ABSTRACT: Biglycan is an extracellular ligand for the dystrophin-associated protein complex (DAPC) that is upregulated in both dystrophic and regenerating muscle. Biglycan also binds to collagen VI, mutations of which cause a congenital muscular dystrophy (Ullrich's; UCMD) that is also characterized by connective tissue abnormalities. The expression of biglycan in early development and postnatal ages has not been well characterized. Here we show that biglycan transcript levels peak at ~21 weeks' gestation in human fetal muscle. Immunocytochemical analysis of developing mouse muscle shows that biglycan can be detected in muscle as early as embryonic day (E)16 and is most abundant between postnatal day (P)1 and P7. Biglycan is also highly expressed in developing tendon, with maximal levels observed at E16–18. This robust tendon expression is correlated with a sharp peak in biglycan transcript levels in the hindlimb. Finally, at E18 collagen VI colocalizes with biglycan in tendon. These results suggest that biglycan has a particularly important function during muscle and connective tissue development. Moreover, biglycan may play a role in the pathogenesis of collagen VI–associated congenital muscular dystrophies.
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DEVELOPMENTAL REGULATION OF BIGLYCAN EXPRESSION IN MUSCLE AND TENDON

BEATRICE E. LECHNER, MD,[1] JAE H. LIM,[2] MARY LYNN MERCADO, PhD,[2] and JUSTIN R. FALLON, PhD[2]

[1] Department of Pediatrics, Women and Infants Hospital, Providence, Rhode Island, USA
[2] Department of Neuroscience, Brown University, 190 Thayer St., Box 1953, Providence, Rhode Island 02912, USA

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Mutations in genes encoding or modifying components of the dystrophin-associated protein complex (DAPC) are the cause of most muscular dystrophies. Mutations of DAPC components such as dystrophin or any of the four sarcoglycans cause certain muscular dystrophies (Duchenne's; limb-girdle muscular dystrophy 2-D, -E, -C, and -F), which most often present clinically in early childhood. These mutations typically result in loss of the protein product, disruption of DAPC assembly and function, and ultimately damage and death of myofibers.[9] Congenital muscular dystrophies present at birth and are more severe. Many of these also result from insults to the DAPC, including mutations in the basal lamina component laminin-2 (congenital muscular dystrophy 1A),[9] and α-dystroglycan glycosylation,[22] a modification necessary for its association with the basal lamina. Mutations in collagen VI, which to date have not been linked to the DAPC, are the cause of Ullrich's congenital muscular dystrophy (UCMD),[6] which is characterized not only by muscular dystrophy but also by joint hyperlaxity and contractures. Thus, collagen VI is likely to play important roles in both muscle and connective tissue.[3]

Biglycan is a small leucine-rich proteoglycan (SLRP) that is a component of the extracellular matrix (ECM) in a variety of tissues including muscle, bone, and connective tissue.[28,35] Defects in biglycan expression are associated with osteoporosis, osteoarthritis, tendon abnormalities, and corneal diseases.[2] Dysfunctional biglycan expression is associated with the pathogenesis of atherosclerosis[23] and renal fibrosing disease.[27] Biglycan is comprised of a 38-kDa polypeptide core containing 10 leucine-rich repeats flanked by two cysteine-rich domains and two chondroitin or dermatan sulfate side chains. Non–glycosaminoglycan-bearing biglycan is also expressed in some tissues.[16] Biglycan is likely to play

Abbreviations: DAPC, dystrophin-associated protein complex; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; SLRP, small leucine-rich proteoglycan; UCMD, Ullrich's congenital muscular dystrophy

Key words: biglycan; collagen VI; development; dystrophin-associated protein complex; muscular dystrophy

Correspondence to: J. R. Fallon; e-mail: Justin_Fallon@brown.edu

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both signaling and structural roles. For example, in mouse bone biglycan is required for bone morphogenic protein 4 binding and osteoblast activation, and in *Xenopus* embryos biglycan improves the ability of chordin to inhibit bone morphogenic protein 4.[8,21] Finally, biglycan associates with matrix molecules, including collagen VI.[31,32]

Several observations suggest that biglycan plays an important role in muscle and perhaps in muscular dystrophy. We have previously demonstrated that biglycan binds to α-dystroglycan, a component of the DAPC.[5] Biglycan also binds, via its polypeptide core, to α- and γ-sarcoglycan. These two sarcoglycans are also selectively and transiently reduced in early postnatal development (Rafii et al., submitted). Biglycan-null mice also display tendon abnormalities.[1] Moreover, biglycan is upregulated in regenerating adult mouse muscle and biglycan-deficient mice display delayed regeneration following injury.[7] Finally, biglycan is upregulated in dystrophic mouse muscle as well as in human muscle,[5,13,37,36] and mice overexpressing biglycan exhibit aberrant eyelid muscle development.[14]

We have previously characterized the developmental pattern of biglycan in mouse muscle from 2 weeks postnatally,[7] and others have also characterized biglycan expression at certain points of development in muscle[4,12,7] and connective tissue[10,25,24,20,17] as well as in the whole embryo during the early stages of development.[33] However, a systematic study of prenatal and early postnatal pattern of biglycan expression has not been made in either humans or mice. This knowledge is particularly important in view of the interaction of biglycan with collagen VI,[31] mutations in which cause UCMD. Here, we show that biglycan is present at the murine sarcolemma and perimysium as early as the embryonic period, is strongly developmentally regulated, and is highly expressed in developing tendon. Furthermore, human fetal muscle demonstrates developmental regulation in skeletal muscle as well as the diaphragm. These results suggest that the role of biglycan may be especially prominent during muscle and tendon development.

MATERIALS AND METHODS

RNA and cDNA Preparation. Wildtype C3H mouse muscle at three prenatal and five postnatal time points [embryonic days (E)14, E16, E18, postnatal days (P)1, P7, P14, P21, and P35] was dissected in 0.1 M phosphate-buffered saline (PBS), pH 7.4, then transferred to a microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at −80°C. For the E14 and E16 time-points, the entire hindlimb minus the foot was harvested. For the E18 time-point the entire hindlimb minus foot and overlying skin was harvested. For each subsequent time-point, the quadriceps femoris muscle was harvested. Experiments were performed according to institutional IACUC guidelines. Human fetal muscle of various ages was harvested at autopsy and snap-frozen in liquid nitrogen. Institutional Review Board approval was obtained. RNA extraction was performed using the Trizol method (Invitrogen, Carlsbad, California). Pooling of six pairs of hindlimbs for the murine embryonic ages, three pairs of quadriceps femoris muscles for the P1 point, and one individual quadriceps femoris muscle for each of the subsequent ages was performed. Genomic DNA was removed by incubating the RNA sample with DNase I (Invitrogen) for 30 min at 37°C with subsequent RNA reextraction with Trizol (Invitrogen). The purified RNA was converted to cDNA using the Superscript III First-Strand Synthesis System Kit (Invitrogen).

Quantitative PCR Analysis. Quantitative polymerase chain reactions (qPCR) were performed on the ABI PRISM 7300 real-time thermocycler using the SYBR-Green method (Invitrogen) for human biglycan and the LUX primer method (Invitrogen) for mouse biglycan experiments. Primers were designed using DS Gene primer design software (Accelrys, San Diego, California). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizer (human GAPDH primer; Maxim Biotech, South San Francisco, California).

Mouse biglycan and GAPDH primers were labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxy-fluorescein (JOE), respectively. Data analysis was performed using the standard curve method for the SYBR-Green experiments and the comparative Ct method with a validation experiment for the mouse biglycan experiments. Experiments were performed in triplicate.

Primer Sequences. Mouse biglycan LUX forward (labeled with FAM): 5′-GAA CAA CTG CCA CCG CCA TTG-3′; mouse biglycan reverse: 5′-CAA GCA GAG CCC AGG AGA GC-3′; mouse GAPDH LUX forward (labeled with JOE): 5′-CAA CAG CAA CTC CCA TTC TTC C-3′; mouse GAPDH reverse: 5′-AAG GGC ATC CTG GGC TAC AC-3′; human biglycan forward: 5′-TGT GTG TGT GTC TTG TGC TT-3′; human biglycan reverse: 5′-AGT GAA AGG GAC AGG CGA AG-3′.

Immunohistochemistry. Wildtype C3H mouse tissue was harvested at three prenatal and four postnatal time points (E14, E16, E18, P1, P7, P14, and P21). For the E14 through P1 points, the entire embryo was harvested. For the P7 through P21 points, the quadriceps femoris muscle was dissected out individually. The specimens were then flash-frozen in isopentane and stored at −80°C. The frozen tissue was cryostat sectioned to 10-μm thickness, mounted on slides, and stored at −20°C. Sections were fixed in 1% paraformaldehyde and stained with primary antibodies using the Mouse on Mouse immunostaining kit for monoclonal antibodies (Vector Laboratories, Burlingame, California), then incubated with primary antibody overnight at 4°C and with secondary antibody for 30 min at room temperature. Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). The following antibodies were used: monoclonal antimouse developmental myosin heavy chain antibody (Novocastra, Newcastle upon Tyne, UK), monoclonal antimouse biglycan antibody (Fallon Laboratory), polyclonal antirabbit collagen VI antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania), and polyclonal antirabbit dystrophin antibody (Abcam, Cambridge, Massachusetts). Secondary antibody labeling was performed with goat antimouse IgG conjugated to Alexa 488 (Molecular Probes, Eugene, Oregon) for biglycan and developmental myosin heavy chain, goat antimouse IgG conjugated to CY3 (Jackson Immunoresearch, West Grove, Pennsylvania) for biglycan for the coimmunostaining experiments, goat antirabbit IgG conjugated to CY3 (Jackson Immunoresearch) for collagen VI, and goat antirabbit IgG conjugated to Alexa 488 (Molecular Probes) for dystrophin. Mouse IgG and rabbit IgG (Vector Laboratories), respectively, were used as controls.

Light Microscopy. Light, phase contrast, and fluorescent microscopy was performed using a Carl Zeiss Axiovert 200M light/fluorescence microscope (Göttingen, Germany) or a Nikon Eclipse E800 microscope (Melville, New York). Images were acquired with Scanalytics IP Lab Spectrum software (Fairfax, Virginia) or Carl Zeiss Axiovision 4.2 software (Göttingen, Germany).

Electron Microscopy. Transmission electron microscopy was performed as follows. The E18 mouse hindlimb, with skin removed, was fixed in Karnovsky's fixative for several days. Limb cross-sections, ~1 mm thick, were prepared and rinsed in 0.15 M sodium cacodylate buffer. Tissue was postfixed with 1% osmium tetroxide for 1 h at 4°C. Samples were rinsed in buffer, dehydrated through a graded acetone series, and infiltrated with Spurr's epoxy resin. Tissue blocks were allowed to polymerize overnight in a 70°C oven. Using a Reichert Ultracut S, ultrathin sections were prepared and placed on 300 mesh thin bar copper grids. The sections were stained with 5% uranyl acetate in methanol followed by lead citrate. These sections were examined using a Morgagni 268 electron microscope.

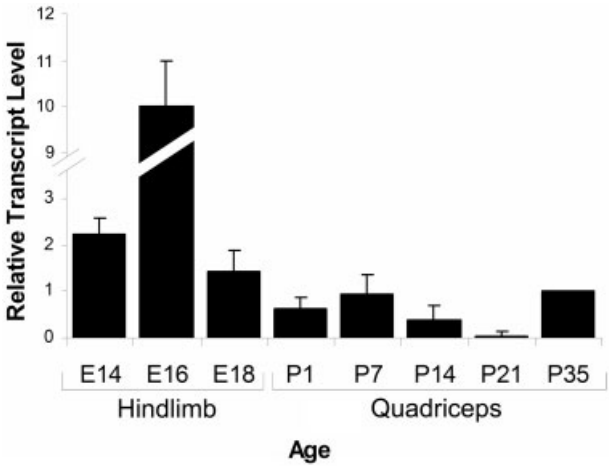

FIGURE 1. Expression of biglycan transcript in mouse hindlimb (E14–18) and isolated muscle (P1–35). Transcript levels were determine by quantitative real-time PCR and are normalized to ATP synthase. Expression is shown relative to that at P35. Note the striking spike in expression at E16 followed by a decrease in biglycan transcript expression during subsequent embryonic and postnatal development.

RESULTS

Biglycan mRNA and Protein Expression in Developing Mouse Hindlimb Muscle. We first determined the expression of biglycan mRNA during development. Quantitative real-time PCR (qRT-PCR) experiments were performed on RNA extracted from tissue at eight time-points: E14, 16, and 18 (entire hindlimb) and P1, 7, 14, 21, and 35 (isolated quadriceps). This period corresponds to the development of the muscle and connective tissue elements through adulthood. As shown in Figure 1, biglycan transcript was present at E14 and exhibited striking 5-fold upregulation at E16. By E18, the transcript levels dropped 5-fold and then diminished gradually until reaching their lowest levels at P21. This transient upregulation in biglycan mRNA in E16 animals was observed in three embryos from separate litters.

We used immunohistochemistry to determine the localization of biglycan protein expression in developing muscle. At E14, biglycan protein expression was not detected in muscle. At E16, intracellular

biglycan staining in close proximity to the sarcolemma was detectable. At E18 and P1, no sarcolemmal biglycan expression was detected. At P7, minimal sarcolemmal expression of biglycan was detectable, which increased at each subsequent timepoint. By P21, although sarcolemmal biglycan expression was not present in all myofibers, it was evident in many fibers (Fig. 2). Furthermore, biglycan expression is also evident at two other locations within muscle tissue. At E16, biglycan shows localization to the perimysium and the ECM. At E18, prominent biglycan expression was detected in the ECM surrounding the sarcolemma as well as at the perimysium. The level of expression in the ECM peaked at P1 and subsequently diminished. Thus, two patterns of biglycan expression become evident. First, peak total biglycan expression in muscle (including the sum of ECM and sarcolemmal staining) occurred perinatally. Second, a shift from biglycan ECM expression to sarcolemmal expression occurred during the course of development.

Although the levels of biglycan in muscle increased from E16 through P7, we did not detect any burst in biglycan protein expression in this tissue that would be consistent with the mRNA expression profile observed in whole hindlimb (Fig. 1). We therefore examined biglycan protein expression in the whole hindlimb at E16–18. Figure 3a,c shows that at E18 prominent biglycan immunoreactivity was observed in the periosteum as well as in circumscribed populations of cells intimately associated with the muscle. At these stages the signal was much stronger in these connective tissue structures than in the muscle itself. A similar pattern was observed at E16.

We sought to identify the biglycan-rich structures that were closely associated with the muscle. Figure 3a,b shows that these areas, even when within the boundaries of the muscle mass, did not express developmental myosin heavy chain, a marker of muscle fibers. Further, analysis of sequential sections for biglycan immunoreactivity and with trichrome stain revealed that the intramuscular, biglycan-rich regions showed the blue/purple staining characteristic of connective tissue (Fig. 3c,d). Phase contrast microscopy at E18 showed that these regions were comprised of mononucleated cells and lacked myofibers (Fig. 4c). Ultrastructural analysis showed that this juxta-muscular tissue was comprised of fibroblasts surrounded by collagen fibers. Further, the myofiber sarcolemma directly apposed to these cells showed thickening characteristic of myotendinous junctions (Fig. 5). Together, these findings indicate that this nonmuscle, biglycan-rich tissue was developing tendon.

Biochemical studies have shown that collagen VI binds to biglycan.[31] Therefore, we double-labeled E18 hindlimb with antibodies to biglycan and collagen VI. In muscle, collagen VI was detected around the sarcolemma of virtually all myofibers. In contrast, biglycan immunoreactivity at this stage encircled groups of myofibers that often were comprised of a single large and two or three smaller fibers (Fig. 4), which are likely to correspond to primary and secondary myofibers, respectively. Biglycan immunoreactivity at the sarcolemma was weaker and only present around individual myofibers. Finally, biglycan expression was prominent in the tendon, where it colocalized with collagen VI.

Biglycan Is Developmentally Regulated in Human Fetal Diaphragm and Skeletal Muscle. We examined the developmental pattern of biglycan in human muscle. We analyzed gene expression of biglycan in skeletal muscle and diaphragm. In each muscle type we examined samples between 17 weeks and 36 weeks gestational age. Both skeletal muscle and diaphragm displayed a similar pattern of expression: high biglycan transcript levels early in the second trimester, which subsequently fell to low relative levels by the end of the third trimester, suggesting developmental regulation (Fig. 6).

DISCUSSION

We have demonstrated that biglycan is developmentally regulated in muscle and is also strongly expressed in embryonic tendon. Understanding the developmental time course of biglycan expression in muscle and connective tissue is important for understanding the role of biglycan in development. Moreover, these results are pertinent to our understanding of congenital muscular dystrophies, such as those caused by collagen VI mutations, which show both muscle and connective tissue abnormalities.

Biglycan expression in mouse muscle shows a complex pattern (Fig. 2). Prenatally, it is most prominent intracellularly, in the ECM, perimysium, and in the tendon (Figs. 2, 4). Postnatally, while still present in the ECM, biglycan invests individual myofibers and reaches peak expression at the sarcolemma at P14–21. Although total biglycan expression in muscle (connective tissues and sarcolemma) declines after a perinatal peak, it remains detectable at the adult rodent[5,7] and human[36,37] sarcolemma. The high expression of biglycan in early postnatal stages suggests that it may play a particular role at this time,

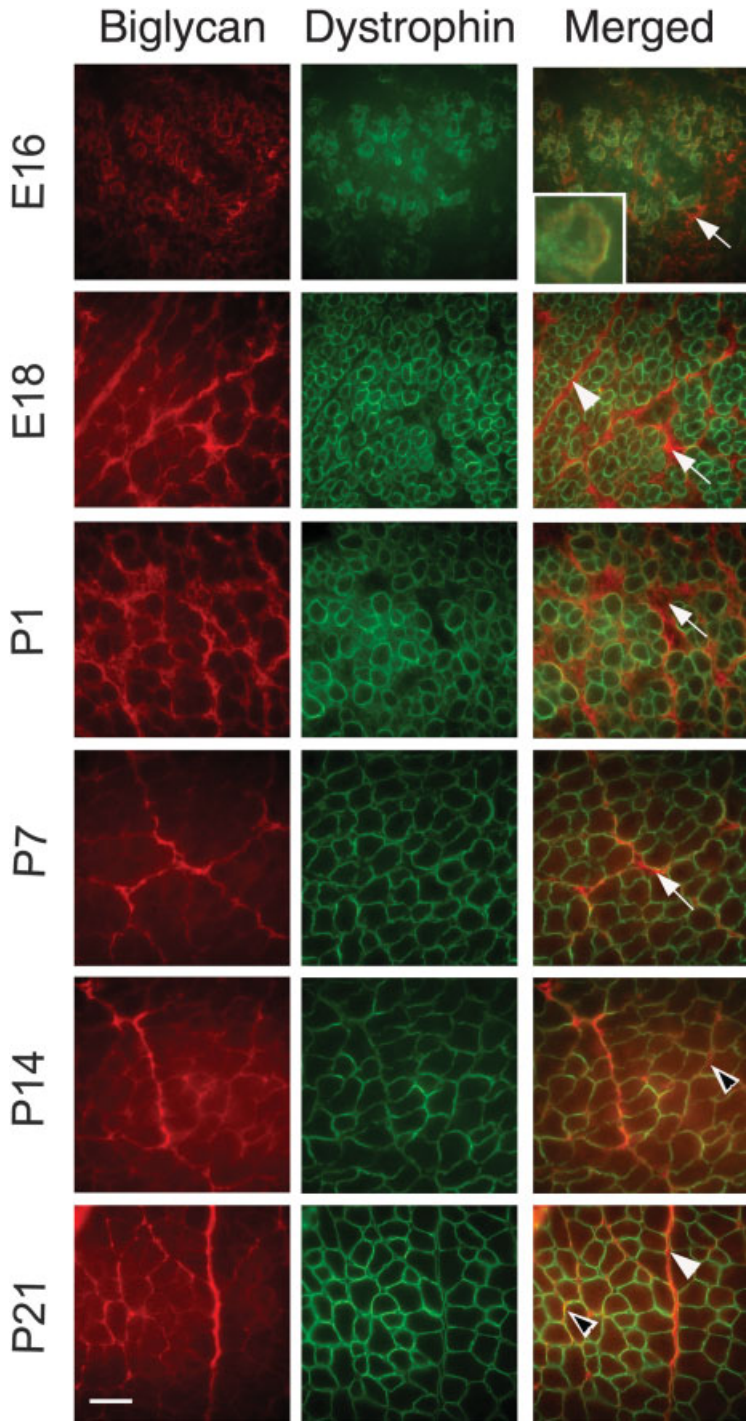

FIGURE 2. The time-course of expression and localization of biglycan protein in developing mouse muscle. Sections from hindlimb of the indicated age were double-labeled with antibodies to biglycan and to dystrophin (to visualize the sarcolemma). Biglycan immunoreactivity is not detected at E14 (data not shown). At E16 biglycan staining is prominent in the connective tissue and in the muscle cells intracellularly in close proximity to the sarcolemma (inset: a magnified myofiber). At E18, immunostaining is present in the perimysium as well as in the extracellular matrix between small groups of muscle fibers. This pattern persists at P1. By P7, biglycan staining is present at the sarcolemma of a subgroup of fibers. At P14 and P21, biglycan staining is evident at the sarcolemma of most fibers. Total biglycan expression in muscle (connective tissue and sarcolemma) peaks at E18 to P1. $\times$40. Scale bar, 50 μm. Arrows indicate ECM staining. Arrowheads indicate perimysial staining. Open arrowhead, sarcolemmal staining.

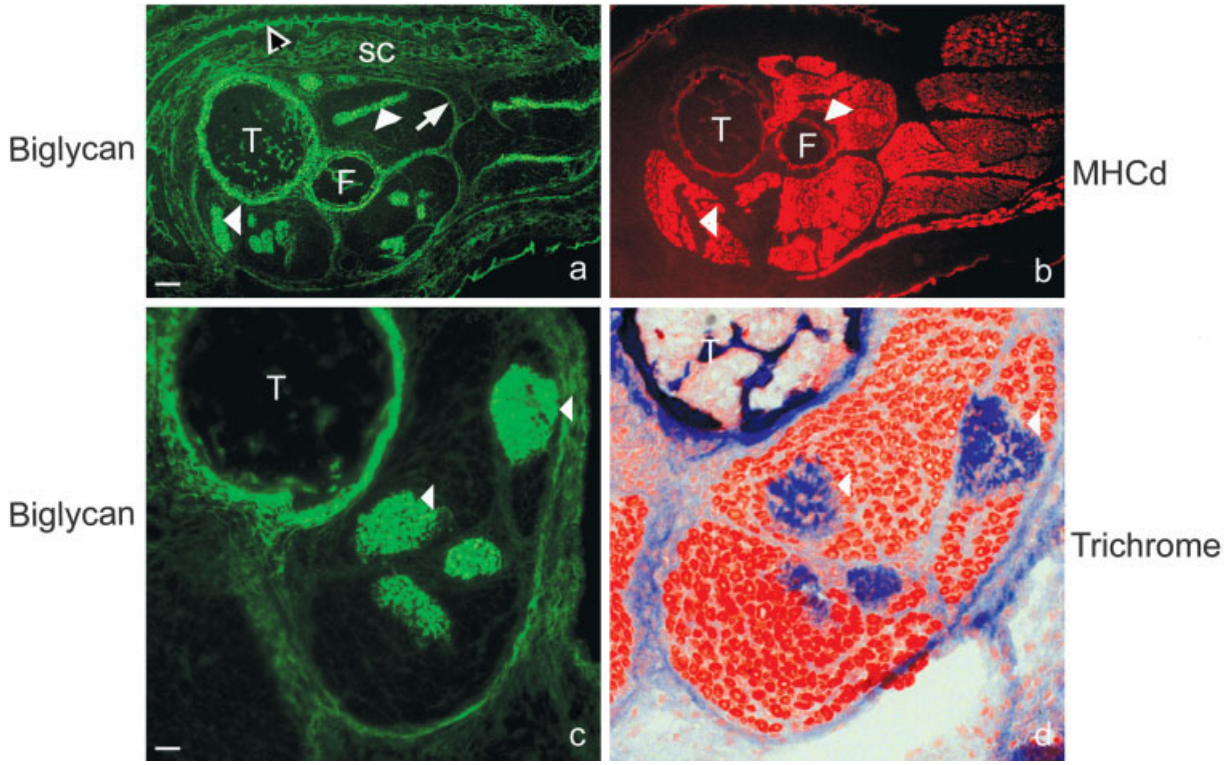

FIGURE 3. Biglycan is expressed in developing tendon (E18). **(a)** Prominent biglycan immunoreactivity is present in distinct "islands" (arrowheads) that are within the anatomical boundary of these immature muscles. Biglycan immunoreactivity is also observed in bone (e.g., T,F), skin (open arrowhead), perimysium (arrow), and subcutaneous connective tissue (SC). Magnification ×5. **(b)** Developmental myosin heavy chain antibody (MHCd) is present throughout muscle bundles, but is excluded from the domains (arrowheads) of intense biglycan immunoreactivity (compare with **a**). Both **(a)** and **(b)** are consecutive sections. Magnification ×5. Scale bar = 100 μm. **(c)** Higher magnification of anti-biglycan stained "islands" (arrowheads) at the edge of the muscle bundles. Magnification ×20. **(d)** Gomori's trichrome histochemistry demonstrating purple staining (arrowheads) corresponding to anti-biglycan stained "islands" in the consecutive section. Purple staining indicates collagen-rich tissue. Both **(c)** and **(d)** are consecutive sections. Magnification ×20. Scale bar, 20 μm. T, tibia, F, fibula.

raising the possibility that biglycan could play a prominent role in the immature, dystrophin-independent DAPC that is expressed up to ~P21.[11]

Biglycan is highly expressed in developing tendon. At E16–18, tendons are prominent and run the entire length of muscle (Fig. 3) and likely represent the striking upregulation of biglycan transcript seen at E16 (Fig. 1). We confirmed the identity of these structures by: (1) their lack of sarcomeric myosin expression (Fig. 3); (2) their high expression of the ECM molecules biglycan and collagen VI (Fig. 4); (3) their apposition to myotendinous junctions; and (4) their characteristic cellular (fibroblast) and extracellular (collagen fibrils) composition (Fig. 5). Moreover, these structures are similar in appearance and disposition to those expressing scleraxis, a transcription factor that marks the tendon lineage.[26] Although the precise function of biglycan in tendons is not known, it could serve both a structural and a signaling role. For example, collagen fibrils are disordered in biglycan null mice.[1] Moreover, biglycan can modulate bone morphogenic protein signaling,[21] which plays important roles in regulating tendon development.[26]

Several observations suggest that the coexpression of biglycan and collagen VI in developing tendon and muscle is of functional importance. Biochemically, biglycan and collagen VI have been shown to bind one another.[31,32] Moreover, collagen VI gene expression in muscle shows a similar developmental pattern to that which we have observed for biglycan.[19] These two molecules are also colocalized in tendon and muscle.[30,34,29,18] Taken together with the genetic studies discussed above, these findings raise the possibility that biglycan and collagen VI may cooperate in muscle and tendon development.

Ullrich's congenital muscular dystrophy is caused by mutations in collagen VI and is associated with muscle wasting and connective tissue anomalies such as hyperlaxity. However, in a subset of patients with

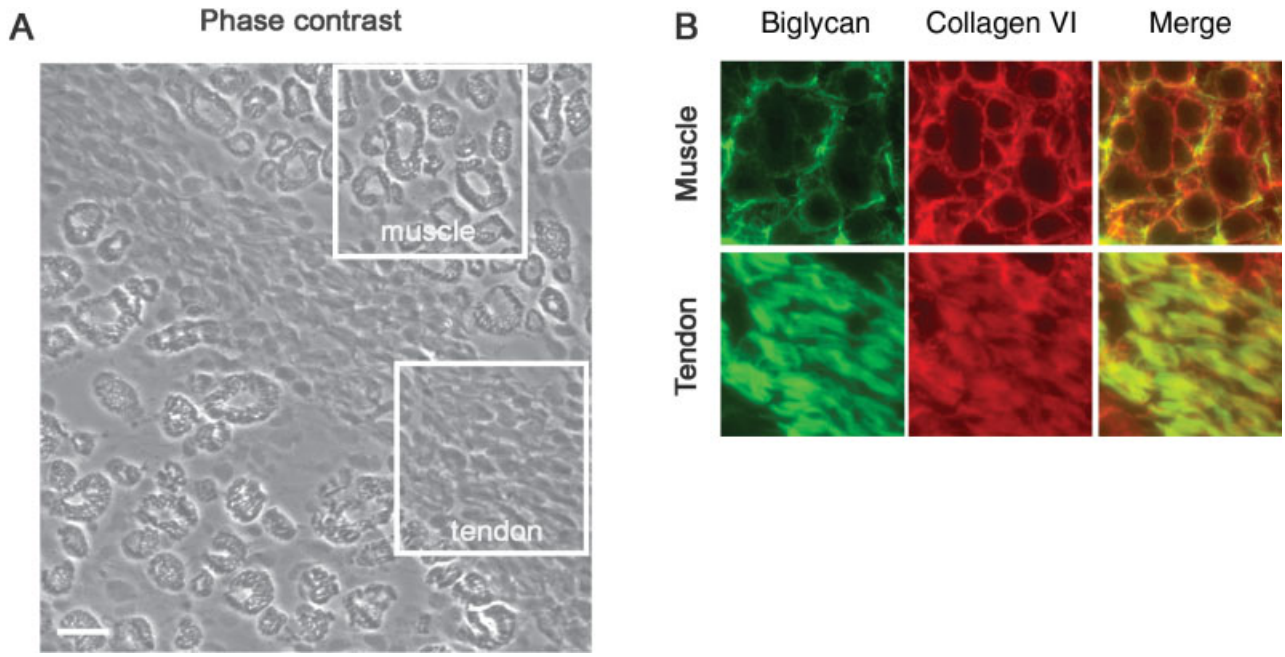

FIGURE 4. Biglycan and collagen VI staining in E18 tendon and muscle. **(A)** A tendon is shown coursing between two domains of muscle by phase contrast microscopy, demonstrating distinct morphology of the cellular subgroup that represents tendon. **(B)** Biglycan immunoreactivity in muscle demonstrates the presence of biglycan in the ECM surrounding groups of myofibers, as well as the subtle presence at the sarcolemma of a few myofibers, while collagen VI is present at the sarcolemma of all fibers, as well as in the ECM, where it colocalizes with biglycan. Biglycan shows much stronger immunoreactivity in tendon as compared to muscle at this stage, where it colocalizes with collagen VI. Magnification ×40. Scale bar, 20 μm.

UCMD, collagen VI is expressed but not appropriately localized to the sarcolemma.[15] We have shown that biglycan is developmentally regulated in human muscle, is developmentally expressed at the protein level in mouse muscle, is strongly expressed in embryonic tendon, and is associated with collagen VI in

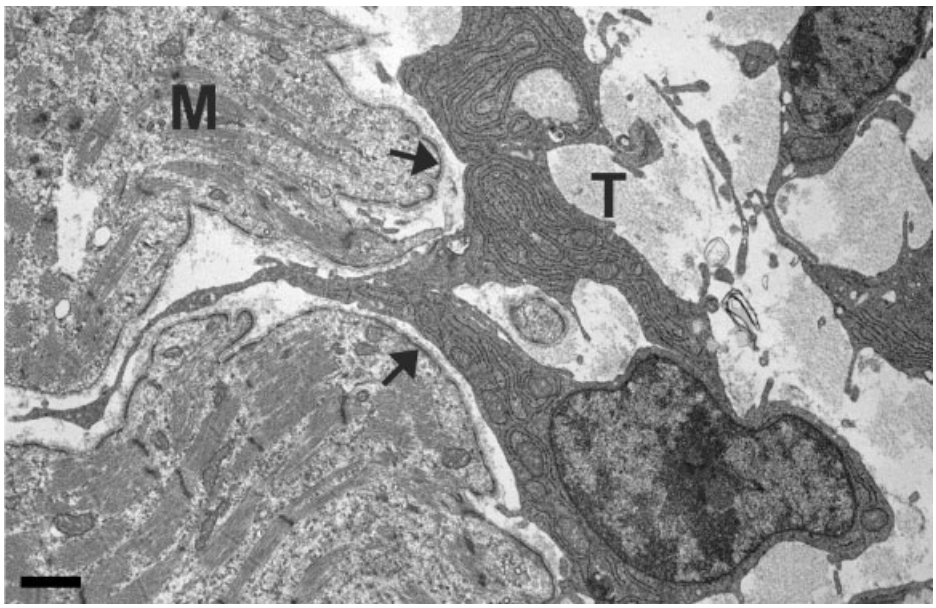

FIGURE 5. Electron microscopy of the developing tendon and myotendinous junction. Fibroblasts that are surrounded by collagen fibers are intercalated with myofibers. Note also the thickened, electron-dense material at the sarcolemmal surface that is characteristic of the myotendinous junction. T, tendon. M, muscle. Arrow indicates neuromuscular junction. Magnification ×4,400. Scale bar, 2 μm.

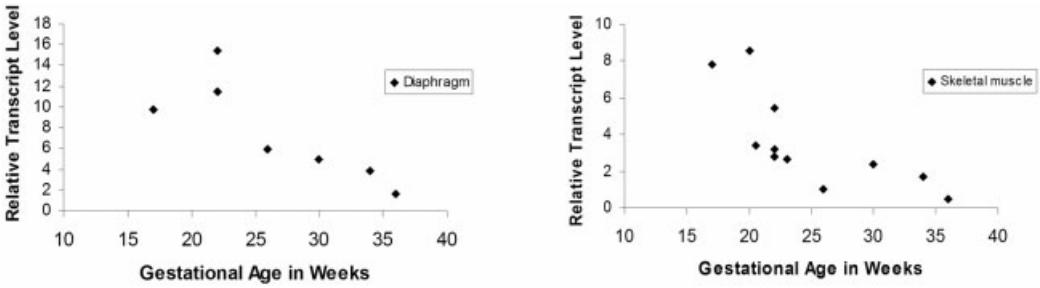

FIGURE 6. Levels of mRNA encoding biglycan in human fetal diaphragm and skeletal muscle. Transcript levels were determined using quantitative real-time PCR and normalized to GAPDH. Levels are shown relative to one specific sample (26-week psoas). A steady decline in biglycan gene transcription is present during the course of fetal development from the early second through the late third trimester.

the embryonic tendon. Although primary biglycan mutations have not been observed, our results suggest that such mutations may be the underlying defect in phenotypically Ullrich-like congenital muscular dystrophies.

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