Thrombospondin 2 Modulates Collagen Fibrillogenesis and Angiogenesis

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Thrombospondin 2 (TSP2)-null mice, generated by targeted disruption of the *Thbs2* gene, display a complex phenotype that is characterized, in part, by a variety of connective tissue abnormalities and increased vascular density in skin and subcutaneous tissues. In this paper we summarize the evidence that TSP2 functions as a matricellular protein to influence cell function by modulating cell–matrix interactions, rather than acting as an integral component of the matrix. Thus, the structurally abnormal collagen fibrils detected in skin appear to be the consequence of the defective adhesion demonstrated by dermal fibroblasts in culture that, in turn, result from increased matrix metalloproteinase 2 (MMP2, gelatinase A) production by these cells. Corroborating evidence for such a mode of action comes from transmission electron microscopic images of developing flexor muscle tendons that show distinct abnormalities in fibroblast–collagen fibril interactions in TSP2-null tissue. The increased vascular density seen in skin of TSP2-null mice can be reproduced in a number of models of injury, including subcutaneous implantation of polyvinyl alcohol sponges and silicone rubber discs, and excisional skin wounds. Experiments are proposed to distinguish between a primarily endothelial cell *versus* an extracellular matrix origin for the increased angiogenesis in TSP2-null mice. *Key words: cell adhesion/cell–matrix interactions/extracellular matrix/matricellular protein/matrix metalloproteinase. Journal of Investigative Dermatology Symposium Proceedings 5:61–66, 2000*

Thrombospondin (TSP) 2 is a member of a group of functionally related, extracellular matrix (ECM) proteins that also includes TSP1, osteopontin, tenascin C, SPARC, and probably other proteins. These proteins do not subserve a primary structural role, in the sense that most collagens, elastins, and laminins are structural proteins. Matricellular proteins, however, interact with structural proteins and heparan sulfate proteoglycans, as well as with cytokines, proteases, and cell-surface receptors to function contextually as adaptors and modulators of cell–matrix interactions (Bornstein, 1995). The complex nature of their function derives from their ability to interact with multiple cell-surface receptors, to bind and activate cytokines, to inhibit the effects of growth factors, and to serve as competitive inhibitors of proteases. The contextual nature of this function thus reflects the composition of the matrix, the availability of cytokines and proteases, and the expression of integrins and other receptors in a given cellular environment (Bornstein, 1995). An additional level of complexity results from the ability of some matricellular proteins to undergo limited proteolytic cleavage to reveal cryptic functions, e.g., SPARC (Sage, 1997) and osteopontin (Smith *et al*, 1996). Whereas we believe that the conceptual distinctions between structural and matricellular proteins are useful, they should not be considered to be absolute. Thus, it is clear that structural proteins such as collagens and laminins can influence cell function by interacting with integrin receptors and activating cell-signaling pathways. On the other hand, in some circumstances, matricellular proteins may affect the structure of the matrix directly, e.g., the ability of osteopontin to inhibit calcification in smooth muscle cell cultures (Wada *et al*, 1999), and possibly in soft tissues *in vivo*.

In view of the structural similarity between TSP2 and TSP1 (Bornstein and Sage, 1994), it is likely that many of the macromolecular interactions that have been established for TSP1 (Frazier, 1991; Bornstein, 1995) also apply to TSP2. Indeed, TSP2, like TSP1, has been shown to bind heparan sulfate proteoglycans, low-density lipoprotein-related receptor protein, and the integrin $\alpha v\beta 3$ (Chen *et al*, 1994, 1996). TSP2 has also been shown to inhibit the angiogenic activity of bFGF in a corneal assay (Volpert *et al*, 1995), and mitogenesis and formation of focal adhesions in bovine aortic endothelial cells (Murphy-Ullrich *et al*, 1993; Panetti *et al*, 1997). In the main, however, the functional properties of TSP2 *in vivo* have not been elucidated.

In an attempt to gain a better understanding of the biologic role of this matricellular protein, TSP2-null mice were generated by disruption of the *Thbs*2 gene in murine embryonic stem (ES) cells, and the mutant ES cells were used to generate TSP2 knockout mice by use of now well-established techniques (Koller and Smithies, 1992). Mice that lack TSP2 develop a complex phenotype characterized by abnormalities in connective tissues, changes in fibroblast adhesion, increased endosteal bone growth, an increase in vascular density, and a bleeding diathesis (Kyriakides *et al*, 1998a). In this paper we describe the changes in collagen fibril structure and the increased angiogenesis that have been found in

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Abbreviations: TSP, thrombospondin; EC, endothelial cells; ECM, extracellular matrix.

TSP2 knockout mice, and discuss the possible mechanisms that might be responsible for these changes. Our findings support the concept that the functions of TSP2 do not result from its physical presence in the ECM, but rather derive from the modulation of cell–matrix interactions and cell function by the protein.

RESULTS AND DISCUSSION

Collagen fibrillogenesis Previously published findings indicated that the skin of TSP2-null mice was fragile and had reduced tensile strength, and histologic analysis of skin showed that collagen fibers were disorganized and lacked the normal, predominantly parallel orientation to the epidermal surface (Kyriakides *et al*, 1998a). In addition, electron microscopic examination revealed the presence of abnormally large collagen fibrils with irregular contours in tissues from mutant mice (Kyriakides *et al*, 1998a). These findings could result from a role of TSP2 as a collagen fibril-associated protein, because similar findings had been reported in mice with deficiencies of the fibril-associated proteins type V collagen, decorin, and fibromodulin (Andrikopoulos *et al*, 1995; Danielson *et al*, 1997; Svensson *et al*, 1999). The presence of TSP2 as an integral component of collagen fibrils, however, could not be documented by light microscopic immunohistochemistry in either the developing or the adult mouse (Kyriakides *et al*, 1998b).

If TSP2 does not affect collagen fibrillogenesis by its physical presence in fibrils, as suggested by the matricellular hypothesis, the protein could still do so by influencing fibroblast function and cell–matrix interactions, or the function of another cell that secondarily affects fibroblast function. Alternatively, TSP2 could serve as a nucleation site for initial assembly of fibrils, either at the cell surface or by interaction with procollagen during the secretory process.

It has been shown by transmission electron microscopy that fibroblasts compartmentalize the pericellular space by extension of long cellular processes. Nascent collagen fibrils subsequently assemble in the resulting extracellular compartments, in close association with the cell surface (Birk and Trelstad, 1986; Birk and Linsenmayer 1994). It seems likely that cells interact with the fibril during this process and that such interactions are compromised in TSP2-null mice. Indirect evidence for a mode of action at the level of cell–matrix interactions was obtained by examination of tendons from early postnatal control and TSP2-null mice. In these experiments, the hind limb flexor tendons from postnatal day 4 and day 8 TSP2-null and wild-type (WT) littermates were cut in cross section and examined by transmission electron microscopy. This analysis of matrix development demonstrated that fibril-forming channels, fiber-forming compartments, and larger fibroblast-defined compartments were well defined at postnatal day 4 (**Fig 1**). The structure of the fibril-forming channels was unaltered in TSP2-null mice relative to WT controls; however, the fiber-forming compartments and larger, fibroblast-defined compartments were significantly less organized in the TSP2-null mice. The long

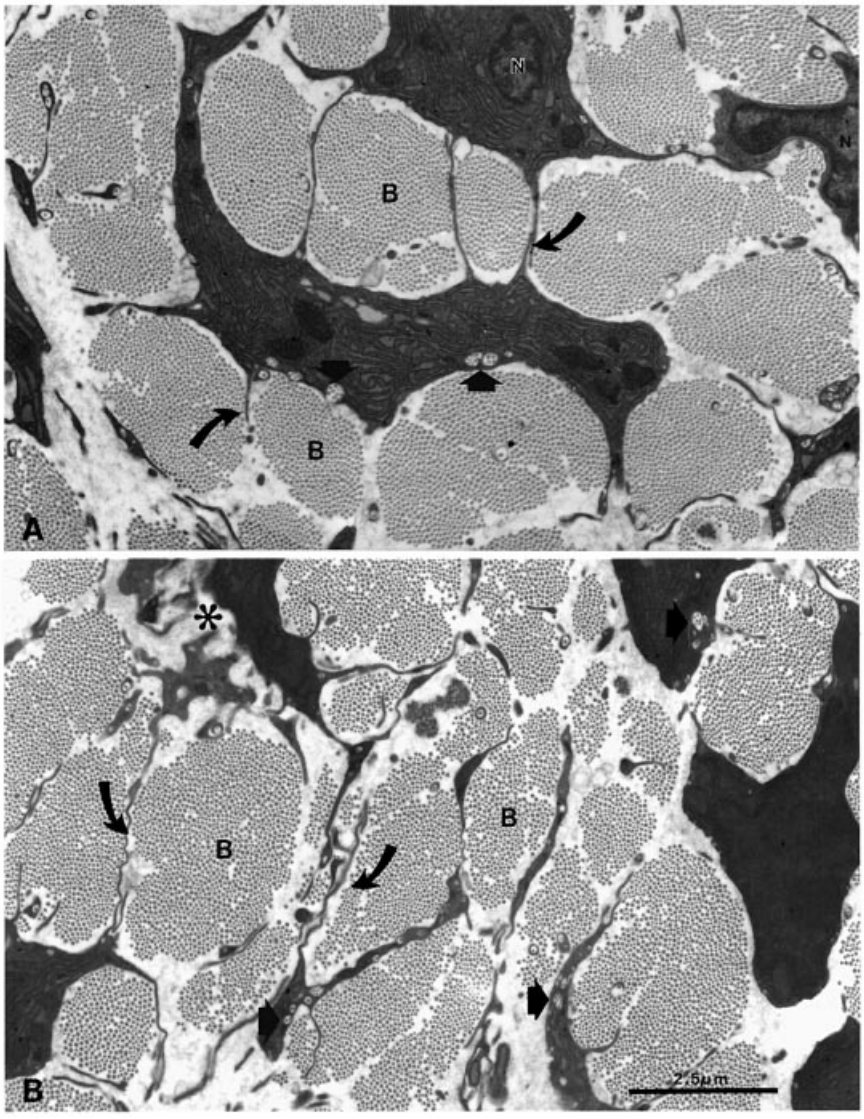

Figure 1. Compartmentalization of the developing tendon matrix. Hind limb flexor tendons from postnatal day 4 littermates were cut in cross-section and examined by transmission electron microscopy. Wild-type (*A*) and TSP2-null (*B*) tendons show a compartmentalization of the extracellular space. Both show extracytoplasmic channels (*arrowheads*) where fibrils assemble and a second compartment where fibrils coalesce into fibril bundles/small fibers (labeled B). As development proceeds the cytoplasmic processes defining these compartments retract (*curved arrows*) and larger fibers characteristic of the mature tendon form. The latter two levels of compartmentalization are disrupted in tendons from TSP2-null mice. Thus, the cytoplasmic processes defining developing fibers are less regular in TSP2-null tendon. The orientation of the fibers and fibril packing within the fibers are also less regular in mutant tendons. Abnormal fibroblasts that are apparently apoptotic (*) are often seen in fields of normal-appearing tendon fibroblasts in TSP-2 null tendons. Such cells are rarely seen in control tissue. N, nucleus. Section stained with uranyl acetate and phosphotungstic acid. *Scale bar*: 2.5 μm.

cytoplasmic processes that define these compartments were less regular in orientation and less extended in the TSP2-null mice (**Fig 1**). This disruption of cellular compartmentalization of the extracellular matrix was associated with fiber disorganization in the mutant mice, relative to WT controls. Fibers had regular orientation in the normal mice, but this was partially lost in the mutant. Collagen fibril packing, within the fibers, was less regular in the TSP2-null tendon, and the fibers from mutant mice had more spaces than in the wild type (**Fig 2**). Even at these early stages of development, there was a tendency toward larger diameter fibrils in the TSP2-null tissue, but this was not as dramatic as seen in adult tissues (Kyriakides *et al*, 1998a).

During these early developmental periods, fibroblasts and their associated matrix normally begin the processes involved in tendon fascicle development. These cell–cell and cell–matrix interactions are apparently abnormal in the TSP2-null tendons because fascicle formation was disrupted relative to the WT controls (**Fig 2**). In addition to these changes, fibroblasts that appear histologically to be apoptotic were frequently observed in fields of normal fibroblasts in the TSP2-null mice (**Figs 1**, **2**). Such cells were virtually absent from normal tendons.

Cell adhesion Additional, more direct evidence for a cellular etiology for the abnormal collagen fibril structure in TSP2-null mice was obtained by examining the functions of dermal fibroblasts in culture. TSP2-null dermal fibroblasts were found to aggregate more readily in monolayer cultures and were more sensitive to trypsinization from tissue culture plastic than were control cells (Kyriakides *et al*, 1998a). These findings suggested that TSP2-null cells have a defect in cell–matrix interactions, and ran counter to expectations, because TSP2 had been shown to destabilize focal adhesions in endothelial cells *in vitro* (Murphy-Ullrich *et al*, 1993). We have now characterized the fibroblast adhesive defect in greater detail. In this study, cell attachment assays were performed in the absence of serum and the behavior of dermal fibroblasts, derived from both WT and TSP2-null mice, was compared. On tissue culture plastic, attachment of TSP2-null cells was significantly decreased compared with normal cells. Coating of the surface with fibronectin increased the attachment of both WT and TSP2-null cells, but did not completely correct the adhesive defect of TSP2-null cells (**Fig 3*A***). Similar results were observed with vitronectin and type I collagen (data not shown), as well as with TSP2 itself (**Fig 3*B***).

The failure to correct the attachment defect of TSP2-null fibroblasts in culture by addition of TSP2 to the substratum raised the possibility that this defect was a secondary consequence of the TSP2-deficient state. Stable transfections of dermal fibroblasts with TSP2 cDNA were attempted, but were unsuccessful because selected cells soon became senescent, or occasionally became transformed. We therefore generated TSP2-deficient mice that carried the conditionally immortalizing SV40 large T antigen, *H*-2k^b-tsA58, as a transgene by crossing TSP2-null mice with

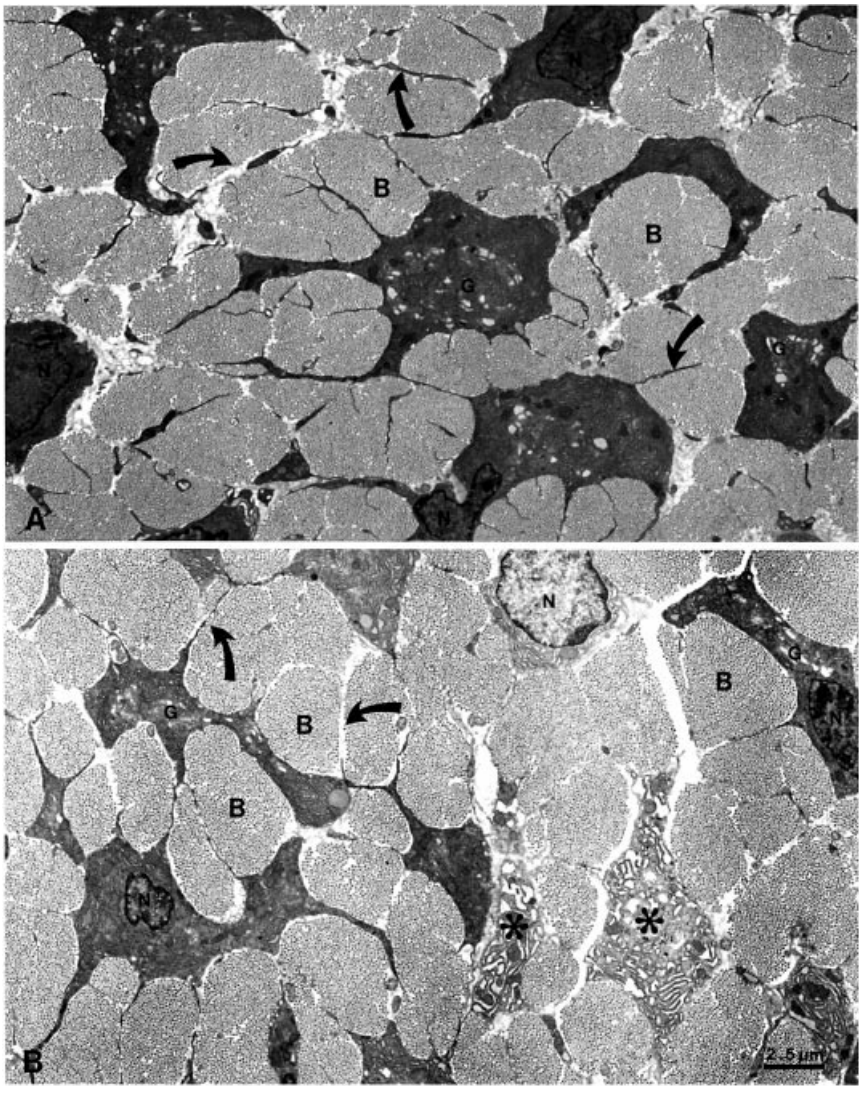

Figure 2. Fibroblast interactions involved in tendon fascicle and fiber development. Transmission electron micrographs of cross-sections of hind limb flexor tendons from postnatal day 8 littermates. Wild-type (*A*) and TSP2-null (*B*) tendons. As tendon development proceeds, TSP-2 null mice continue to show disorganization of fibroblasts and fibers. The cytoplasmic processes defining compartments are less prominent and more disorganized in mutant tendons. The fibers and packing of fibrils within the fibers also are less organized in mutant tendons relative to WT controls. Abnormal fibroblasts that are apparently apoptotic (*) are often seen in fields of normal-appearing tendon fibroblasts in TSP-2 null tendons, but are virtually absent from control tissue. B, fibers or fibril-bundles; curved arrows, cytoplasmic processes defining the fiber-forming compartments; N, nucleus; G, Golgi. Stained with uranyl acetate and phosphotungstic acid. *Scale bar*: 2.5 μm.

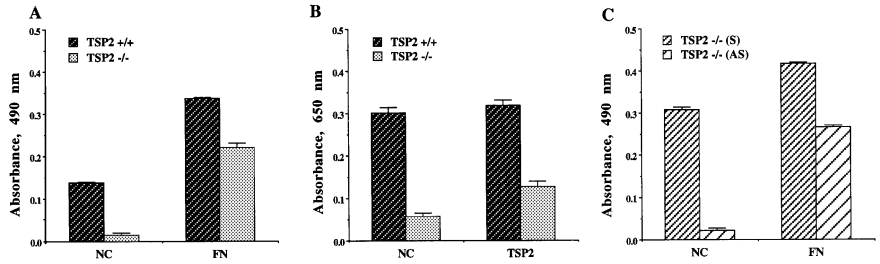

Figure 3. Fibroblast attachment assays. (*A*) Comparison of attachment of WT and TSP2-null skin fibroblasts. Skin fibroblasts were suspended in serum-free medium and allowed to attach to uncoated (NC), or to fibronectin (FN, 5 μg/ml)-coated tissue culture plastic surfaces for 60 min at 37°C. TSP2-null fibroblasts showed a defect in adhesion to tissue culture plastic. Coating of tissue culture plates with fibronectin did not completely correct the attachment defect. Attached cells were determined by the Cell Titer 96 Assay. The differences are significant ($p < 0.005$). (*B*) Coating of tissue culture plastic with TSP2 (5 μg/ml) does not correct the attachment defect in TSP2-null cells. Experiments were performed as described in (*A*) but attachment was quantified by colorimetric measurement of cell-adsorbed methylene blue. (*C*) Rescue of the adhesive defect in TSP2-null dermal fibroblasts by transfection with mTSP2 cDNA. Immortalized dermal fibroblasts derived from TSP2-null mice were transfected with sense and antisense mTSP2 cDNA expression vectors. The adhesive defect of TSP2-null fibroblasts was corrected by transfection with sense mTSP2 cDNA (S), but not with antisense mTSP2 cDNA (AS). Experiments were performed as described in (*A*).

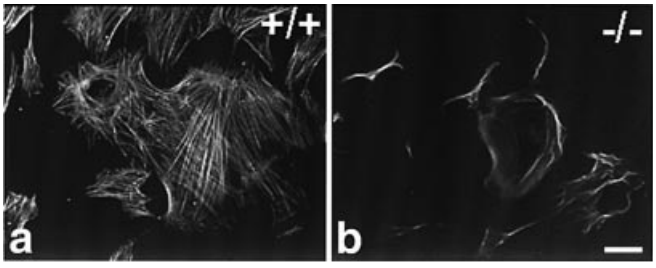

Figure 4. Cytoskeletal morphology of fibroblasts *in vitro*. Skin fibroblasts from control (left) and TSP2-null (right) mice were plated on chamber slides for 24 h in the presence of serum. The cytoskeleton was visualized by staining with phalloidin. Control cells appeared well spread, with clearly defined stress fibers, whereas mutant cells showed little spreading and a peripheral deposition of actin. *Scale bar*: 100 μm.

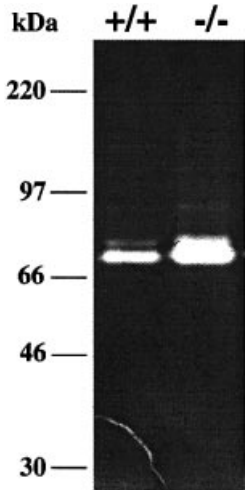

Figure 5. Zymography of conditioned media from mouse dermal fibroblasts. Serum-free conditioned media from mouse dermal fibroblast cultures were applied to SDS-PAGE/0.1% gelatin under nonreducing conditions for zymographic analysis. A 72-kDa gelatinase (MMP2) was found in the conditioned media of mouse dermal fibroblasts. The gelatinolytic activity was significantly increased in media from TSP2–/– fibroblasts compared with those from TSP2+/+ cells. Markers of molecular mass are indicated on the left margin.

transgenic Immortomice (Jat *et al*, 1991). Dermal fibroblasts derived from these mice could be grown indefinitely at the permissive temperature of 33°C, but acquired normal characteristics when grown at 37°C for a few days. The attachment of non-transfected, immortalized TSP2-null fibroblasts resembled that of nonimmortalized TSP2-null cells and was reduced relative to WT cells (data not shown). Thus, at least as regards attachment, we can assume that immortalization with SV40 large T antigen, followed by culture of cells under nonpermissive conditions, does not change the properties of these cells. Cell attachment assays showed that transfection with TSP2 cDNA rescued the adhesive defect of immortalized TSP2-null fibroblasts, whereas the attachment of cells transfected with antisense TSP2 cDNA was comparable with that of nontransfected TSP2-null fibroblasts (**Fig 3*C***).

During the course of attachment assays, and even after replating TSP2-null fibroblasts, it was noted that these cells spread far more slowly than normal cells. When dermal fibroblasts were plated on chamber slides in the presence of serum and stained with phalloidin, WT cells appeared well spread with clearly defined actin-containing stress fibers, whereas TSP2-null cells showed little spreading and a peripheral deposition of actin (**Fig 4**). Thus, the morphology of the actin cytoskeleton in TSP2-null fibroblasts is abnormal, a finding that is indicative of compromised cell–matrix interactions and establishes that these cells are defective in cell spreading as well as in attachment. The morphology of the actin cytoskeleton reverted to normal in TSP2-null cells that had been stably transfected with sense TSP2 cDNA (data not shown), a finding that supports our conclusion that the spreading defect in TSP2-null cells results from a lack of TSP2.

Recent studies have shown that TSP2-null fibroblasts in culture produce twice as much matrix metalloproteinase 2 (MMP2) protein as do normal fibroblasts (**Fig 5**; Yang *et al*, 2000). Importantly, the increased MMP2 observed in the conditioned media of TSP2-null cells was reduced to a level approaching that in conditioned media from WT cells by transfection with TSP2 cDNA. Quantification of the gelatinolytic activity in conditioned media of TSP2-transfected TSP2-null cells indicated that 76% of the difference between the activities of WT and TSP2-null cells had been restored by replacement of the TSP2 cDNA gene. Furthermore, the attachment of TSP2-transfected cells was restored to normal levels (Yang *et al*, 2000). These findings suggest that increased metalloproteinase activity contributes to the attachment defect in TSP2-null cells and, possibly, to the abnormal collagen fibrillogenesis in TSP2-null mice. TSP2-null fibroblasts were also found, by FACS analysis, to display decreased levels of α1 and increased levels of β1 integrins on their cell surfaces (Yang *et al*, unpublished observations), but the functional significance of these changes is not known because at least the level of α1 integrin did not revert to normal in TSP2-transfected cells.

Angiogenesis TSP2, like TSP1, was shown to be antiangiogenic in cell migration and rodent corneal assays (Volpert *et al*, 1995). This function was confirmed in TSP2-deficient mice in which it

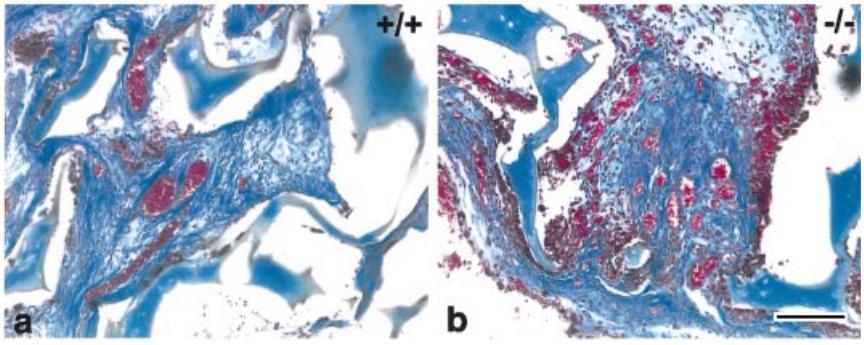

Figure 6. Vascular density of the fibroplastic reaction to polyvinyl alcohol sponges. Sponges were implanted subcutaneously into a WT(+/+) and a TSP2-null(–/–) mouse and were harvested 21 days later. Sections were stained with the Masson Trichrome stain. A significant increase in vascularity and fibroplasia is apparent in the sponge removed from the TSP2-null mouse. *Scale bar*: 100 μm.

could be shown that a number of tissues, including dermis, adipose tissue, and thymus, contained a significantly increased density of small blood vessels (Kyriakides *et al*, 1998a). Because vascular density increased with age when embryonic, neonatal, and adult tissues were compared, it was of interest to determine whether an increase in vascularization could also be demonstrated in newly formed tissue in adult animals. For this purpose, polyvinyl alcohol sponges were inserted subcutaneously in the backs of normal control and TSP2-null mice and removed 3 weeks later. On gross examination, sponges from TSP2-null mice were clearly more vascularized than control sponges, and this impression was confirmed by histologic examination, which shows that the number of blood vessels invading the fibroplastic tissue in the interstices of the sponge is increased (**Fig 6**). Similar results were recently obtained in studies of the foreign body reaction and in wound healing experiments with TSP2-null mice. Thus, the vascular density of the foreign body capsule surrounding subcutaneous silicone rubber implants was six times greater in mutant than in WT mice (Kyriakides *et al*, 1999a), and the vascularity of resorbing granulation tissue in full thickness excisional skin wounds was also significantly increased in mutant mice (Kyriakides *et al*, 1999b).

There are several possible explanations for the angiogenic phenotype of TSP2-deficient mice. Dawson *et al* (1997) have shown that the type I repeats in TSP1 interact with the CD36 receptor on endothelial cells (EC) and that this interaction is likely to participate in the antiangiogenic activity of TSP1. In view of the homology between TSP1 and TSP2, similar interactions might mediate the antiangiogenic function of TSP2; however, the functional consequences of activation of the CD36 receptor are not known, and other cell-surface receptors could be involved. For example, Gao *et al* (1996) suggest that the concerted interaction of TSP1 with integrin αvβ3 and integrin-associated protein (IAP; CD 47) could modulate endothelial cell properties such as cell spreading, migration, and chemotaxis, and the binding sites for these receptors are preserved in TSP2. If a lack of normal TSP2–EC interactions leads to the development of intrinsically defective EC in the TSP2-null mouse, as would appear to be the case for fibroblasts, then some property of EC, e.g., adhesion, migration, or proliferation, should be demonstrably abnormal in culture. We are currently testing this possibility with EC isolated from TSP2-null Immortomice. Alternatively, inhibition of EC function by fibroblast-derived TSP2 might be lacking in TSP2-null mice, resulting in a net shift towards a proangiogenic environment in TSP2-null tissues.

The ECM, and collagen fibrils in particular, are thought to provide important morphogenetic cues for the growth of capillaries by angiogenesis (Polverini, 1996; Sage, 1996). Since there are clear abnormalities in collagen fibril structure in TSP2-null mice, the possibility also exists that abnormal ECM–EC interactions, apart from direct TSP2–EC interactions, contribute to the increased vascularity in these mice. Some support for this conjecture is provided by the observation that vascular density is highest in tissues in mutant mice in which collagen fibrils appear most abnormal, i.e., skin and subdermis (Kyriakides *et al*, 1998a). In order to test this hypothesis, we plan to implant acellular dermal matrix strips, obtained from WT and TSP2-null mice, subcutaneously into immunodeficient transgenic thrombomodulin-lacZ mice. Thrombomodulin-lacZ mice have been generated by a targeted mutation in which a functional lacZ gene was inserted in one allele at the thrombomodulin locus (Weiler-Guettler *et al*, 1996). Implants will be removed at different times and the extent of invasion of blood vessels from the host will be measured, as judged by the number of β-galactosidase-positive vessels per high power-field in randomly chosen sections from WT and TSP2-null implants. A higher density of blood vessels in TSP2-null implants would support the hypothesis that the organization or composition of dermal matrix from TSP2-null mice provides a stimulus for angiogenesis.

CONCLUSIONS

Our findings are consistent with the view that matricellular proteins, such as TSP2, function by modulating cell–matrix interactions, and thereby cell function. Thus we provide evidence in this paper that TSP2-null mouse dermal fibroblasts produce higher than normal levels of MMP2 and that this proteolytic activity is responsible for the defect in adhesion displayed by these cells. Although it remains to be proven, increased MMP2 activity could also account for the abnormal interactions of mouse tendon fibroblasts with adjacent growing collagen fibrils, observed in developing tendon, and the abnormally shaped collagen fibrils seen in mature mouse skin and tendon. Whereas the increased angiogenesis detected in some tissues in the TSP2-null mouse is consistent with the antiangiogenic activity of TSP2 *in vitro*, the molecular and cellular basis for this activity is less certain. Possibilities include the ability of TSP2 to bind and sequester angiogenic factors such as bFGF or VEGF, changes in cell signaling that result from the failure of TSP2 to interact with its several cell-surface receptors, and more complex mechanisms that implicate an abnormal extracellular matrix in increased angiogenesis.

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