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Matrix Pathobiology

Skeletal Abnormalities in Mice Lacking Extracellular Matrix Proteins, Thrombospondin-1, Thrombospondin-3, Thrombospondin-5, and Type IX Collagen

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Thrombospondin-5 (TSP5) is a large extracellular matrix glycoprotein found in musculoskeletal tissues. TSP5 mutations cause two skeletal dysplasias, pseudoachondroplasia and multiple epiphyseal dysplasia; both show a characteristic growth plate phenotype with retention of TSP5, type IX collagen (Col9), and matrillin-3 in the rough endoplasmic reticulum. Whereas most studies focus on defining the disease process, few functional studies have been performed. TSP5 knockout mice have no obvious skeletal abnormalities, suggesting that TSP5 is not essential in the growth plate and/or that other TSPs may compensate. In contrast, Col9 knockout mice have diminished matrillin-3 levels in the extracellular matrix and early-onset osteoarthritis. To define the roles of TSP1, TSP3, TSP5, and Col9 in the growth plate, all knockout and combinatorial strains were analyzed using histomorphometric techniques. While significant alterations in growth plate organization were found in certain single knockout mouse strains, skeletal growth was only mildly disturbed. In contrast, dramatic changes in growth plate organization in TSP3/5/Col9 knockout mice resulted in a 20% reduction in limb length, corresponding to similar short stature in humans. These studies show that type IX collagen may regulate growth plate width; TSP3, TSP5, and Col9 appear to contribute to growth plate organization; and TSP1 may help define the timing of growth plate closure when other extracellular proteins are absent. ***(Am J Pathol 2008, 172:1664–1674; DOI: 10.2353/ajpath.2008.071094)***

Thrombospondin-5 (TSP5), also known as cartilage oligomeric matrix protein, is the fifth member of the TSP gene family that includes trimeric (TSP1 and TSP2) and pentameric (TSP3, TSP4, and TSP5) protein members.[1] First identified in cartilage and considered to be cartilage specific, TSP5 was subsequently found in other musculoskeletal tissues and is used as a marker for osteoarthritis and joint injury.[2–9] TSP5 has been intensely studied, because mutations in this gene were shown to cause pseudoachondroplasia and multiple epiphyseal dysplasia (MED/EDM1).[10–17] Mutations result in a dominant-negative effect on TSP5 causing massive retention of mutant TSP5 and other extracellular matrix (ECM) proteins, including types II and IX collagen and matrillin-3 (MATN3), in large rough endoplasmic reticulum *cis*-ternae.[15,18–20] Recent studies show that these ECM proteins associate with TSP5 to form a structured intracellular matrix network.[20] Interestingly, mutations in MATN3 (MED/EDM5) and all three type IX collagen (Col9) genes also cause MED phenotypes (MED/EDM2, -3, and -6) that are typically milder than the MED condition caused by TSP5 mutations.[21]

Different approaches have attempted to define the role of TSP5. Recent work suggests that TSP5 stimulates chondrocyte proliferation through the binding of granulin-epithelin precursor, an autocrine growth factor.[22] Binding experiments demonstrate that TSP5 interacts with types I, II and IX collagens, MATN3, aggrecan, granulin-epithelin

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Table 1. Mouse Knockout Strains Used and Control Background Strains

Strain	Background
TSP1	C57BL/6
Col9	
TSP3	129SvJ
TSP5	129SvJ × C57BL/6
TSP3/5	
TSP5/Col9	(129SvJ × C57BL/6) × C57BL/6
TSP1/3/5	
TSP1/5/Col9	
TSP3/5/Col9	
TSP1/3/5/Col9	

precursor, and integrins.[22–28] The presence of an ordered intracellular matrix network in pseudoachondroplasia chondrocytes and colocalization of these proteins in the normal ECM suggest that TSP5 has a significant role in the ECM.[20] These observations support a model in which TSP5, type IX collagen, and MATN3 interact and function to establish and maintain the homeostasis of the ECM.[29] Furthermore, TSP1 and TSP3 are present in the growth plate and may play similar and/or redundant roles with TSP5 in ECM.

TSP1, TSP3, TSP5, and type IX collagen knockout mice were previously evaluated in other studies.[30–34] Interestingly, loss of type IX collagen, TSP1, TSP3, TSP5, or MATN3 reportedly causes minor or no overt phenotypic changes, and life spans were normal. Type IX collagen knockout mice have early-onset osteoarthritis, suggesting that the loss of this protein affects matrix integrity and permits articular cartilage erosion.[31] TSP1- and TSP2-null mice have wound-healing abnormalities[30] and minor craniofacial dysmorphism,[35] whereas TSP3 knockout mice show accelerated endochondrial ossification and increased trabecular bone in the femoral head.[32,36] The MATN3 knockout mouse has higher bone mineral density and increased prevalence of arthritis.[37] In contrast, TSP5 knockout mice show no reported phenotypic abnormalities.[33]

The TSP genes have been implicated in different disease processes, not limited to the growth plate. TSP3 is highly expressed in osteosarcomas and associated with metastasis.[38,39] Single nucleotide polymorphic variants in TSP2 and TSP4 are associated with early-onset coronary disease,[40] with the TSP4 A387P variant specifically associated with atherosclerosis.[41] Only TSP5 mutations have been found to cause defined disease pathology, whereas variation in the other TSP genes contributes to the development of complex human disorders.[39–42]

In this study, growth plates from single and combinatorial knockout mouse strains were systematically evaluated to elucidate the roles of type IX collagen and TSP1, TSP3, and TSP5 in the growth plate. We show that the loss of individual proteins affects growth plate size and organization, whereas compound loss exacerbates growth plate disorganization and causes short limb length.

Materials and Methods

Knockout Mice

Four previously described knockout mouse lines, TSP1, TSP3, TSP5, and Col IX, were evaluated.[30–34] Controls and compound knockout strains, TSP5/Col9, TSP3/5, TSP1/3/5, TSP3/5/Col9, TSP1/5/Col9, and TSP1/3/5/Col9, were generated and are shown in Table 1. Because the genetic background was not the same for all strains, controls were bred for each strain. Genotyping primers specific for each knockout strain are shown in Table 2 as is the original reference describing the knockout strain.

Histology and Immunohistochemistry

Hind limbs from control and knockout mice were obtained from 1- and 2-month-old mice, and the growth plate from upper tibias were analyzed in all studies. The limbs were fixed in Carson Millonig, pH 7.4 (10% formalin), or ethanol, and decalcified for 1 week in Immunocal formic acid decalcifier solution (American Master Tech Scientific, Inc., Lodi, CA). After embedding in paraffin, 5-μm sections were stained with H&E, following standard protocols.

For immunohistochemistry, the sections were digested with 1 mg/ml pepsin A (Sigma-Aldrich, St. Louis, MO) in 0.1 N HCl for 30 minutes at room temperature for all antibodies except type X collagen. Sections for type X collagen staining were digested in 1 mg/ml hyaluroni-

Table 2. Genotype Primer Sequences

Strain	Primer	Reference
Tsp1	5′-GAGTTTGCTTGTGGTGAACGCTCAG-3′ 5′-AGGGCTATGTGGAATTAATATCGG-3′ 5′-TGCTGTCCATCTGCACGAGACTAG-3′	Agah et al[30]
Tsp3	5′-CTGTGGCAGAGAAGATTCGGA-3′ 5′-ATCGTGTGTTGTCTTGGCGAG-3′ 5′-GTGGATGTGGAATGTGTGCGA-3′	Hankenson et al[32]
Tsp5	5′-TTGCTCGTTAGGGACTCCAT-3′ 5′-CAGAAGCAAAGGTCGTGGAA-3′ 5′-CGCCTTCTTGACGAGTTCTT-3′	Svensson et al[33]
Col9	5′-CCAGTGAACTCCCCTTCCATT-3′ 5′-CTGGCCCTTTCTAACAACTCA-3′ 5′-GATCTCCTGTCATCTCACCT-3′ 5′-AGAACTCGTCAAGAAGGCGA-3′	Nakata et al[43]

dase in PBS containing 0.05% Tween 20 for 1 hour at room temperature. The sections were rinsed in distilled H_2O and then washed in PBS containing 0.05% Tween 20 for 5 minutes, followed by 20 minutes incubation with 10% donkey serum in PBS containing 0.05% Tween 20 to reduce nonspecific antibody binding. All sections were then incubated overnight at 4°C with the primary antibody. An affinity-purified goat polyclonal MATN3 antibody was used at 1:500 (R&D Systems, Minneapolis, MN) and goat polyclonal type II collagen antibody at 1:200. Affinity-purified donkey anti-goat biotin-conjugated secondary antibody (Chemicon International, Temecula, CA) was used at a dilution of 1:500 (for MATN3) or 1:300 (for type II collagen) for 30 minutes, followed by horseradish peroxidase-conjugated streptavidin (Chemicon International) for 30 minutes. The results were detected using 3.3′ diaminobenzidine (Sigma-Aldrich). A polyclonal rabbit primary TSP5 antibody was used at a 1:200 dilution (Kamiya Biomedical Co., Thousand Oaks, CA), and rabbit polyclonal type X collagen antibody was used at 1:200 (gift from Dr. Lunstrum, Shriners Hospital for Children, Portland, OR). Affinity-purified donkey anti-rabbit biotin-conjugated secondary antibody (Chemicon International) was used at a dilution of 1:400 (for both TSP5 and type X collagen) for 30 minutes, followed by horseradish peroxidase-conjugated streptavidin (Chemicon International) for 30 minutes. The results were also detected using 3.3′ diaminobenzidine (Sigma-Aldrich). For type IX collagen staining, a mouse monoclonal at 1:150 (Hybridoma bank at the University of Iowa) was incubated with sections overnight and detected using the Histomouse kit (Zymed Laboratories, South San Francisco, CA). All sections were examined, and images were captured using an Olympus BX51 light microscope (Olympus, Melville, NY) attached to a Spot RT camera Ver 4.6.4.2.

Growth Plate Analysis

To control for any possible strain differences, all growth plates from each strain were compared with their respective control background strain listed in Table 1. Upper tibial growth plates were obtained from at least three different mice of each strain. Growth plate organization was blindly assessed using the following methods. Sections were examined at ×100 magnification, and cells were designated as being arranged in columns or clusters using Image Pro (Media Cybernetics, Silver Spring, MD). Lone cells or cells arranged in small groups were classified as clustered to differentiate them from cells organized in columns (see Figure 2A). The growth plate was digitally outlined at ×40 magnification, and the area was computed using Image Pro. Measurements of the area were calibrated using a microscopic grid. Chondrocyte density in the growth plate was calculated by dividing the total number of chondrocytes by the area of the growth plate per 100 μm^2. The chondrocyte density and counts were averaged from at least three sections from different limbs from each strain and their respective controls. Similarly, five measurements spaced along the width of the growth plate were taken from three sections of different limbs using Image Pro software, and the measurements were then averaged (see Figure 3A).

Limb Length Measurements

Soft tissue was removed from hind limb, and the resulting skeletal tissue was fixed in 70% ethanol and then subjected to radiographical examination. All limbs were attached to a grid to ensure consistency and accuracy of measurements, and at least 10 hind limbs were measured for each strain. Measurements were made from end-to-end of both long bones in the hind limb (see example in Figure 4A, below) using Image Pro software. The measurements were converted to millimeters by measuring an object of known length and calculating the pixel-to-millimeter ratio.

Exercise Protocol

Two to four male mice from TSP5, Col9, and TSP5/Col9 knockout strains and the controls were subjected to an exercise protocol in which they were allowed to run from age 6 weeks to 5 months. The rodent wheel was held in place by a heavy metal plate, and the distance run by each mouse was recorded using a VDO C10+ magnetic bicycle monitor (Cytec, Rohrbach, Germany). Daily distances were recorded both manually and digitally from Monday through Friday, and distance traveled on Saturday and Sunday was recorded with the digital monitor only. All housing conditions and experiments were conducted in compliance with University of Texas Medical School at Houston animal care standards (protocol no. 02-022 and 05-020).

Results

Growth Plate Abnormalities

These experiments were performed to assess the roles of TSP1, TSP3, and TSP5, and type IX collagen in the growth plate. We first asked whether the loss of one or more of these extracellular proteins affected development and longevity. All offspring were liveborn, premature death was not observed, and there was no deviation from Mendelian expectations.

We next investigated what effect the loss of individual TSP proteins and type IX collagen had on growth plate organization and size. We evaluated the growth plates at 1 and 2 months of age because these time spans represent periods of rapid growth, after which the mouse growth plate is essentially quiescent and limb growth ceases. Chondrocyte growth plate disorganization was observed in all of the individual knockout strains at both ages with the least effect seen in the TSP1 strain (Figure 1A). In general, the columnar patterning was disrupted, and there was more clustering of chondrocytes. The TSP3/5-null growth plate was similar to those of the TSP3- and TSP5-null mice at 1 month (Figure 1A, panels E, L, G, and N, and 1B, panels B and F). More disorganization

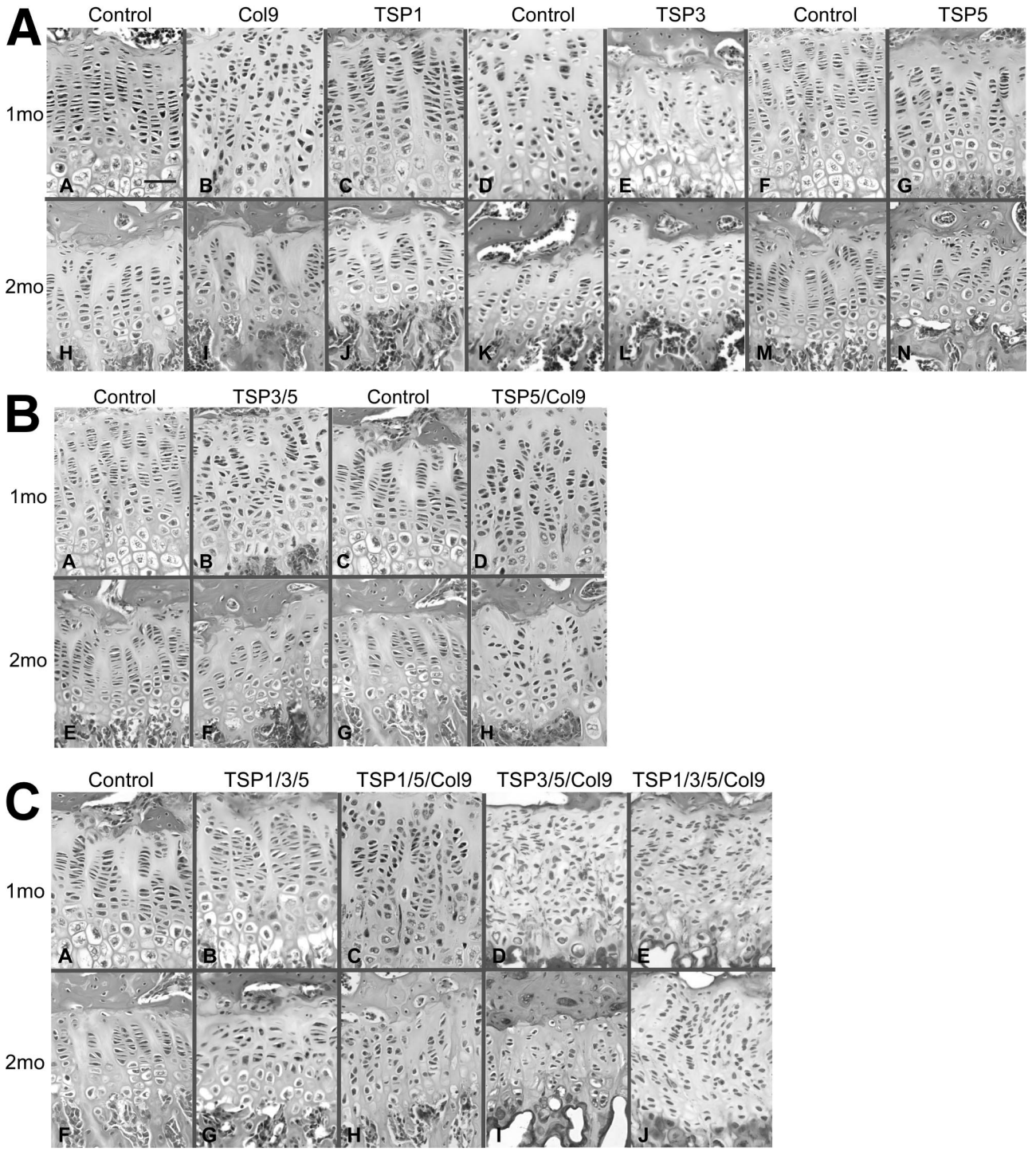

Figure 1. Growth plates from single and compound knockout mice. **A:** H&E-stained upper tibial growth plates from single and compound knockout mice at 1 and 2 months of age are shown. Control growth plates (**A**, **D**, **F**, **H**, **K**, and **M**) are on the left for each strain. The loss of type IX collagen (**B** and **I**), TSP3 (**E** and **L**), or TSP5 (**G** and **N**) causes disturbance of growth plate organization. The Col9 growth plate appears wider and the chondrocytes columns are not as well defined as in the control growth plates. Loss of TSP5 (**G** and **N**) causes minor disruption of columnar patterning, whereas loss of TSP1 (**C** and **J**) has little effect. **B:** Control growth plates (**A**, **C**, **E**, and **G**) are on the left for each strain. The double knockout of TSP5/Col9 genes (**D** and **H**) produces growth plate pathology that is similar to that of the single Col9 knockout growth plate [**A** (**B** and **I**)], but loss of TSP3/5 (**B** and **F**) causes more growth plate disorganization than TSP5 alone [**A** (**G** and **N**)]. **C:** Control growth plates (**A** and **F**) are on the left. Loss of TSP1/3/5 (**B** and **G**) is similar to the TSP3/5 knockout growth plate [**B** (**B** and **F**)] but loss of type IX collagen in combination with loss of TSP3/5 (**D** and **I**) and TSP1/3/5 (**E** and **J**) has a significant impact on the organization of the growth plate and the hypertrophic zone. Loss of type IX collagen in combination with loss of TSP1/5 is shown in **C** and **H**. Scale bar = 100 μmol/L [**A** (**A**)]. Upper tibial growth plates were used in all studies.

was observed in the TSP5/Col9 knockout growth plate (Figure 1B, panels, D and H) compared with either null strain alone (Figure 1A, panels B, I, G, and N). The most dramatic disorganization was observed in the TSP3/5/Col9 knockout growth plate (Figure 1C, panels D and I). This disorganization is similar to that observed when β1-integrin is inactivated in chondrocytes, resulting in chondrocyte rotation.[44] The additional loss of TSP1 with

any of the other combinations did not appear to contribute to chondrocyte disorganization (Figure 1).

The organization of the chondrocytes in the growth plate was quantified by measuring the number of chondrocytes in columns or clusters. At 1 and 2 months, the controls had 65 to 85% of the chondrocytes in columns, compared with the knockout strains, which had 10 to 42% in columns (Figure 2). The TSP3/5/Col9 knockout had very few chondrocytes in columns (Figures 1C, panels D and I, and 2). These results suggest that TSP3, TSP5, and

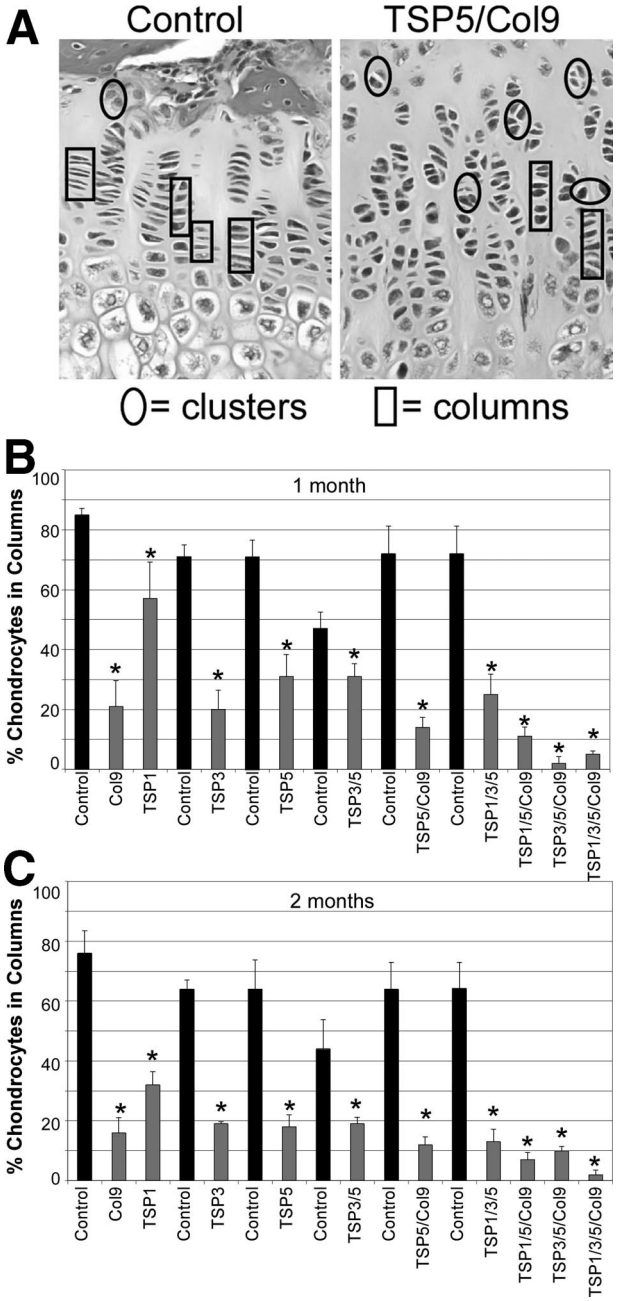

Figure 2. Comparison of growth plate chondrocyte organization in knockout mice and controls. **A:** The method used in the classification of chondrocytes is shown and described in Materials and Methods. **B:** Comparison of percent chondrocytes organized into columns in all knockout strains and respective controls at 1 month of age. **C:** Comparison of percent chondrocytes organized into columns in all knockout strains and respective controls at 2 months of age. Black and gray bars are measurements for controls and strains, respectively. All knockout strains showed disrupted chondrocyte columns, with the TSP1 knockout the least affected and the TSP3/5/Col9 and TSP1/3/5/Col9 knockout strains the most affected. $^*P < 0.05$. Upper tibial growth plates were used in all studies.

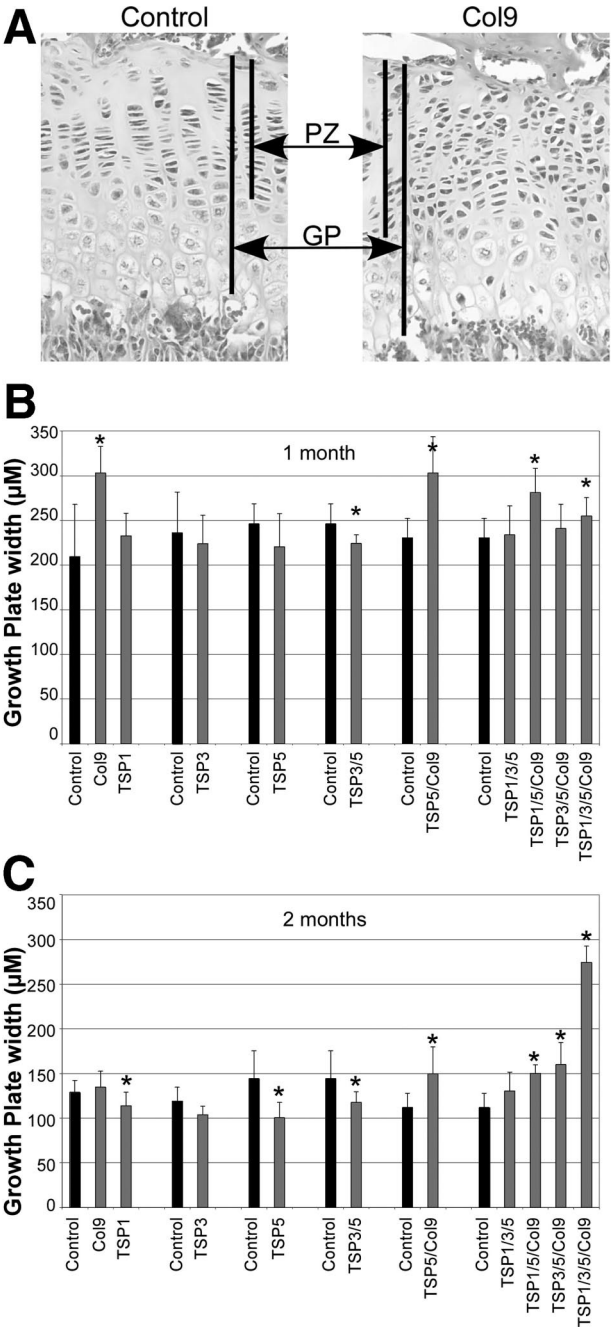

Figure 3. Loss of type IX collagen affects the width of growth plate. **A:** Measurements of growth plates was performed as shown on the images and described in Materials and Methods. PZ, proliferative zone: GP, growth plate. **B:** Comparison of growth plate width at 1 month. Black and gray bars are measurements for controls and strains, respectively, and the **asterisk** indicates a significant difference from the control ($P < 0.05$). The overall growth plate of the Col9 and combinatorial knockout strains lacking Col9 tend to be wider compared with the controls. **C:** By 2 months, there is no difference in the width of the Col9 growth plate compared with the control; however, the TSP1, TSP5, and TSP3/5 growth plates are all more narrow than the control, whereas the growth plates of the other combinatorial mouse strains are wider than the control. $^*P < 0.05$. Upper tibial growth plates were used in all studies.

type IX collagen play a role in the chondrocyte organization in the growth plate.

The widths of all of the growth plates are shown in Figure 3. Significantly wider growth plates were observed at 1 month in mouse strains lacking type IX collagen (Col9, TSP5/Col9, TSP1/5/Col9, and TSP1/3/5/Col9) (Figure 3B). However, by 2 months, only the growth plates from combinatorial knockout strains lacking type IX collagen (TSP5/Col9, TSP1/5/Col9, TSP3/5/Col9, and TSP1/3/5/Col9) remain wider (Figure 3C). These results suggest that type IX collagen plays a role in determining growth plate width. Interestingly, the growth plate of the compound quadruple knockout, TSP1/3/5/Col9, is twice the width of the control at 2 months (Figure 3C). Here, the additional loss of TSP1 has a significant impact on the width of the growth plate, suggesting that TSP1 loss, together with loss of other ECM proteins, may interfere with growth plate closure (Figure 1C). In contrast, the TSP3/5 growth plate is thinner than the control at both ages, whereas this observation was made in TSP1 and TSP5 knockout growth plates only at 2 months (Figure 3, B and C).

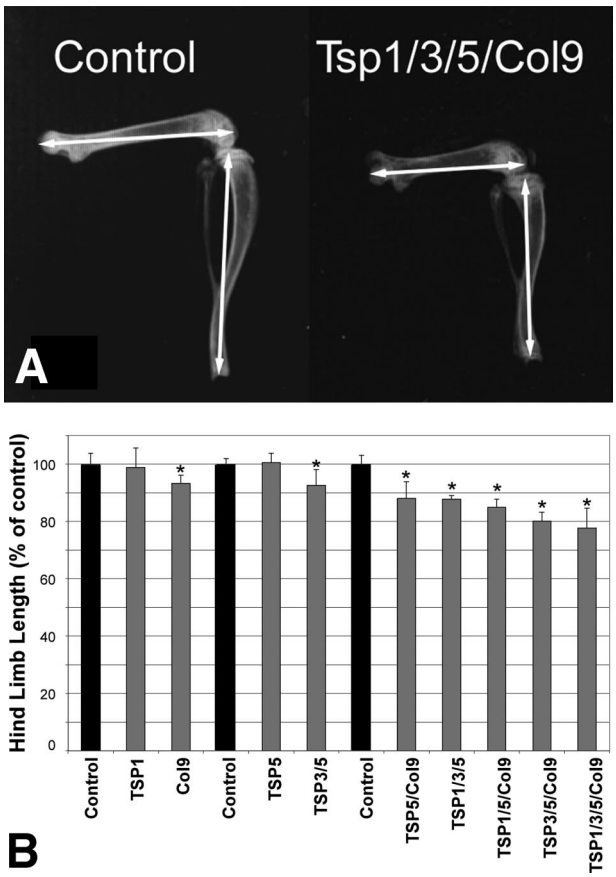

Figure 4. Measurement of bone length. **A:** Measurement of the TSP1/3/5/Col9 limb compared with control limb is shown and described in Materials and Methods. **B:** Comparison of hind limb length from all knockout strains. Black and gray bars are measurements for controls and strains, respectively, and the **asterisk** indicates a significant difference ($P > 0.05$) from the control. The long bones of the hind limb are significantly shorter in Col9, TSP3/5, TSP5/Col9, TSP1/3/5, TSP1/5/Col9, TSP3/5/Col9, and TSP1/3/5/Col9 mice. The combinatorial loss of TSP3, TSP5, and type IX collagen has the greatest effect on limb length. $^*P < 0.05$.

To assess the limb lengths in the single and compound knockout mice, limbs were collected at 1 month, and the long bones of the hind limbs were measured by radiographical examination, as shown in Figure 4A. Loss of type IX collagen resulted in a 5% reduction in limb length. An 8% reduction was observed with loss of both TSP3/5. The combinatorial knockout strains showed the most dramatic reductions ranging from 11 to 21%. Loss of TSP1 alone or in combination did not contribute to limb length reduction (Figure 4B).

We next asked whether the quantity and distribution of TSP5, types II and IX collagens, and MATN3 protein in growth plate were altered. All sections were immunostained simultaneously. MATN3 was diminished in type IX knockout growth plates and in all combinatorial Col9 knockout strains (Figure 5). MATN3 incorporation into ECM was not affected by loss of TSP1, TSP5, and TSP3/5 (Figure 5). TSP5 staining was mildly decreased in the Col9 knockout growth plate, which showed limited patchy staining in the hypertrophic zone (data not shown). These observations are consistent with previous findings.[29] No intracellular protein retention was observed. Immunolocalization of types II and IX collagens were similar in null and control mice strains.

Effect of Exercise on Articular Cartilage

Previous studies showed that 9-month-old type IX collagen knockout mice develop arthritis more often than controls.[31] To determine whether TSP5 knockout mice were more prone to arthritis, Col9, TSP5, and TSP5/Col9 knockout mice were subjected to exercise (Figure 6; Table 3). The mice ran an average of 960 ± 405 miles (range, 277 to 1488 miles) from 6 weeks to 5 months of age. There was no correlation between genotype and the distance the mice ran. Compared with the control (Figure 6A), the Col9 knockout nonrunner mice had flat articular cartilage (Figure 6D; Table 3). However, exercise further accentuated the flattening of the articular surface (Figure 6G). The TSP5 runner had very mild flattening (Figure 6H) compared with nonrunner and control (Figure 6, E and B) articular cartilage (Table 3). TSP5/Col9 knockout articular cartilage had cartilage flattening similar to Col9 single knockout mice, but in the absence of both TSP5 and Col9, fibrillation of the articular cartilage surface was also observed (Figure 6, F and I). Cartilage fibrillation is a hallmark of cartilage degeneration.[45] These findings suggest that a combination of TSP5 and Col9 deficiency results in development of exercise-associated cartilage degeneration.

Discussion

Previous observations suggest that individual loss of TSP1, TSP3, TSP5, and type IX collagen causes modest to no alteration in murine skeletal development.[30–33,35] In this work, we focused on the combinatorial role(s) that TSP1, TSP3, TSP5, and type IX collagen plays in the growth plate and on whether their functions are redundant. Toward this goal, we compared several parameters in single and combinatorial knockout mice: growth plate

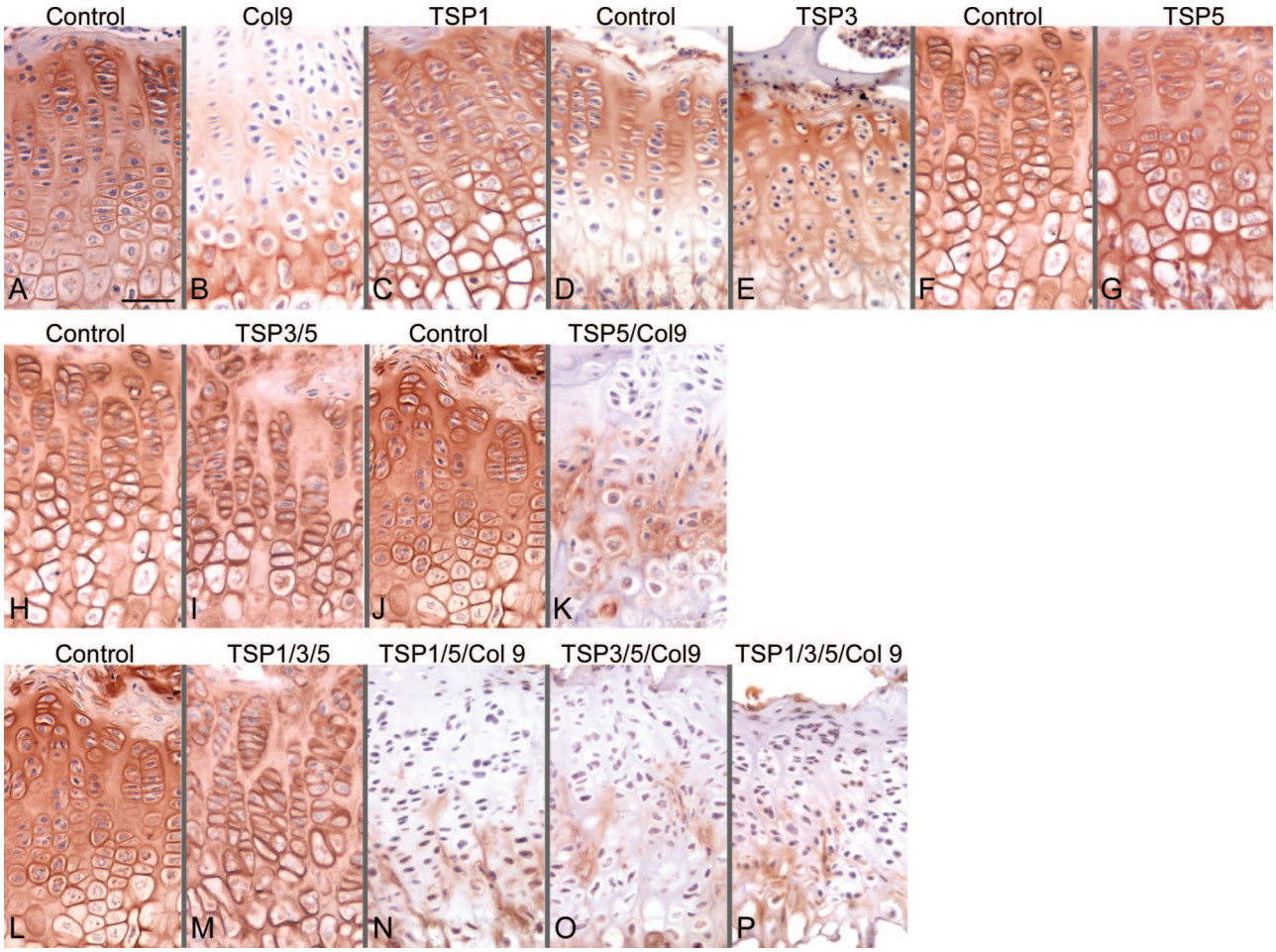

Figure 5. Distribution of MATN3 in knockout growth plates. Growth plates were simultaneously immunostained with an antibody to MATN3, as described in Materials and Methods. Control sections are to the left for all strains (**A**, **D**, **F**, **H**, **J**, and **L**). MATN3 was markedly diminished in the Col9 knockout growth plate and in all compound knockout growth plate strains that do not synthesize type IX collagen (**B**, **K**, **N**, **O**, and **P**). Interestingly, the Col9 knockout growth plate shows light immunostaining throughout the proliferative zone and darker staining in the hypertrophic zone (**B**). Loss of TSP1, TSP3, and TSP5 does not affect the quantity or distribution of MATN3 (**C**, **E**, and **G**). Loss of TSP3/5 (**I**) and TSP1/3/5 (**M**) are also shown. Scale bar = 100 μmol/L. Upper tibial growth plates were used in all studies.

organization, chondrocyte density, growth plate width, and relative presence and distribution of TSP5, types II and IX collagen, and MATN3 in the ECM.

These studies led to several novel observations. First, a deficiency in any one of these individual proteins disturbs the columnar arrangement of chondrocytes in the growth plate, although the lack of TSP-1 only mildly perturbed chondrocyte organization. Increased growth plate width was associated with loss of type IX collagen at only 1 month of age and was found in combination strains lacking type IX collagen. In contrast, the growth plate is significantly narrower at 1 and 2 months in the TSP3/5 knockout strain, and their hind limbs were 8% shorter than the controls. A 20% reduction in limb length and almost complete loss of chondrocyte columnar patterning was observed in TSP3/5/Col9 knockout mice. Taken together, these results show that the loss of individual ECM proteins and combinatorial loss significantly impact growth plate organization and long bone growth.

TSP1 single- and TSP1/2 double-null mice recruit fewer macrophages and produce less monocyte chemoattractant protein in wounds, whereas both TSP2 and TSP3 knockout mice have thicker cortical bone.[30,32,36] Additionally, TSP3 knockout mice show earlier ossification and more trabecular bone in the femoral head.[32] Previous work with these null mice focused on assessing gross skeletal parameters, and identified subtle skeletal abnormalities. Here, a detailed study of growth plates and long bones has revealed both subtle alterations in single-null strains and more dramatic alterations in compound-null mice. The TSP1-null growth plate shows the least disorganization followed by that in TSP3-null growth plates (Figure 2). In contrast to a previous report that TSP5 knockout mice had no growth plate abnormalities,[33] using quantitative morphometry, we demonstrate significant growth plate disorganization (Figure 3). However, none of the single TSP knockout mice showed any reduction in limb length, despite an abnormal growth plate organization. This finding suggests that the individual loss of any of these three TSP proteins is not sufficient to disrupt long bone development or growth.

In contrast to the TSP-null mouse strains, previous studies of type IX collagen knockout mice showed early-onset osteoarthritis,[31,43] less MATN3 incorporation into

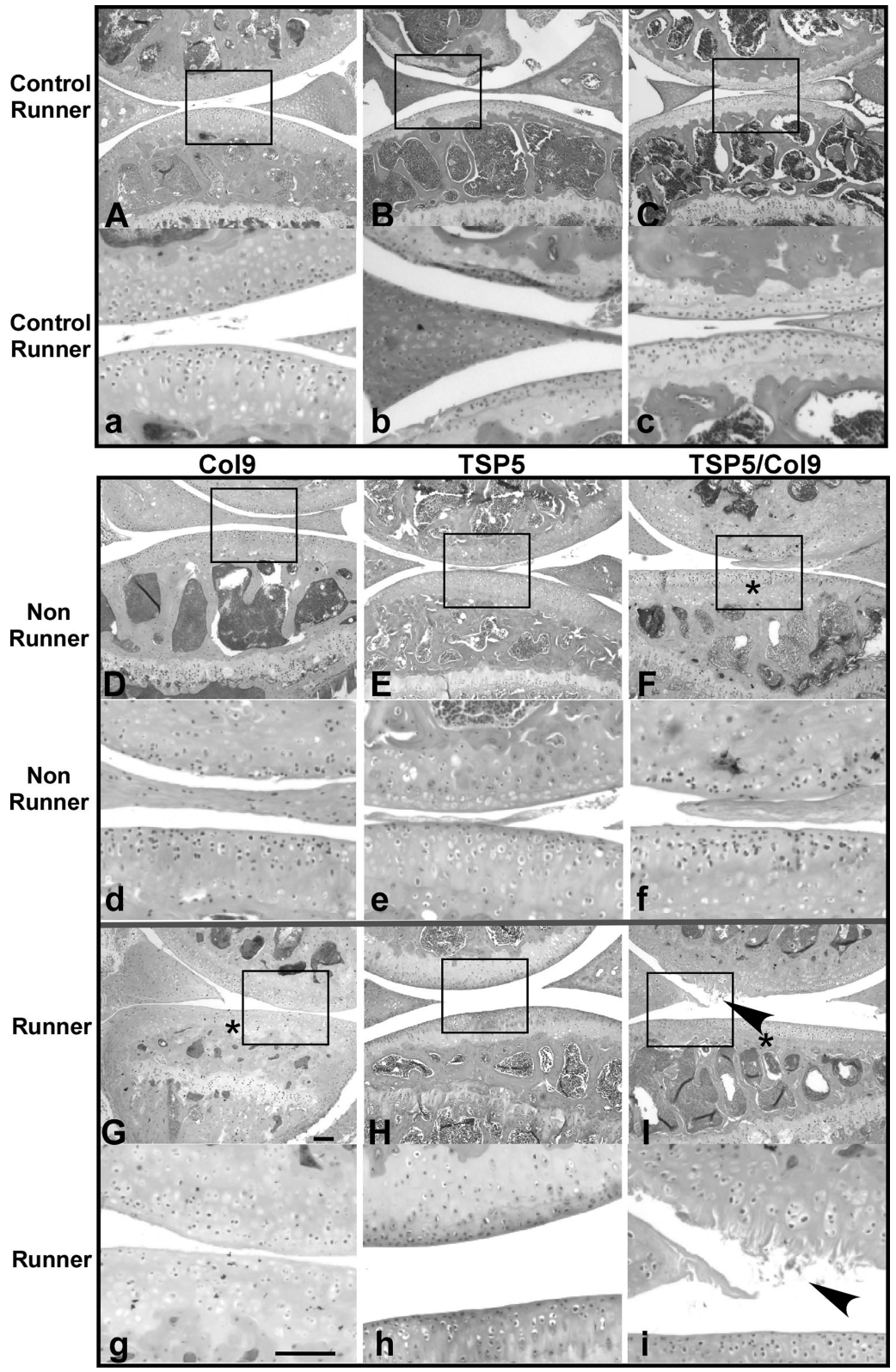

Figure 6. Articular cartilage from hind limbs from knockout mice subjected to exercise regimen. C0l9, TSP5, TSP5/Colp knockout, and control mice were subjected to a running exercise regimen, as described in Materials and Methods. The H&E-stained articular cartilage sections are shown. All of the wild-type running mice (**A**, **B**, and **C**) had normal articular cartilage. The nonrunner TSP5 knockout mice (**E**) had relatively normal articular cartilage, whereas the articular cartilage of the runners (**H**) was mildly flattened. At the end of the exercise regimen, the Col9 knockout nonrunner (**D**) had mild flattening, whereas the runner (**G**) had severe flattening (**asterisk**) of the articular cartilage. Interestingly, the TSP5/Col9 knockout nonrunner (**F**) had flattened articular cartilage (**asterisk**), and exercise caused additional flattening (**asterisk**) and fraying (**arrow**) of the cartilage in the runner (**I**). A closer view of the articular cartilage cap is shown below each panel (**a–i**). Scale bar = 100 μmol/L.

Table 3. Effects on Articular Cartilage from Hind Limbs from Knockout Mice Subjected to Exercise Regimen

	Nonrunner		Runner	
	Flattening	Fibrillation	Flattening	Fibrillation
Control	No	No	No	No
Col9	+	No	++	No
TSP5	No	No	+	No
Col9/TSP5	++	No	++	Yes

the matrix,[29] degraded type II collagen fibrils,[46] and slower fracture repair.[47] We observed similar findings and, in addition, found wider growth plates and significant limb shortening at 1 month of age. Lower chondrocyte density was found in these mice, although the growth plate was wider, suggesting that although there is a similar number of chondrocytes, they are distributed over a larger area. This may indicate that there is more interterritorial matrix or less dense matrix because of absent matrix proteins in these growth plates. Furthermore, the ratio of the proliferative zone to the total width of the growth plate is similar to that of the controls, indicating that chondrocyte proliferation is not increased in these mice (Figure 3A; data not shown). This increase in growth plate width does not lead to longer limbs but rather to significantly shorter hind limbs (Figure 4). These results suggest that loss of type IX collagen has a more significant impact, compared with loss of any of the individual TSP proteins.

Loss of type IX collagen also affects MATN3 incorporation into the interterritorial matrix of the growth plate (Figure 5), a finding that is consistent with previous observations.[29] The loss of MATN3 immunostaining does not obviously correlate with chondrocyte organization. For example, when comparing control with TSP1/3/5 growth plates, no appreciable differences in MATN3 immunostaining are observed despite a significant difference in chondrocyte organization (Figures 5, L and M, and 2).

MATN3 is a member of a family of proteins proposed to be adaptor molecules that bind to the collagen network,[48] and it colocalizes with and binds to TSP5.[27] MATN3 binds directly to type IX collagen[29]; therefore decreased incorporation of MATN3 is expected in the absence of type IX collagen. No appreciable decrease in MATN3 immunostaining was found in the ECM of TSP5-null mice, suggesting that the interaction between MATN3 and TSP5 may be of lower affinity than the interaction between MATN3 and type IX collagen. Because individual losses of TSP1, TSP3, TSP5, and type IX collagen all cause perturbation of the growth plate, the next set of studies was undertaken to determine whether these ECM proteins are functionally redundant. Both TSP3 and TSP5 proteins are expressed in the growth plate and are pentameric members of the same gene family. Loss of both proteins does not disturb chondrocyte organization more than the loss of an individual protein (Figures 1 and 2) but has a significantly increased impact on growth plate width (Figure 3) and hind limb length, which is reduced by 8% (Figure 4). This finding suggests that TSP3 and TSP5 do not play completely redundant or compensatory roles. In contrast, the loss of both TSP5 and type IX collagen does not have a substantial impact on the growth plate parameters that is more than the loss of type IX collagen alone (Figures 1 to 3). These results suggest that type IX collagen plays a more significant role in the growth plate than members of the TSP family and that the latter proteins most likely function in same pathway.

With the exception of the TSP1/3/5-null strain, all triple- and quadruple-null strains have approximately 10% or less of their chondrocytes organized into columns. The TSP3/5/Col9 knockout strain showed the most significant growth plate disorganization and limb length reduction (20%; Figures 1 to 4). The loss of TSP1 has a minor impact on the organization of the growth plate and hind limb lengths. However, the TSP1/3/5/Col9 knockout strain has a much wider growth plate at 2 months when compared with the control or the TSP3/5/Col9 strain. This finding suggests that TSP1 could play a role in timing of growth plate closure when other ECM proteins are absent (Figure 3C). Whereas few hypertrophic chondrocytes are observed in the TSP5/Col9, TSP1/5/Col9, TSP3/5/Col9, and TSP1/3/5/Col9 strains at 1 month of age, significant numbers are present at 2 months of age (Figure 1). Type X collagen immunostaining was used to identify the hypertrophic zone (data not shown). This observation may be indicative of delay in chondrocyte maturation, despite the presence of TSP1 in some of the strains. Altogether, these observations suggest a role for TSP1 in the regulation of growth plate closure and a role for TSP5 and type IX collagen in maturation of chondrocytes. These findings are consistent with recent work showing slow cartilage maturation and bone formation during fracture repair in type IX collagen knockout mice.[47]

To evaluate the integrity of the articular cartilage matrix, TSP5, Col9, and TSP5/Col9 knockout strains were subjected to an exercise protocol from 6 weeks to 5 months of age. We found that exercise-induced flattening of the articular cartilage superficial zone cap was most pronounced in the hind limbs of the type IX collagen knockout mice but was also present, to a lesser extent, in the TSP5 knockout mice. Exercise had the most dramatic effect on the articular cartilage of Col9 knockout mice by exacerbating joint erosion. Interestingly, mice lacking both type IX collagen and TSP5 have a flattened articular cartilage even without exercise, and exercise caused fraying of the articular cartilage surface. Osteoarthritic changes were not observed in the wild-type mice with exercise (Figure 6). These results suggest that both TSP5 and type IX collagen play a role in maintaining homeostasis of the articular cartilage. These findings also have implications for the human skeletal dysplasias caused by mutations in TSP5 and type IX collagen genes that are associated with joint erosion and early-onset osteoarthritis.[17,42,49] These abnormalities are likely related to diminished amounts of these proteins in the matrix.[13,50,51] However, other factors, such as the presence of mutant protein could contribute to the disease phenotype.

The results of these studies indirectly suggest different and nonredundant roles for TSP1, TSP3, TSP5, and type

IX collagen in the growth plate. TSP1 appears to primarily affect the timing of growth plate closure. TSP3, TSP5, and type IX collagen all participate in growth plate organization that directly modulates linear growth. Additionally, TSP5 and type IX collagen affect chondrocyte maturation and articular cartilage homeostasis. These findings can be extrapolated to the growth plate pathology observed in skeletal dysplasias caused by mutations in TSP5, type IX collagen, and MATN3 genes. These skeletal dysplasias results from chondrocyte pathology related to cellular protein retention and to loss of one or more of these proteins in the ECM. The results of this study suggest that loss of these proteins from the matrix affects organizational regulation and may significantly contribute to the growth plate pathology observed in pseudoachondroplasia and MED. In summary, we suggest that TSP3, TSP5, and type IX collagen operate in concert and are important for proper growth plate organization and that TSP1 functions in a minor or accessory role in the growth plate.

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References

1. Adams J, Tucker RP, Lawler J: The thrombospondin gene family. New York, Springer-Verlag, 1995
2. Andersson ML, Thorstensson CA, Roos EM, Petersson IF, Heinegard D, Saxne T: Serum levels of cartilage oligomeric matrix protein (COMP) increase temporarily after physical exercise in patients with knee osteoarthritis. BMC Musculoskelet Disord 2006, 7:98
3. Délot E, Brodie SG, King LM, Wilcox WR, Cohn DH: Physiological and pathological secretion of cartilage oligomeric matrix protein by cells in culture. J Biol Chem 1998, 273:26692–26697
4. Fang C, Johnson D, Leslie MP, Carlson CS, Robbins M, Di Cesare PE: Tissue distribution and measurement of cartilage oligomeric matrix protein in patients with magnetic resonance imaging-detected bone bruises after acute anterior cruciate ligament tears. J Orthop Res 2001, 19:634–641
5. Koelling S, Clauditz TS, Kaste M, Miosge N: Cartilage oligomeric matrix protein is involved in human limb development and in the pathogenesis of osteoarthritis. Arthritis Res Ther 2006, 8:R56
6. Lohmander LS, Saxne T, Heinegard DK: Release of cartilage oligomeric matrix protein (COMP) into joint fluid after knee injury and in osteoarthritis. Ann Rheum Dis 1994, 53:8–13
7. McKeand J, Rotta J, Hecht JT: Natural history study of pseudoachondroplasia. Am J Med Genet 1996, 63:406–410
8. Neidhart M, Hauser N, Paulsson M, DiCesare PE, Michel BA, Hauselmann HJ: Small fragments of cartilage oligomeric matrix protein in synovial fluid and serum as markers for cartilage degradation. Br J Rheumatol 1997, 36:1151–1160
9. Neidhart M, Muller-Ladner U, Frey W, Bosserhoff AK, Colombani PC, Frey-Rindova P, Hummel KM, Gay RE, Hauselmann H, Gay S: Increased serum levels of non-collagenous matrix proteins (cartilage oligomeric matrix protein and melanoma inhibitory activity) in marathon runners. Osteoarthritis Cartilage 2000, 8:222–229
10. Briggs MD, Chapman KL: Pseudoachondroplasia and multiple epiphyseal dysplasia: mutation review, molecular interactions, and genotype to phenotype correlations. Hum Mutat 2002, 19:465–478
11. Briggs MD, Mortier GR, Cole WG, King LM, Golik SS, Bonaventure J, Nuytinck L, De Paepe A, Leroy JG, Biesecker L, Lipson M, Wilcox WR, Lachman RS, Rimoin DL, Knowlton RG, Cohn DH: Diverse mutations in the gene for cartilage oligomeric matrix protein in the pseudoachondroplasia-multiple epiphyseal dysplasia disease spectrum. Am J Hum Genet 1998, 62:311–319
12. Hecht JT, Deere M, Putnam E, Cole W, Vertel B, Chen H, Lawler J: Characterization of cartilage oligomeric matrix protein (COMP) in human normal and pseudoachondroplasia musculoskeletal tissues. Matrix Biol 1998, 17:269–278
13. Hecht JT, Hayes E, Haynes R, Cole WG: COMP mutations, chondrocyte function and cartilage matrix. Matrix Biol 2005, 23:525–533
14. Hecht JT, Hayes E, Snuggs M, Decker G, Montufar-Solis D, Doege K, Mwalle F, Poole R, Stevens J, Duke PJ: Calreticulin, PDI, Grp94 and BiP chaperone proteins are associated with retained COMP in pseudoachondroplasia chondrocytes. Matrix Biol 2001, 20:251–262
15. Hecht JT, Montufar-Solis D, Decker G, Lawler J, Daniels K, Duke PJ: Retention of cartilage oligomeric matrix protein (COMP) and cell death in redifferentiated pseudoachondroplasia chondrocytes. Matrix Biol 1998, 17:625–633
16. Hecht JT, Nelson LD, Crowder E, Wang Y, Elder FF, Harrison WR, Francomano CA, Prange CK, Lennon GG, Deere M, Lawler J: Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. Nat Genet 1995, 10:325–329
17. Posey KL, Hayes E, Haynes R, Hecht JT: Role of TSP-5/COMP in pseudoachondroplasia. Int J Biochem Cell Biol 2004, 36:1005–1012
18. Hecht JT, Makitie O, Hayes E, Haynes R, Susic M, Montufar-Solis D, Duke PJ, Cole WG: Chondrocyte cell death and intracellular distribution of COMP and type IX collagen in the pseudoachondroplasia growth plate. J Orthop Res 2004, 22:759–767
19. Merritt TM, Alcorn JL, Haynes R, Hecht JT: Expression of mutant cartilage oligomeric matrix protein in human chondrocytes induces the pseudoachondroplasia phenotype. J Orthop Res 2006, 24:700–707
20. Merritt TM, Bick R, Poindexter BJ, Alcorn JL, Hecht JT: Unique matrix structure in the rough endoplasmic reticulum cisternae of pseudoachondroplasia chondrocytes. Am J Pathol 2007, 170:293–300
21. Chapman KL, Mortier GR, Chapman K, Loughlin J, Grant ME, Briggs MD: Mutations in the region encoding the von Willebrand factor A domain of matrilin-3 are associated with multiple epiphyseal dysplasia. Nat Genet 2001, 28:393–396
22. Xu K, Zhang Y, Ilalov K, Carlson CS, Feng JQ, Di Cesare PE, Liu CJ: Cartilage oligomeric matrix protein associates with granulin-epithelin precursor (GEP) and potentiates GEP-stimulated chondrocyte proliferation. J Biol Chem 2007, 282:11347–11355
23. Chen FH, Herndon ME, Patel N, Hecht JT, Tuan RS, Lawler J: Interaction of cartilage oligomeric matrix protein/thrombospondin 5 with aggrecan. J Biol Chem 2007, 282:24591–24598
24. Chen FH, Thomas AO, Hecht JT, Goldring MB, Lawler J: Cartilage oligomeric matrix protein/thrombospondin 5 supports chondrocyte attachment through interaction with integrins. J Biol Chem 2005, 280:32655–32661
25. Halasz K, Kassner A, Morgelin M, Heinegard D: COMP acts as a catalyst in collagen fibrillogenesis. J Biol Chem 2007, 282:31166–31173
26. Holden P, Meadows RS, Chapman KL, Grant ME, Kadler KE, Briggs MD: Cartilage oligomeric matrix protein interacts with type IX collagen, and disruptions to these interactions identify a pathogenetic mechanism in a bone dysplasia family. J Biol Chem 2001, 276:6046–6055
27. Mann HH, Ozbek S, Engel J, Paulsson M, Wagener R: Interactions between the cartilage oligomeric matrix protein and matrilins: implications for matrix assembly and the pathogenesis of chondrodysplasias. J Biol Chem 2004, 279:25294–25298
28. Thur J, Rosenberg K, Nitsche DP, Pihlajamaa T, Ala-Kokko L, Heinegard D, Paulsson M, Maurer P: Mutations in cartilage oligomeric matrix protein causing pseudoachondroplasia and multiple epiphyseal dysplasia affect binding of calcium and collagen I, II, and IX. J Biol Chem 2001, 276:6083–6092
29. Budde B, Blumbach K, Ylostalo J, Zaucke F, Ehlen HW, Wagener R, Ala-Kokko L, Paulsson M, Bruckner P, Grassel S: Altered integration of matrilin-3 into cartilage extracellular matrix in the absence of collagen IX. Mol Cell Biol 2005, 25:10465–10478
30. Agah A, Kyriakides TR, Lawler J, Bornstein P: The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. Am J Pathol 2002, 161:831–839
31. Fässler R, Schnegelsberg PN, Dausman J, Shinya T, Muragaki Y,

McCarthy MT, Olsen BR, Jaenisch R: Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. Proc Natl Acad Sci USA 1994, 91:5070–5074
32. Hankenson KD, Hormuzdi SG, Meganck JA, Bornstein P: Mice with a disruption of the thrombospondin 3 gene differ in geometric and biomechanical properties of bone and have accelerated development of the femoral head. Mol Cell Biol 2005, 25:5599–5606
33. Svensson L, Aszodi A, Heinegard D, Hunziker EB, Reinholt FP, Fassler R, Oldberg A: Cartilage oligomeric matrix protein-deficient mice have normal skeletal development. Mol Cell Biol 2002, 22:4366–4371
34. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO: Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 1998, 101:982–992
35. Nishiwaki T, Yamaguchi T, Zhao C, Amano H, Hankenson KD, Bornstein P, Toyama Y, Matsuo K: Reduced expression of thrombospondins and craniofacial dysmorphism in mice overexpressing Fra1. J Bone Miner Res 2006, 21:596–604
36. Hankenson KD, Bain SD, Kyriakides TR, Smith EA, Goldstein SA, Bornstein P: Increased marrow-derived osteoprogenitor cells and endosteal bone formation in mice lacking thrombospondin 2. J Bone Miner Res 2000, 15:851–862
37. van der Weyden L, Wei L, Luo J, Yang X, Birk DE, Adams DJ, Bradley A, Chen Q: Functional knockout of the matrilin-3 gene causes premature chondrocyte maturation to hypertrophy and increases bone mineral density and osteoarthritis. Am J Pathol 2006, 169:515–527
38. Adolph KW, Bornstein P: The human thrombospondin 3 gene: analysis of transcription initiation and an alternatively spliced transcript. Mol Cell Biol Res Commun 1999, 2:47–52
39. Dalla-Torre CA, Yoshimoto M, Lee CH, Joshua AM, de Toledo SR, Petrilli AS, Andrade JA, Chilton-MacNeill S, Zielenska M, Squire JA: Effects of THBS3, SPARC and SPP1 expression on biological behavior and survival in patients with osteosarcoma. BMC Cancer 2006, 6:237
40. McCarthy JJ, Parker A, Salem R, Moliterno DJ, Wang Q, Plow EF, Rao S, Shen G, Rogers WJ, Newby LK, Cannata R, Glatt K, Topol EJ: Large scale association analysis for identification of genes underlying premature coronary heart disease: cumulative perspective from analysis of 111 candidate genes. J Med Genet 2004, 41:334–341
41. Stenina OI, Desai SY, Krukovets I, Kight K, Janigro D, Topol EJ, Plow EF: Thrombospondin-4 and its variants: expression and differential effects on endothelial cells. Circulation 2003, 108:1514–1519
42. Unger S, Hecht JT: Pseudoachondroplasia and multiple epiphyseal dysplasia: new etiologic developments. Am J Med Genet 2001, 106:244–250
43. Nakata K, Ono K, Miyazaki J, Olsen BR, Muragaki Y, Adachi E, Yamamura K, Kimura T: Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing αl(IX) collagen chains with a central deletion. Proc Natl Acad Sci USA 1993, 90:2870–2874
44. Aszodi A, Hunziker EB, Brakebusch C, Fassler R: Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. Genes Dev 2003, 17:2465–2479
45. Mankin HJ, Dorfman H, Lippiello L, Zarins A: Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips: II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg 1971, 53:523–537
46. Hu K, Xu L, Cao L, Flahiff CM, Brussiau J, Ho K, Setton LA, Youn I, Guilak F, Olsen BR, Li Y: Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. Arthritis Rheum 2006, 54:2891–2900
47. Opolka A, Ratzinger S, Schubert T, Spiegel HU, Grifka J, Bruckner P, Probst A, Grassel S: Collagen IX is indispensable for timely maturation of cartilage during fracture repair in mice. Matrix Biol 2007, 26:85–95
48. Wagener R, Ehlen HW, Ko YP, Kobbe B, Mann HH, Sengle G, Paulsson M: The matrilins: adaptor proteins in the extracellular matrix. FEBS Lett 2005, 579:3323–3329
49. Lachman RS, Krakow D, Cohn DH, Rimoin DL: MED, COMP, multilayered and NEIN: an overview of multiple epiphyseal dysplasia. Pediatr Radiol 2005, 35:116–123
50. Dinser R, Zaucke F, Kreppel F, Hultenby K, Kochanek S, Paulsson M, Maurer P: Pseudoachondroplasia is caused through both intra- and extracellular pathogenic pathways. J Clin Invest 2002, 110:505–513
51. Posey KL, Davies S, Bales ES, Haynes R, Sandell LJ, Hecht JT: In vivo human cartilage oligomeric matrix protein (COMP) promoter activity. Matrix Biol 2005, 24:539–549