transitional matrix” has been described as being at work during newt limb regeneration (Calve et al., 2010). This extracellular matrix exists transiently throughout the blastema, and its extent is refined as regeneration proceeds, similar to the *tsp-4* expression domains. One proposed function for the transitional matrix is to infiltrate stump tissues such as muscle, facilitating their breakdown and entry into the blastema, and sustaining their less-differentiated state once there (Calve et al., 2010). Transitional matrix markers include Fibronectin and Tenascin (Calve et al., 2010). Interestingly, earlier work on Tenascin in newt regeneration revealed its expression to be virtually identical to the expression reported here for *tsp-4* (Onda et al., 1990). Thus, we postulate that *tsp-4* is a novel marker of the transitional matrix. Future experimentation will elucidate any potential role for *tsp-4* in the establishment or maintenance of the blastema. Should *tsp-4* or *tsp-1* play a functional role in any of these

processes, their apparent nerve-independence will need to be explored in more detail as other factors controlling blastema growth, such as newt Anterior Gradient protein, have been shown to be nerve-dependent (Kumar et al., 2007). This study also highlights an analogous expression between the single invertebrate *thrombospondin* and *tsp-4*. In *Drosophila*, *thrombospondin* is also expressed in tendon progenitors (Adams et al., 2003). This similarity in expression, and perhaps in function, is intriguing as the vertebrate and invertebrate tendons are not believed to be evolutionarily homologous tissues, and hence the thrombospondins appear to have been independently recruited in the two cases, perhaps for the same purpose. This could have occurred by cooption of an established regulatory cassette. In *Drosophila*, expression in the developing embryo at segment boundaries is downstream of Hedgehog signaling (Chanana et al., 2007). Regenerating limbs express Sonic hedgehog from their posterior margins just as limb buds do, and perhaps the persistent expression of *tsp-4* in the posterior region of the blastema as digits regenerate might be dependent on Shh.

Future work will be necessary to distinguish between these and other potential roles of Thrombospondins in limb regeneration. A foremost consideration is whether or not Thrombospondins are required for urodele limb regeneration. If they are required, identifying molecules that interact with TSP-1 and TSP-4 in the regenerating limb could reveal additional novel players in this process. Structure/function analysis might reveal whether any of the sequence differences highlighted in this work modulate the binding of TSP-1 or TSP-4 to their putative interacting proteins, such as heparin, and whether these interactions impinge upon regeneration. It will be important to determine if *tsp-1/tsp-4* are expressed and required in other regenerating axolotl tissues. Moreover, it will be interesting to investigate the expression of these molecules in regenerating tissues of non-urodeles such as *Xenopus* tadpole tails and legs or mouse digit tips. Further experimentation might also uncover the roles Thrombospondins play in contexts where regeneration does not occur following amputation, and how Thrombospondins might link to scarring pathways in these situations.

EXPERIMENTAL PROCEDURES

Animal Husbandry

All axolotl experimentation was performed in accordance with Harvard University's IACUC. Axolotls were bred in-house or obtained from the Ambystoma Genetic Stock Center, Lexington, KY. All axolotls measured 4–5 cm snout-to-tail unless otherwise specified and were white or albino mutants. Axolotls in figure 2 measured approximately 7 cm in length.

Amputations and blastema collections

All amputations were performed while axolotls were anesthetized with 0.1% tricaine. Animals were amputated at the mid-humerus level unless otherwise specified using a pair of fine dissecting scissors. Following amputation, exposed bone was trimmed. Blastemas were collected in Trizol (for RNA purification) or 4% PFA in DEPC-treated PBS (for histology). Denervation was performed immediately following amputation by snipping the nerves at the base of the limb with fine dissecting scissors and removing them through the amputation site using forceps.

cDNA synthesis

Total RNA was purified by Trizol/chloroform extraction. First-strand cDNA was prepared using random hexamers and oligo-dT as primers and Roche Transcriptor polymerase.

Cloning axolotl thrombospondins

Thrombospondin cDNA fragments were initially cloned by degenerate PCR from mixed-stage regenerating limb preparations. The original *tsp-4* fragment was amplified using primers 5'-CGGCTGCATCCGGAAYTNTAYAT-3' and 5'-TGGCAGGCGGCGTANCCYTGYTG-3', and the original *tsp-1* fragment using 5'-CGACGAGGACGGCCAYCARAAYAA-3' and 5'-CACTCGTACTTTCATGTTCGAGAAARAANACCAT-3'. The *tsp-3* fragment was recovered using primers 5'-GGCGACGCCTGCGAYAAITGYCC-3' and 5'-CGTCGGTACGGTGTTCACRTGRAANGT-3'. Initial PCR products were subcloned into pGEM-T-easy (Promega) and sequenced. 5' RACE (Invitrogen) was used to amplify the most 5' ends of the ORFs. The 3' end of the *tsp-1* ORF was recovered by probing a cDNA library (Stratagene) with a previously recovered fragment. The Oligo(dT) primed axolotl cDNA library (Salamander Larvae Lambda cDNA Library, Stratagene cat #937670) was screened by plaque lift hybridization with a radiolabeled annealed overlapping oligo probe to *tsp1* (*tsp1*_cDNA-OV a GTTGTAAATGCACGAGGGCAAGAAG, *tsp1*_cDNA-OVb CAGAGTCTGCCATGATCTTCTTGC). Recovery of the *tsp1* positive clone was followed by in vivo excision of the phagemid from the Uni-ZAP XR vector into pBluescript for DNA sequencing. The 3' end of the *tsp-4* ORF was recovered using a forward primer (5'-TGGATTATATTTGGCTATCA-3') in a previously identified fragment and a reverse, partially degenerate oligo-dT primer anchored in the polyA tail (5'-TTTTTTTTTTTTTTTTTTTTTTTTNNNN-3'). Full-length *tsp-4* cDNA was recovered from a single animal blastema preparation via Phusion polymerase with primers 5'-GCGGCCGCGCCACCATGCTGCTGCCCGGAAGGAACG-3' and 5'-GCGGCCGCTTACTCGCTCTCACTGCCGAAGT-3'. Full-length *tsp-1* cDNA was recovered from a single animal wound epidermis preparation via Phusion polymerase with primers 5'-GCGGCCGCGCCACCATGGCCTTGTGTTGGGGGGCTCTTTC-3' and 5'-GCGGCCGCTTATGTATCTCTGCACTCATATTTGAGATCCGAGA-3'. Full cDNAs were subcloned into pGEM-T-easy and sequenced.

Protein alignments

Sequences with the following accession numbers were used to generate the alignment data. For TSP-1, *X. tropicalis*: XP_002937245; *D. rerio*: XP_690395; *H. sapiens*: NP_003237; *M. musculus*: NP_035710. For TSP-4, *X. tropicalis*: NP_001072673; *D. rerio*: NP_775333; *H. sapiens*: NP_003239; *M. musculus*: NP_035712. Alignments were created with AlignX (Vector NTI), using default settings to create a similarity plot based on a ClustalW algorithm.

In situ hybridization

In situ probes were generated using the initial clones in pGEM by PCR with T7 and Sp6 primers followed by sequencing and transcription with Sp6 or T7 polymerase (Roche). Probes span bases 2636–3395 in the *tsp-1* cDNA sequence, 797–1649 in the *tsp-4* cDNA sequence, and the entire *tsp-3* cDNA sequence fragment that was recovered. Amputations were performed on axolotl forelimbs at mid-humerus, and tissue was harvested at 24 hours, 1 week, 2 weeks, 3 weeks, and 4 weeks post-amputation. Intact axolotl limb tissue was also harvested. Fixed tissues were sectioned and subjected to in situ hybridization. Section in situ hybridization was performed as described in (Murtaugh et al., 2001) with the following modifications. Limbs were fixed for 1 hr 45 min at room temperature, and sections were cut at 16-µm thickness.

qRT-PCR

Total RNA was prepared by Trizol/chloroform extraction from intact forelimb skin and from wound epidermis at 60 hrs post-amputation. Samples were normalized for total RNA and cDNA was synthesized as described above. qRT-PCR was performed using a Roche LightCycler 2.0 machine, reagents, and software. GAPDH (used for normalization) primers were 5'-GACGCTGGTGCAGGCATTGCC-3' and 5'-ACCATCAGGTCCACAACACGCTGAC-3'; Tsp1 primers were 5'-CGCCGAGCACCTGCGCAATG 3' and 5' CAGCCTGTGTGACGTGGATCGTG-3'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Thrombospondin-1

Thrombospondin-4

Fig. 1.

Alignment of axolotl TSP-1 and TSP-4 with other vertebrate TSPs. Alignment insets: species code: Am, *Ambystoma mexicanum*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*; Hs: *Homo sapiens*; Mm: *Mus musculus*. Shading code: black: identical across all species; dark grey: conservative substitutions; light grey: similar amino acids; white: no identity; dash: introduced gap. **A:** Domain structure of TSP-1. **B:** An identity plot of the ClustalW alignment of axolotl TSP-1 with other selected vertebrates shows the proteins are very highly conserved. Selected divergent sequences are shown in insets below plot. **C:** Domain structure of TSP-4. **D:** Identity plot of axolotl TSP-4 with other selected vertebrates shows the proteins are highly conserved. Selected divergent sequences are shown as in B.

Fig. 2.

Expression of *tsp-1* and *tsp-4* in intact axolotl limbs. Section in situ hybridization was performed on intact juvenile limbs. Panels A and B are longitudinal sections, anterior at top; C-L are transverse sections, dorsal at top. Magnifications: 5x: A, B, G, H; 10x: C-F; 20x: I-L. **A:** *Tsp-1* is expressed in digit chondrocytes, but is not detectable in the developing ossification centers (oc) or joints (j). **B:** *Tsp-4* is expressed in connective tissue between muscle and skin (arrowhead), but is not detectable in cartilage. **C:** Transverse section through an ossification center within a digit. *Tsp-1* is not expressed within the ossification center (asterisk), but is expressed in some periosteum cells (arrowheads) and in dorsal and ventral tendons (arrows). **D:** Transverse section through a cartilaginous portion of a digit. *Tsp-1* is expressed in chondrocytes (asterisk), some perichondrial cells (arrowheads), and in dorsal and ventral tendons (arrows). **E:** Serial section immediately proximal to (C) showing *tsp-4* is expressed in connective tissue just outside the periosteum (arrowheads) and in dorsal and ventral tendons (arrows). *Tsp-4* is not detectable in the ossification center (asterisk). **F:** Serial section immediately proximal to (D) showing *tsp-4* is expressed in perichondrium and associated connective tissue (arrowheads) and in dorsal and ventral tendons. **G-H:** *Tsp-1* and *tsp-4* expression in metacarpal-level sections. **I-L:** Magnified views of selected points from G and H. **I:** *Tsp-1* is expressed in chondrocytes and perichondrium. **J:** *Tsp-1* is expressed in ventral tendons. **K:** *Tsp-4* is expressed in perichondrium, associated connective tissue, and tendons (arrowhead). Muscle is denoted by "m." **L:** *Tsp-4* is robustly expressed in ventral tendons.

Fig. 3.

Expression of *tsp-1* in regenerating axolotl limbs. Section in situ hybridization for *tsp-1* was performed at various time points following amputation. Amputations were performed at mid-humerus level unless otherwise noted. Low-magnification (5x) views for each time point are shown in A–D. Higher magnification views are shown of inset areas (10x in G, 20x in all others). Anterior at left, posterior at right, distal at top. **A, E:** At 24 hours post-amputation, *tsp-1* expressing cells can be seen covering the stump in the newly-grown wound epidermis (WE) (A, E, arrowheads). High levels of expression can also be seen among scattered stump cells, especially toward the distal end (A). **B, F:** The *tsp-1* expression persists in the basal layer of the WE at one week post-amputation (arrowheads). **C, G:** Limb regenerating from a mid-radius/ulna amputation. At two weeks post-amputation, wound epidermal expression persists but is waning (compare arrowhead to arrow in G). **D, H–L:** By three weeks post-amputation, chondrocytes in the regenerate begin to condense while expressing *tsp-1*. **H:** Maturing digits express *tsp-1* within condensing chondrocytes. **I:** Less mature digits located posteriorly continue to express *tsp-1* in scattered blastema cells. **J:** *Tsp-1* expression within the basal cells of the epidermis is still detectable along the posterior edge. **K:** The distal end of the regenerating humerus expresses *tsp-1* in the proliferating chondrocytes. **L:** An adjacent section shows robust *tsp-1* expression within the central portion of the cartilage of the regenerating humerus, just distal to the amputation plane.

Fig. 4.

Expression of *tsp-4* in regenerating limbs. Section in situ hybridization was performed at various time points following amputation at mid-humerus level. Low-magnification (5x) views are shown in A–D. 20x views are shown of inset areas. Anterior at left, posterior at right, distal at top. **A, E:** At 24 hours post-amputation, the wound epidermis (WE) has formed, but WE cells do not express *tsp-4*. **B, F:** At one week post-amputation, an early blastema expressing *tsp-4* has formed. Note that *tsp-4* expression is throughout the blastema mesenchyme but is excluded from the wound epidermis. **C, G:** At two weeks post-amputation, *tsp-4* expression is intense throughout the blastema. **H:** At two weeks post-amputation, some tendons on the stump express *tsp-4*. **H–D, I–L:** At three weeks post-amputation, *tsp-4* expression in the regenerate begins to become restricted to the developing perichondrium in earliest-forming digits. **D:** Three weeks post-amputation, 5x view, insets I–K outlined. Arrowheads denote connective tissue spanning length of limb. **I:** *Tsp-4* expression in perichondrium overlying first digit. **J:** *Tsp-4* expression in perichondrium in a section straight through chondrocyte layer (compare to Fig. 3H). **K:** Less mature digits along posterior edge continue to express *tsp-4* throughout mesenchyme (compare to Fig. 3I). **L:** Regenerating cartilage expresses *tsp-4* in the central portion.

Fig. 5.

Expression of *tsp-1* and *tsp-4* in developing larval limbs. Panels A, B, E, and F are 20x magnification. All other panels are 10x magnification. **A:** *Tsp-1* is expressed in the epidermis overlying the entire early limb bud, stage 44. Arrowheads denote examples of epidermal cells expressing *tsp-1*. Torso was sectioned coronally, anterior/posterior axis denoted. **B:** A more anterior section of the same limb bud as (A), showing robust expression of *tsp-1* throughout the epidermis (e). **C:** *Tsp-1* expression intensifies within condensing chondrocytes (c) as identified by their shape and position within the limb in stage 46.5. **D:** Chondrocytic expression of *tsp-1* in the developing humerus (h) and radius/ulna (r/u), stage 50. **E:** *Tsp-4* expression is not detectable above background levels in larval limb bud mesenchyme sectioned transversely. **F:** In ventral coronal sections, *tsp-4* is not detectable at stage 45. **G:** In more dorsal coronal sections, *tsp-4* expression begins to be detectable near the base of the limb bud outgrowth, in the proximal posterior region (arrowhead). **H:** *Tsp-4* expression in the distal mesenchyme, stage 48. **I–K:** Skeletal condensations outlined with dashed lines. **I:** *Tsp-4* expression in ventral mesenchyme suggests tendon progenitors. **J:** Stage 51, distal limb, transverse section, showing ventral expression of *tsp-4* in developing tendons. **K:** Stage 51, more proximal transverse section, showing *tsp-4* expression consistent with tendon. **L:** Stage 52: *Tsp-4* expression persists within the mesenchyme of the less-developed, posterior digits (arrowhead).

Fig. 6.

Expression of *tsp-1* and *tsp-4* is independent of innervation. Forelimbs were amputated bilaterally proximally, leaving only 1 mm stump. Right limbs were denervated, while left limbs were not. Limbs were harvested three weeks later and subjected to in situ hybridization. Magnifications: A, B, E, F: 5x; C, D, G, H: 20x. **A:** The control limb is regenerating normally and shows basal-layer expression in the wound epidermis characteristic of *tsp-1*. **B:** *Tsp-1* is expressed in the basal layer of the epidermis in denervated limbs whose regeneration has stopped progressing. **C:** Area outlined in (A) showing robust *tsp-1* expression in control basal layer of epidermis. **D:** Area outlined in (B) showing *tsp-1* is still enriched in basal epidermal cells in denervated limbs. **E:** The control limb shows normal *tsp-4* expression. **F:** In denervated limbs, *tsp-4* is expressed throughout the stunted blastema, similar to its expression in normal regenerates at earlier time points. **G:** Area outlined in (E) showing distal, anterior portion of control limb. **H:** Area outlined in (F) showing robust expression of *tsp-4* in mesenchyme of denervated, stunted regenerate.