Latent TGF-β binding protein 4 promotes elastic fiber assembly by interacting with fibulin-5

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Elastic fiber assembly requires deposition of elastin monomers onto microfibrils, the mechanism of which is incompletely understood. Here we show that latent TGF-β binding protein 4 (LTBP-4) potentiates formation of elastic fibers through interacting with fibulin-5, a tropoelastin-binding protein necessary for elastogenesis. Decreased expression of LTBP-4 in human dermal fibroblast cells by siRNA treatment abolished the linear deposition of fibulin-5 and tropoelastin on microfibrils. It is notable that the addition of recombinant LTBP-4 to cell culture medium promoted elastin deposition on microfibrils without changing the expression of elastic fiber components. This elastogenic property of LTBP-4 is independent of bound TGF-β because TGF-β–free recombinant LTBP-4 was as potent an elastogenic inducer as TGF-β–bound recombinant LTBP-4. Without LTBP-4, fibulin-5 and tropoelastin deposition was discontinuous and punctate in vitro and in vivo. These data suggest a unique function for LTBP-4 during elastic fibrogenesis, making it a potential therapeutic target for elastic fiber regeneration.

connective tissue | development | extracellular matrix

Elastic fibers are ubiquitous extracellular matrix (ECM) components responsible for tissue elasticity. Elastic fibers are composed of an amorphous core of cross-linked elastin and a mantle of fibrillin-rich microfibrils (1, 2). Degradation of elastic fibers causes emphysema, arterial stiffness, and loose skin. The turnover rate of elastic fibers is very low, and there is no known method to effectively regenerate elastic fibers. The process of elastic fiber assembly involves synthesis and secretion of tropoelastin (elastin monomer), formation of tropoelastin microaggregates, initial cross-linking of elastin, deposition of microaggregated elastin on microfibrils, followed by late cross-linking of elastin by lysyl oxidase (2–4). Microfibrils are composed of polymers of fibrillin-1 and -2 as well as several associated molecules, such as microfibril-associating glycoproteins and latent TGF-β–binding proteins (LTBPs) (2, 5, 6), but the roles of these associated molecules in elastic fiber assembly remain obscure. Although microfibrils provide a scaffold for elastin deposition, microfibrils can also form macroaggregates devoid of elastin (5). Therefore, a key question for elastic fiber formation and regeneration is what promotes elastin deposition on microfibrils.

Several microfibril-associating molecules have been implicated in elastogenesis, including LTBP-4. Mice lacking LTBP-4S, the major form of LTBP-4 in lung and intestine (7), were reported to show severe defects in elastic fiber formation in the intestine, lung, and pulmonary artery (8, 9), indicating crucial function of LTBP-4 in elastic fiber assembly. LTBPs are large ECM proteins that share structural homology with fibrillins, and play major roles in regulating TGF-β bioavailability. Although the TGF-β

precursor protein is cleaved into the N-terminal prodomain called latency-associated peptide (LAP) and the C-terminal mature TGF-β, TGF-β is kept inactive by continued noncovalent association with LAP (TGF-β–LAP). In addition, LAP is bound to LTBPs through disulfide bonds; thus TGF-β is secreted from cells in the form of a large latent complex consisting of TGF-β–LAP-LTBP (6, 10, 11). Among the four members of the LTBP family, LTBP-1, -3, and -4 bind LAP and target latent TGF-β to extracellular matrix. LTBP-1, -2, and -3 are not considered necessary for elastogenesis as judged by the phenotypes of mice or humans deficient in these genes (6). *LTBP4* homozygous mutations in humans cause defective elastic fiber formation, resulting in cutis laxa and emphysema (12). However, how LTBP-4 is involved in elastic fiber assembly is largely unknown.

We and others previously reported that the secreted protein fibulin-5 is necessary for elastic fiber assembly (13, 14). *Fbln5* (the fibulin-5 gene) knockout mice exhibit stiff and tortuous aortae, emphysematous lungs, and loose skin due to disorganized elastic fibers. Fibulin-5 interacts with both tropoelastin (14) and lysyl oxidase–like enzymes (15, 16), and facilitates elastin deposition onto microfibrils and subsequent cross-linking (16). To understand the molecular mechanism of fibulin-5–dependent elastin deposition on microfibrils, we examined the binding of fibulin-5 with microfibril-associated molecules. In addition to tropoelastin and lysyl oxidase–like enzymes, fibulin-5 interacts with several other molecules, such as integrins (13, 17, 18), elastin microfibril interface located protein (EMILIN) (19), apolipoprotein(a) (20), extracellular superoxide dismutase (21), LTBP-2 (22), and fibrillin-1 (23). However, these interactions do not account for the fiber organizing function of fibulin-5, because mice deficient in these molecules do not phenocopy *Fbln5* knockout mice (24–27).

Here we report that LTBP-4 orchestrates elastin deposition onto microfibrils by binding with fibulin-5, which is bound to tropoelastin. We show that LTBP-4 interacts with fibulin-5 specifically through the four-cysteine domains of LTBP-4 and the C-terminal domain of fibulin-5. Knockdown of *LTBP4* by siRNA in human dermal fibroblasts abolished linear deposition of fibulin-5

and elastin on microfibrils, yielding a small number of punctate aggregates of these proteins. It is intriguing to note that the addition of recombinant LTBP-4 to *LTBP4* knockdown cell cultures strongly induced elastic fiber assembly to a level surpassing that observed in control cell cultures. We also demonstrate that the elastogenic activity of LTBP-4 is not dependent on associated TGF-β, but is attributed to LTBP-4 itself.

Results

LTBP-4 Interacts with Fibulin-5. Elastic fibers have been reported to be defective in lungs, intestines, skin, and pulmonary arteries of *Ltbp4S*$^{-/-}$ mice (8, 9). We also found tortuous and stiff aortae with severely disorganized elastic lamellae in *Ltbp4S*$^{-/-}$ mice (Fig. S1 *A–D*). These phenotypes, together with the emphysematous lungs, were very similar to the phenotypes of *Fbln5* knockout mice (13, 14). The phenotypic resemblance of *Ltbp4* and *Fbln5* knockout mice led us to study the interaction of fibulin-5 and LTBP-4. We found that LTBP-4 colocalizes with fibulin-5 in mouse lung and skin tissues (Fig. 1). In vitro binding assays using recombinant proteins of FLAG-tagged LTBP-4 and Myc-tagged fibulin-5 revealed interaction of these proteins (Fig. 2 *A–C*). When LTBP-4S and LTBP-4L, a transcript variant in the N-terminal region, were divided into five fragments, only the N-terminal fragments of LTBP-4S and -L interacted with fibulin-5 (Fig. 2*B*). We further subdivided the binding domain of LTBP-4 and fibulin-5 and found that the four-cysteine domains at the N terminus of LTBP-4S and L specifically interact with fibulin-5 (Fig. 2*C*). On the other hand, the C-terminal domain of fibulin-5 is involved in this interaction, because deletion of the C-terminal domain abolished the binding, and a C-terminal domain-Fc fusion protein was sufficient to bind with LTBP-4S (Fig. S2 *A–C*). These data demonstrate the specificity of binding between fibulin-5 and LTBP-4. The binding of fibulin-5 and LTBP-4 was confirmed with a solid-phase binding assay (Fig. S3*A*, and the purity of recombinant proteins was shown by SDS/PAGE in Fig. S4 *A and B*).

Recombinant LTBP-4S Protein Promotes Elastic Fiber Assembly. To study the function of LTBP-4 in elastic fiber assembly, we used neonatal human dermal fibroblasts (HDFs) that develop an elastic fiber meshwork when maintained at confluence (We use the term "elastic fibers" to refer to fibers containing linearly assembled and insoluble elastin as well as microfibrils that serve as scaffolds for linear deposition of elastin). We confirmed that gene knockdown with *LTBP4* siRNA transfection achieved more than 90% reduction of *LTBP4* mRNA expression at day 7 of culture, and remained at greater than 80% reduction at day 14 (Fig. S5*A*). Control siRNA transfection did not affect elastic fiber development (Fig. S6 *A and B*), as elastin was deposited linearly along LTBP-4–positive microfibrils in the ECM of control cells (the specificity of antibodies is shown in Fig. S4 *C–E*).
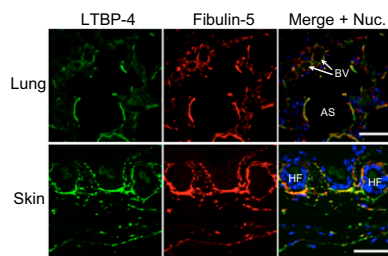

Fig. 1. LTBP-4 colocalizes with fibulin-5 in mouse tissues. Immunostaining of WT mouse lung at P0 (sagittal section) and mouse skin at 8 wk using anti–LTBP-4 antibody and anti–fibulin-5 antibody, indicating colocalization of these molecules. The lung figure presents LTBP-4 and fibulin-5 localization in terminal air sacs (AS). The circular structures present small blood vessels (BV). (Scale bars, 50 μm.) HF, hair follicle.
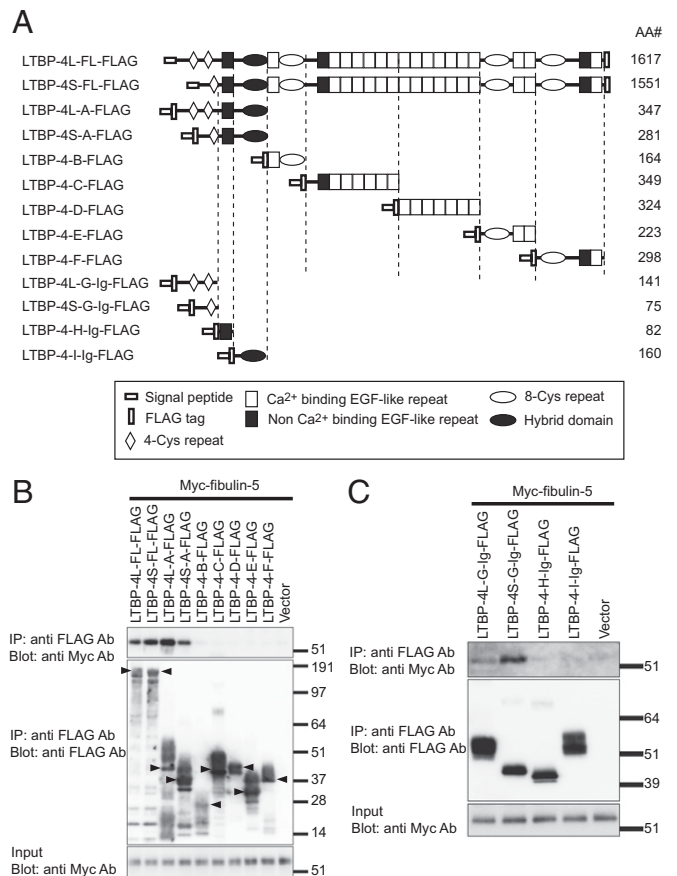

Fig. 2. In vitro binding assay, showing interaction of LTBP-4 with fibulin-5. (*A*) Domain structure of the full-length LTBP-4 and the LTBP-4 truncation mutants. These mutants were expressed as N-terminal FLAG-tagged proteins flanked by the preprotrypsin signal sequence. LTBP-4L-G, LTBP-4S-G, LTBP-4-H, and LTBP-4-I were constructed as C-terminal fusion proteins with the Fc region of human IgG. The amino acid lengths (AA#) of the constructs except IgG are indicated. The expression vectors were independently transfected into 293T cells. Transfected cells were cultured in serum-free medium for 48 h, and the cell lysates and the conditioned media were harvested. Mixtures of the media and cell lysates (*B*), or the media alone (*C*) were incubated with each other, and these reactants were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitants were separated by SDS/PAGE, and analyzed by Western blotting. (*B*) Interaction of fibulin-5 with full-length LTBP-4L and -4S and with the N-terminal domains of LTBP-4L and -4S, but not with other parts of LTBP-4. Expected bands for the LTBP-4 constructs are indicated by arrowheads. (*C*) Interaction of fibulin-5 with LTBP-4L and -4S fragments containing four-cysteine domains, but not with other N-terminal fragments.

However, *LTBP4* knockdown abolished deposition of elastin (Fig. 3 *A* and *B*). The addition of rLTBP-4S at 6 nM restored linear deposition of elastin on microfibrils, excluding the possibility that the knockdown result was an off-target effect (Fig. 3 *C* and Fig. S7*C*). To our surprise, the addition of rLTBP-4S at higher concentrations induced enhanced elastic fiber assembly in a dose-dependent manner to levels that exceeded assembly observed in control cells (Fig. 3 *C–E*). Elastin staining fully colocalized with LTBP-4 staining, and there was virtually no linear elastin deposition on LTBP-4–negative microfibrils (Fig. 3 *A–E* and Fig. S7 *A–E*). To quantify the amount of mature (i.e., cross-linked) elastic fibers induced by rLTBP-4S, we metabolically labeled newly synthesized elastin with [^{3}H]-valine, and measured the incorporation of [^{3}H]-valine into the culture NaOH-insoluble fraction, which corresponds to the amount of mature elastic fibers (28). As shown in Fig. 3*F*, we detected significant, dose-dependent incorporation of [^{3}H]-valine into the insoluble fraction upon
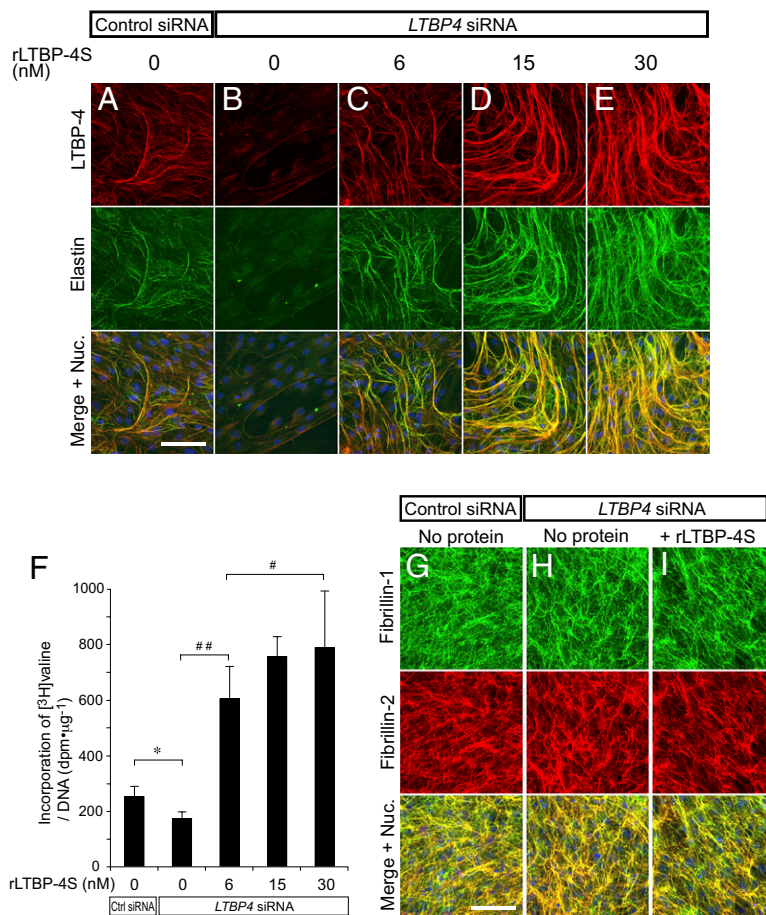
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Fig. 3. Recombinant LTBP-4S protein (rLTBP-4S) promotes elastic fiber assembly without changing the configuration of microfibrils. (*A–E*) HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured in 10% serum-containing media supplemented with rLTBP-4S as indicated. The cells were fixed 13 d after transfection and stained with anti–LTBP-4 and anti-elastin antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. Note even more elastic fiber assembly by rLTBP-4S-supplemented *LTBP4* knockdown (KD) cells than control KD cells, which express a normal level of LTBP-4. (Scale bar, 100 μm.) Nuc., nuclei. (*F*) Quantitation of insoluble (i.e., cross-linked and mature) elastin produced by the knockdown cells cultured with the indicated amounts of rLTBP-4S added to the medium. Cells were metabolically labeled with [³H]valine during the culture period, and the radioactivity of NaOH-insoluble fractions was quantitated. The radioactivity count was corrected by the amount of total DNA of the cells of duplicate plates. Insoluble elastin was significantly decreased in *LTBP4* KD culture (*$P = 0.0006$, Student's *t* test; $n = 6$, Ctrl KD; $n = 8$, *LTBP4* KD). Addition of rLTBP-4S in *LTBP4* KD culture increased insoluble elastin in a dose-dependent manner above the level of Ctrl KD (#$P < 0.05$, ##$P < 0.01$, analysis of variance, Tukey test, $n = 8$). Error bars represent SD; Ctrl, control. (*G–I*) HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured in 10% serum containing media with or without rLTBP-4S (30 nM). The cells were fixed 13 d after transfection and stained with anti–fibrillin-1 and -2 antibodies. (Scale bar, 100 μm.)

adding rLTBP-4 to the medium. This result demonstrated that the elastin-positive fibers induced by rLTBP-4 were mature and cross-linked. No overt differences in the configuration of microfibrils were observed after *LTBP4* knockdown or addition of rLTBP-4S, as indicated by fibrillin-1 and -2 antibody staining (Fig. 3 *G–I*), suggesting that rLTBP-4S turned elastin-negative microfibrils to elastin-positive fibers. Moreover, *LTBP4* knockdown or addition of rLTBP-4S did not affect the mRNA expression levels of elastic fiber components including *FBN1* (fibrillin-1), *FBLN4* and *-5* (fibulin-4 and -5), LOX (lysyl oxidase), and *LOXL1* (lysyl oxidase-like 1), whereas *ELN* (elastin) mRNA modestly increased 4 d after *LTBP4* knockdown (Fig. S5C). Abrogated elastin deposition in the absence of LTBP-4 and enhanced elastic fiber assembly by rLTBP-4S was also observed in cultures of adult HDFs (Fig. S6 *C–E*) and of wild-type and *Ltbp4S$^{-/-}$* mouse embryonic fibroblasts (MEFs) (Fig. S8 *A–C*). These data indicate that a substantial amount of microfibrils were formed and sufficient amounts of other elastic fiber components were expressed even in the absence of LTBP-4: however, microfibrils and elastic fiber components including elastin could not be assembled as elastic fibers without LTBP-4. Upon addition of rLTBP-4S, elastin and added rLTBP-4S colocalized with fibrillin-1 (Figs. S7 and S8I). This suggests that LTBP-4 promoted elastic fiber assembly by facilitating tropoelastin deposition on microfibrils (we term this property of LTBP-4 an "elastogenic" property).

Recombinant LTBP-4S Protein Contains TGF-β–LAP. We next investigated whether the elastogenic property of LTBP-4 is due to the function of LTBP-4 itself or to TGF-β associated with LTBP-4, as TGF-β is reported to increase expression of ECM components including elastic fibers (29), and LTBP-4 is reported to be

a modulator of extracellular TGF-β levels (12, 30). Because we obtained rLTBP-4S from 293T cells stably expressing LTBP-4S, the rLTBP-4S might contain considerable amounts of TGF-β–LAP endogenously expressed by 293T cells, although LTBP-4 is reported to bind much less TGF-β–LAP than do LTBP-1 or -3 (31, 32). To assess the amount of TGF-β bound to rLTBP-4S, we heated rLTBP-4S to release active TGF-β and measured it using a cell-based TGF-β bioassay system (33). We determined that the TGF-β activity bound to 48 nM of rLTBP-4S corresponded to that of 52 pM of active TGF-β1 (Fig. S9A), a concentration sufficient to induce mRNA expression of ECM components (34). Although addition of recombinant LTBP-4S did not affect the expression of elastic fiber components at 4 d of culture (Fig. S5C), it is difficult to know if this is the case throughout the culture period. Instead of monitoring all of the components throughout the culture period, we decided to make recombinant LTBP-4 without TGF-β, thereby eliminating the possible contribution of TGF-β by LTBP-4. To remove TGF-β activity from rLTBP-4S, we coexpressed LTBP-4 and an engineered empty LAP (eLAP) that does not contain TGF-β by introducing a stop codon at the cleavage site between LAP and TGF-β1. We presumed that the overexpressed eLAP in 293T cells would bind rLTBP-4S, excluding the binding of endogenous TGF-β–LAP in a dominant negative manner. As expected, rLTBP-4S purified from eLAP-overexpressing cells (rLTBP-4S + eLAP) did not show TGF-β activity (Fig. S9A).

Elastogenic Activity of LTBP-4 Is Independent from Bound TGF-β. To assess whether TGF-β bound to rLTBP-4S accounts for the elastogenic activity of rLTBP-4S, we first tested the effect of active TGF-β1 on *LTBP4* knockdown HDFs. Addition of active

TGF-β1 to the culture medium at 60 pM, which is a higher concentration of TGF-β than in medium containing rLTBP-4S at 30 nM, did not restore elastic fiber assembly (Fig. S9F); whereas, the addition of 30 nM of rLTBP-4S induced more elastic fiber assembly than that observed in cultures of control cells (Fig. S9D). Next, we added 30 nM of rLTBP-4S + eLAP to the culture medium of *LTBP4* knockdown HDFs, and found that HDFs supplemented with rLTBP-4S + eLAP induced as much elastic fiber assembly as HDFs supplemented with rLTBP-4S (Fig. S9 D and E). These data suggest that the elastogenic property of LTBP-4 is not dependent on associated TGF-β, but can be attributed to LTBP-4 itself.

LTBP-4 Is Required for Linear Deposition of Fibulin-5 and Elastin on Microfibrils. Immunostaining for fibulin-5 and LTBP-4 in the ECM of HDFs revealed that fibulin-5 specifically colocalized with LTBP-4 on microfibrils (Fig. 4A). When *LTBP4* was knocked down by siRNA, deposition of fibulin-5 was abolished (Fig. 4B). The addition of rLTBP-4S not only rescued but augmented the linear deposition of fibulin-5 (Fig. 4C). This was not accompanied by an increase of *FBLN5* mRNA expression (Fig. S5C), which suggests that LTBP-4 is necessary for fibulin-5 deposition on microfibrils. A requirement for LTBP-4 for fibulin-5 deposition was also observed in cultures of adult HDFs (Fig. S6 F–H) and of MEFs from *Ltbp4S*$^{-/-}$ and wild-type mouse embryos (Fig. S8 D–F). On the other hand, fibulin-5 is not necessary for LTBP-4 deposition, because knockdown of *FBLN5* did not affect deposition of LTBP-4 (Fig. 4E). Although *FBLN5* knockdown and *LTBP4* knockdown both resulted in abrogated fibulin-5 deposition, there were clear differences when recombinant fibulin-5 (rFibulin-5) was added in the culture media. The added rFibulin-5 in *FBLN5* knockdown cell cultures deposited linearly on microfibrils positive for LTBP-4 staining (Fig. 4F). However, when rFibulin-5 was added to *LTBP4* knockdown cell cultures, the added protein did not deposit linearly and displayed primarily a punctate pattern (Fig. 4D). The addition of rFibulin-5 to *LTBP4* knockdown cells also caused increased elastin deposition, but only in a punctate pattern (Fig. S10D). We observed this punctate deposition in the ECM of *LTBP4* knockdown cells even without rFibulin-5 addition, although the amount of aggregates was very low (Fig. 3B and Fig. S10B). The punctate deposition of elastin in the absence of LTBP-4 was far less efficient than the linear deposition of elastin in the presence of LTBP-4 found in the ECM of control cells (Fig.

Fig. 4. LTBP-4 is necessary for linear deposition of fibulin-5 to microfibrils. HDFs were transfected with control siRNA (A), *LTBP4* siRNA (B–D), or *FBLN5* siRNA (E, F), and cultured for 13 d in 10% serum containing media with or without recombinant proteins as indicated [rLTBP-4S 30 nM (C) or rFibulin-5 60 nM (D, F)], followed by immunostaining with anti–LTBP-4 and anti–fibulin-5 antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. Note the punctate pattern of rFibulin-5 deposition on microfibrils in *LTBP4* KD culture, in contrast to the linear deposition of rFibulin-5 in the presence of LTBP-4.

S10 A and B). To substantiate and refine the light microscopy evidence of linear and punctuated protein deposition, we examined by electron microscopy the matrix made by *LTBP4* knockdown cell culture, with or without recombinant LTBP-4 or fibulin-5. Tannic acid was used to stain elastin. Fig. S10 E–G shows that microfibrils of *LTBP4* knockdown cultures were not stained by tannic acid and addition of recombinant LTBP-4 caused dark staining of microfibril bundles with fine elastin particles; whereas larger aggregates of elastin were observed in the cultures supplemented with recombinant fibulin-5. As shown in Fig. 5, fibulin-5 immunostaining in the skin and the lung of *Ltbp4S*$^{-/-}$ mice exhibits a punctate pattern, in contrast to the linear staining pattern along microfibrils in wild-type mice tissues. Therefore, loss of LTBP-4 abolishes linear deposition and causes punctate deposition of fibulin-5 both in vitro and in vivo.

LTBP-4 Interacts with Tropoelastin Indirectly Through Binding of LTBP-4 with Fibulin-5 and Fibulin-5 with Tropoelastin. Finally, we examined whether the ability of LTBP-4 to facilitate tropoelastin deposition to microfibrils is mediated by direct interaction of LTBP-4 and tropoelastin. We performed solid-phase binding assays using recombinant tropoelastin as an immobilized protein and rLTBP-4S and rFibulin-5 as soluble ligands. As shown in Fig. S3B, rLTBP-4S did not interact with tropoelastin, whereas rFibulin-5 strongly interacted with tropoelastin. However, rLTBP-4S efficiently bound to the tropoelastin-coated plate in the presence of rFibulin-5 (Fig. 6A), indicating that LTBP-4 binding to tropoelastin is dependent on fibulin-5. Together with the result that LTBP-4 interacts with fibulin-5 (Fig. 2, Figs. S2 and S3A), this result suggests that LTBP-4 does not tether tropoelastin directly, but through interaction of LTBP-4 with fibulin-5 and fibulin-5 with tropoelastin.

Discussion

In this study, we investigated the essential role of LTBP-4 in elastic fiber assembly. Our proposed model for the molecular mechanism of elastic fiber organization by LTBP-4 is illustrated in Fig. 6B. In the presence of LTBP-4, elastin, which is tethered by fibulin-5, deposits linearly on microfibrils through direct interaction of LTBP-4 with fibulin-5. In the absence of LTBP-4, the elastin/fibulin-5 complex cannot linearly deposit on microfibrils, and misplaced aggregates of elastin/fibulin-5 grow to form globular structures. It was reported that subcritical concentrations (120 μg/mL) of tropoelastin spontaneously aggregate to form ~200–300-nm particles at 37 °C (35). Indeed, by electron microscopy we observed 200–300-nm aggregates of elastin in *LTBP4* knockdown HDF cultures supplemented with rFibulin-5 (Fig. S10G). LTBP-4 may prevent this globular aggregation by trapping fibulin-5/elastin complex on microfibrils before the aggregates grow, thereby permitting subsequent coalescence of elastin to occur on microfibrils. This results in thickening of elastic fibers. It is also possible that LTBP-4 controls self-association of elastin by an unknown mechanism. In either case, LTBP-4 is the molecule necessary for linear assembly of elastin on microfibrils through interaction with fibulin-5. It has been reported that elastin appears to be virtually excluded from microfibril bundles in both *Ltbp4S*$^{-/-}$ mice (9, 36) and *Fbln5*$^{-/-}$ mice (37) as revealed by transmission electron microscopy. Similar observations were made in the tissues of human patients homozygous for *LTBP4* and *FBLN5* mutations, both of which yield cutis laxa, a loose skin syndrome (12, 38). Our model explains why LTBP-4 deficiency and fibulin-5 deficiency cause very similar phenotypes even at the electron microscopic level.

It was reported that fibulin-5 directly interacts with the N-terminal fragments of fibrillin-1 with high affinity (23, 39), which seems to be inconsistent with our result that fibulin-5 deposits poorly in a linear configuration on microfibrils in the absence of LTBP-4. However, the binding affinity between fibulin-5 and
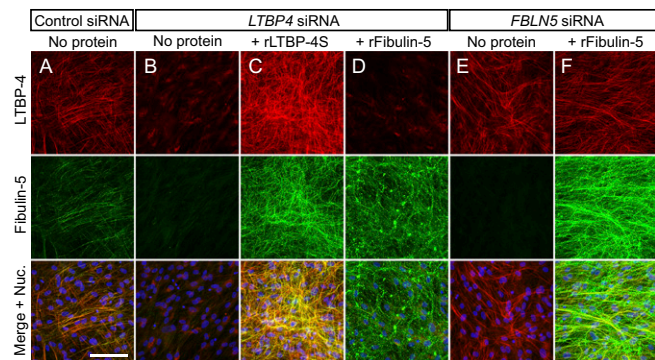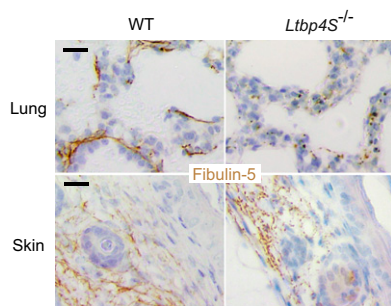
Fig. 5. Punctate pattern of fibulin-5 deposition in the absence of LTBP-4 in vivo. Immunohistochemistry of the lung and the skin tissues from 7-d-old wild-type and *Ltbp4S*⁻/⁻ mice was performed using anti–fibulin-5 antibody. (Scale bars, 20 μm.)

fibrillin-1 fragments may not be proportional to the interaction efficiency between fibulin-5 and fibrillin-1 microfibrils in cell culture, because the N terminus of fibrillin-1 is considered to be folded and bound with the C terminus of a second fibrillin-1 (40), and the binding sites of fibrillin-1 for LTBP-4 and for fibulin-5 are not the same (41). Therefore, the fibrillin-1–binding site for fibulin-5 might be hidden, whereas the binding site for LTBP-4 might be available when assembled as microfibrils. It is also possible that LTBP-4 might interact with microfibrils through binding with an intervening molecule, such as fibronectin, that can interact with fibrillin-1 at a site different from the N terminus (42). Nevertheless, granular aggregates of elastin and fibulin-5 observed in the absence of LTBP-4 seemed to associate with microfibrils, perhaps indicating residual binding of fibrillins and fibulin-5 or of fibrillins and tropoelastin (43).

We previously reported that LTBP-2 interacts with fibulin-5 and regulates elastic fiber assembly (22). In that report, we showed that LTBP-2 prevents fibulin-5 deposition on fibrillin-1–free microfibrils, and promotes fibulin-5 deposition on fibrillin-2–free microfibrils. Unlike LTBP-4, LTBP-2 knockdown in human dermal fibroblasts did not reduce fibulin-5 and elastin deposition on microfibrils, and recombinant LTBP-2 did not promote fibulin-5 and elastin deposition on microfibrils unless fibrillin-2 was knocked down. Therefore, in human dermal fibroblast cultures,
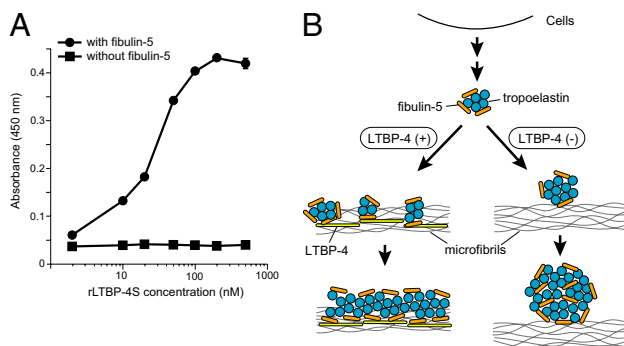
LTBP-2 does not affect fibulin-5 and elastin deposition unless either fibrillin-1 or -2 is knocked down. As we did not knock down fibrillins in our current study, microfibrils made by the cells contain both fibrillin-1 and -2. Therefore, the presence of LTBP-2 did not affect fibulin-5 and elastin deposition.

Among the four LTBP family members, LTBP-1 and -3 strongly interact with LAP, through their third 8-cysteine domain (6). The same domain of LTBP-4 interacts only weakly with LAP (32), and LTBP-2 does not bind with LAP (31). We have shown that a small amount of TGF-β is associated with rLTBP-4S purified from 293T cells overexpressing *LTBP4S* cDNA, consistent with previous reports. Although LTBP-4 does not bind LAP as effectively as LTBP-1 or -3, lung fibroblasts from *Ltbp4S*⁻/⁻ mice and skin fibroblasts from *LTBP4* mutant humans were reported to show either decreased or increased activation of TGF-β, respectively, indicating that LTBP-4 is a modulator of extracellular TGF-β activity (12, 30). Therefore, it is important to distinguish between TGF-β–dependent and independent functions of LTBP-4. Our data showing hyperassembly of elastic fibers after the addition of rLTBP-4S and rLTBP-4S + eLAP, but not TGF-β, to *LTBP4* knockdown cells demonstrate that the elastogenic activity of LTBP-4 is independent from bound TGF-β. These data substantiate the data of Dabovic and coworkers (9) demonstrating that the defects in TGF-β and in elastin observed in *Ltbp4S*⁻/⁻ mice are separable.

In this study, we did not address potential cellular involvement in elastic fiber assembly. It is reported that globular elastin aggregates are actively remodeled by cells to form linear fiber structures (44). In addition, fibrillins are ligands of integrins and cell surface heparan sulfate proteoglycans (45, 46). Further study is needed to reconcile possible cellular involvement and our model for elastic fiber assembly.

Degradation of elastic fibers causes not only loose skin, but also aging-related diseases, such as emphysema, stiff arteries, and pelvic prolapse (47, 48). However, there is no known therapeutic intervention to regenerate elastic fibers. Although TGF-βs were reported to increase the amount of ECM proteins including elastic fibers (29), TGF-βs are multipotent cytokines that also are important modulators of cell growth, inflammation, and apoptosis (49, 50). Moreover, excessive TGF-β activity is reported to be deleterious in many disease states (49–51). LTBP-4S + eLAP provides a way to specifically increase elastic fibers without activating the TGF-β pathway. Our finding that LTBP-4 increases elastic fibers by promoting elastin deposition on microfibrils could be an approach toward regenerative treatment for diseases caused by elastic fiber degradation.

Materials and Methods

Antibodies and Immunodetection. Primary antibodies used were: mouse anti-elastin monoclonal (1:100; Millipore), rabbit anti–fibrillin-1 polyclonal (1:100; Elastin Products Company), mouse anti-fibrillin-2 monoclonal (1:100, provided by L. Sakai), rabbit anti–fibulin-5 polyclonal (1:2,000, provided by T. Sasaki (Max Planck Institute for Biochemistry, Martinsried, Germany), Fig. 1; 1:100, previously described, ref. 16, Fig. 5), mouse anti–fibulin-5 monoclonal (1:200, previously described, ref. 22, Fig. 4; Figs. S4, S6, and S8), goat anti–LTBP-4 polyclonal (1:40 or 1:200; R&D Systems, Fig. 1, Fig. S8), rabbit anti–LTBP-4 polyclonal (1:200, made by immunizing rabbits with recombinant human LTBP-4, Figs. 3 and 4, Figs. S4, S6, S9, and S10) antibodies. See *SI Materials and Methods* for details.

Cell Culture. The 293T cells, HDFs, and MEFs were maintained in DMEM (Invitrogen) supplemented with 2 mM glutamine, 100 units/100 mg·mL⁻¹ penicillin/streptomycin, and 10% FBS at 37 °C in 5% CO_2. HDFs from facial tissue excised for cleft lip repair of a 3-mo-old patient were provided by M.N. Adult HDFs were from a 56-y-old Caucasian (Cell Applications). Studies

Fig. 6. LTBP-4 does not directly interact with elastin, but interacts with fibulin-5-bound elastin. (*A*) Solid-phase binding assays using recombinant tropoelastin as an immobilized protein and rLTBP-4S as a soluble ligand, in the presence (500 nM) or absence of rFibulin-5 (means ± SD, n = 3). (*B*) A proposed model for the role of LTBP-4–fibulin-5 interaction in elastic fiber assembly. In the presence of LTBP-4, microaggregation of tropoelastin, which is tethered by fibulin-5, deposits linearly on microfibrils. Subsequent coalescence of tropoelastin takes place on microfibrils, resulting in thickening of elastic fibers. In the absence of LTBP-4, tropoelastin/fibulin-5 complex cannot linearly deposit on microfibrils, and misplaced aggregates of tropoelastin/fibulin-5 grow to form globular structures.

of human skin samples were approved by the ethical commission of Kansai Medical University and Kyoto University, and patients' informed consent was obtained. See *SI Materials and Methods* for details.

In Vitro Binding Assay. The 293T cells were transfected using LipofectAMINE PLUS (Invitrogen) or FuGENE HD (Promega). After transfection, they were cultured in serum-free DMEM/F12 (Invitrogen). The mixture of conditioned media and cell lysates (Fig. 2*B*, Fig. S2 *B and C*) or conditioned media (Fig. 2*C*) were subjected to immunoprecipitation with anti-FLAG M2 affinity gel followed by Western blotting as described previously (22).

RNAi. Duplex RNA oligonucleotides (Stealth Select RNAi) were purchased from Invitrogen. Control oligonucleotides were designed as a scrambled sequence of protein phosphatase PP2C gamma, a gene irrelevant to the extracellular matrix. Mixed siRNA duplexes (at final concentration 167 pM) were reverse transfected into HDFs using Lipofectamine RNAiMAX (Invitrogen). See *SI Materials and Methods* for details.

Solid-Phase Binding Assays. Various concentrations of recombinant FLAG-tagged LTBP-4S with or without 500 nM (30 μg mL^{-1}) of recombinant Myc-tagged fibulin-5 were used as soluble ligands. Solid-phase binding assays using purified tropoelastin were performed as previously described (22). The primary antibody was anti-FLAG M2 antibody (Sigma-Aldrich), and the secondary antibody was HRP-conjugated anti-mouse IgG antibody (Pierce). Signals were detected with Substrate Reagent Pack (R&D Systems).

Statistics. Results are expressed as the mean ±SD and statistical analysis was performed by Student's t test or Tukey test. $P < 0.05$ was considered significant.

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CELL BIOLOGY

Supporting Information

Noda et al. 10.1073/pnas.1215779110

SI Materials and Methods

Aorta Extensibility Measurements. The diameters of the extirpated aortae and the intraaortic pressure were measured as previously described (1). The slope was calculated from the aortic dimension at 40 and 120 mm Hg.

Electron Microscopy. For transmission electron microscopy, ascending aortae were fixed in 2% glutaraldehyde and 1 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer at 4 °C overnight. Samples were postfixed in 1% osmium tetroxide and processed for Epon embedding. Thin sections (95 nm) were stained with tannic acid and *p*-nitrophenol followed by counterstaining with uranyl acetate and lead citrate. Electron micrographs were taken with JEM-1400A (JEOL).

Cell Culture for Elastic Fiber Development. To develop elastic fibers, confluent HDFs at passage 8 were seeded on noncoated glass coverslips (Fisher) at 8×10^4/well in a 24-well plate, and on the next day the medium was changed to 1 mL/well of DMEM/F12 (Invitrogen) supplemented with 2 mM glutamine, 100 units/100 mg·mL^{-1} penicillin/streptomycin, and 10% FBS. The cells were kept at 37 °C in 5% CO_2 for 12–14 d without changing the medium.

Antibodies. All secondary antibodies conjugated with Alexa 488 or 546 for immunofluorescence, with peroxidase for immunohistochemistry, were purchased from Invitrogen. Paraffin sections of fixed tissues were subjected to immunofluorescence staining (Fig. 1). Immunohistochemical staining of paraffin sections were performed by the avidin-biotin-peroxidase complex method (ABC-Elite; Vector Laboratories) (Fig. 5). Immunofluorescence staining of HDFs (Figs. 3 and 4, Figs. S6, S7, S9, and S10) was performed as previously described (2), and visualized using BZ-9000 microscope (Keyence) with GFP-BP, TRITC, and DAPI-BP filter cubes, CFI Plan Apo 40× NA 0.95 lens (Nikon), and a built-in 2/3 inch, 1.5 megapixel, 12-bit, monochrome cooled CCD camera (Keyence). Image files were taken in TIFF format using the operating software, merged, linearly contrast stretched (with the same setting in each set of experiments) using Photoshop CS5 (Adobe), and imported into Illustrator CS5 (Adobe) for assembly.

Plasmid Construction. The following fragments of *LTBP4S, LTBP4L*, and their fragment cDNAs were amplified by PCR, and subcloned into pEF6/ssFLAG (preprotrypsin signal sequence, an FLAG tag and a 6× His tag at the N terminus), pEF6/FLAG (an FLAG tag and a 6× His tag at the C terminus) or pEF6/Myc (a Myc tag and a 6× His tag at the C terminus) (3): LTBP-4L-A (exons 2–10), LTBP-4S-A (exons 6–10), LTBP-4-B (exons 11–13), LTBP-4-C (exons 14–20), LTBP-4-D (exons 21–27), LTBP-4-E (exons 28–31), LTBP-4-F (exons 32–35), LTBP-4L-G (exon 2–5), LTBP-4S-G (exon 6), LTBP-4-H (exon 7), and LTBP-4-I (exons 8–10). LTBP-4L-G-Ig, LTBP-4S-G-Ig, LTBP-4-H-Ig, and LTBP-4-I-Ig were C-terminally fused with the Fc region of human IgG. Human full-length *FBLN5* cDNA was cloned as previously described (4). The expression vectors of full length-, ΔN1-, ΔN2-, ΔM-, and ΔC-fibulin-5 were prepared as previously described (2). The cDNAs of *N*-, M-, C-, and NM-fibulin-5 fragments were amplified by PCR, and subcloned into pEF6/ssFLAG. These fibulin-5 truncation mutants were C-terminally fused with the Fc region of mouse IgG. The cDNA for eLAP corresponding to 1–278 aa of human TGFβ1 precursor was PCR-amplified from HDF cDNA with additional stop codon at the 3′ end, and subcloned to pLenti 6.3 vector (Invitrogen).

Protein Purification. Recombinant LTBP-4S (rLTBP-4S) with an FLAG tag and a 6x-His tag was purified using TALON affinity resin (Takara) from serum-free conditioned medium of 293T cells stably transfected with pEF6/FLAG-LTBP-4S or pEF6/ssFLAG-LTBP-4S. Purification of recombinant fibulin-5 was described previously (2). To prepare rLTBP-4S + eLAP, tagged LTBP-4S-expressing 293T cells were infected with lentiviral vectors expressing eLAP without a tag, followed by purification with TALON affinity resin from serum-free conditioned medium. Recombinant tropoelastin was expressed in bacteria and purified as previously described (2). The purity of each protein was confirmed by Coomassie blue staining of an SDS/PAGE gel. The protein concentration was quantified from scanned gel images with ImageJ software using BSA as a standard.

RNAi. The oligonucleotides sequences are as follows:
Control sense 5′-AAACCCGGUAUGUCACUCUGUCA-GC-3′,
  antisense 5′-GCUGACAGAGUGACAUACCGGGUUU-3′;
LTBP4-2 sense 5′-ACCGAGACAGCUGAGUACCAGUC-AU-3′,
  antisense 5′-AUGACUGGUACUCAGCUGUCUCGGU-3′;
LTBP4-3 sense 5′-UGUGCAAGAGUGGCGUGUGUGUG-AA-3′,
  antisense 5′-UUCACACACACGCCACUCUUGCACA-3′;
FBLN5-1 sense 5′-CCAGUCAGGACAGUGUUUAGAUA-UU-3′,
  antisense 5′-AAUAUCGAAACACUGUCCUGACUGG-3′;
FBLN5-2 sense 5′-CCCACCACUCUCAGCUCCAAACU-AU-3′,
  antisense 5′-AUAGUUUGGAGCUGAGAGUGGUGGG-3′;
FBLN5-3 sense 5′-GGCCUCUUAUAUGCCGCUUUGGA-UA-3′,
  antisense 5′-UAUCCAAAGCGGCAUAUAAGAGGCC-3′.

qPCR. Total RNA was extracted using RNeasy Plus Mini Kit (QIAGEN) and transcribed to cDNA with random hexamers using SuperScript III First-Strand Synthesis System (Invitrogen). For qPCR, the reaction was performed with QuantiTect SYBR Green PCR Kit (QIAGEN), and the products were analyzed with Rotor-Gene Q (QIAGEN). The following primer sequences were used:
GAPDH forward 5′-AGGTGAAGGTCGGAGTCAACG-3′,
  reverse 5′-GATGACAAGCTTCCCGTTCTCAG-3′;
LTBP4 forward 5′-GAGACAGCTGAGTACCAGTCATT,
  reverse 5′-CTGTGTGTGGTAGTAGTAGCCGT-3′;
FBLN4 forward 5′-TTGATGTGAACGAGTGTGACATGG,
  reverse 5′-CAGAGGTAGCTGGAGTAGCTACAC;
FBLN5 forward 5′-ATACTCACTGTTACCATTCTGGCT-3′,
  reverse 5′-GGTTAACACACATCATGTCTCCTC-3′;
ELN forward 5′-AGTTGGTGGCTTAGGAGTGTCT-3′,
  reverse 5′-TTAACTCCTGCTCCAGTGGGAACT-3′;
LOX forward 5′-GAGTCCTGGCTGTTATGATACCTA-3′,
  reverse 5′-GTATAGTCAGATTCAGGAACCAGG-3′;
LOXL1 forward 5′-TCAAGCGCTATGCATGCACCTCTCA-TA-3′,
  reverse 5′-GATGTCCGCATTGTAGGTGTCATAGCA-3′;
FBN1 forward 5′-CATTAAGTGCACTGATCTGGAC-3′,
  reverse 5′-ATTCAGGTTCTCAGAGCACTCAT-3′.

Quantitative Measurement of Insoluble Elastin. The 8×10^5 HDFs transfected with each siRNA were plated on 60-mm dishes. Three days after plating, 20 mCi of [^{3}H]valine (American Radiolabeled Chemicals) was added to each dish, together with recombinant

LTBP-4S protein. The cultures were incubated at 37 °C in 5% CO_2 for 10 d. The cells were harvested in 0.1 M acetic acid on ice. After centrifugation, the pellets were boiled in 0.1 N NaOH for 15 min followed by centrifugation, and the pellets were again boiled in 0.1 N NaOH for 1 h. Subsequently, the NaOH-insoluble pellets were boiled with 5.7 N HCl for 1 h, mixed with scintillation fluid, and measured for radioactivity with Tri-Carb 2100TR (Packard Bioscience).

TGF-β Activity Assay. Mink lung epithelial cells stably transfected with a luciferase gene driven by TGF-β–responsive plasminogen activator inhibitor-1 promoter sequences (TMLECs) were allowed to attach for 3 h in a 96-well plate. DMEM/0.1% BSA containing TGF-β standards or recombinant proteins were heat-treated and added to TMLECs. After 16 h, TGF-β activities were assessed by measuring luciferase activity in cell lysates. Luciferase activities were measured using ONE-Glo Luciferase Assay System (Promega).

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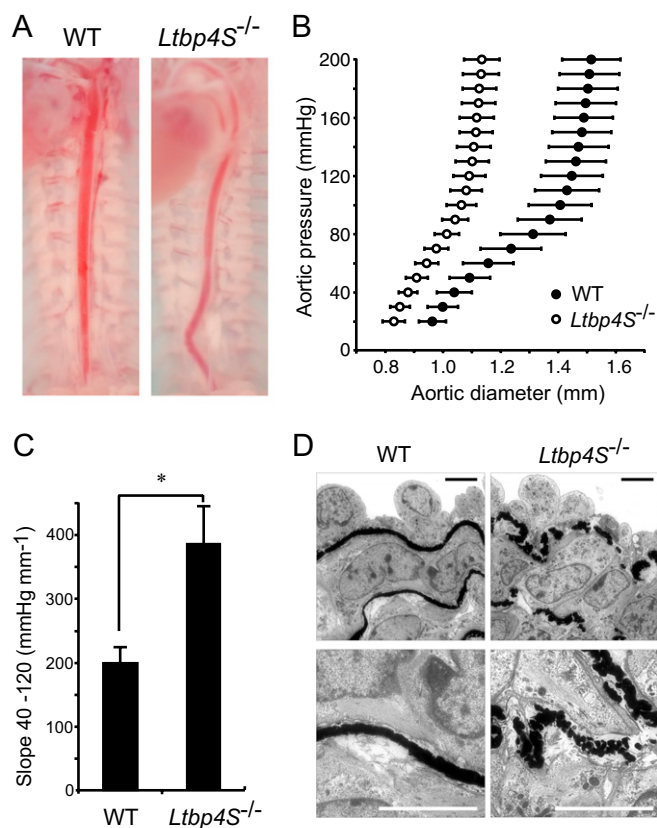
Fig. S1. Vascular phenotypes of *Ltbp4S*^{−/−} mice. (*A*) Aortae of WT and *Ltbp4S*^{−/−} mice at P5. Note tortuosity of the aorta of *Ltbp4S*^{−/−} mice. (*B*) Pressure–diameter relationship of aorta explants of 4-wk-old mice (*n* = 6 for each genotype). Aortic diameter was measured while continuously changing the intraluminal pressure. (*C*) Slopes of pressure–diameter curves between 40 and 120 mm Hg. Aortae of *Ltbp4S*^{−/−} mice were significantly less extensible than control aortae in the range of physiological pressure (*P = 0.00003, Student's *t* test, *n* = 6 for each genotype). Error bars represent SD. (*D*) Transmission electron micrographs of ascending aortae from WT and *Ltbp4S*^{−/−} mice at P2. Note disorganized elastic lamellae stained in black by tannic acid, in *Ltbp4S*^{−/−}. (Scale bars, 5 μm.)

Fig. S2. In vitro binding assay, showing interaction of LTBP-4 with fibulin-5. The expression vectors were independently transfected into 293T cells. Transfected cells were cultured in serum-free medium for 48 h, and the cell lysates and the conditioned media were harvested. Mixtures of the media and cell lysates were incubated with each other, and these reactants were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitants were separated by SDS/PAGE, and analyzed by Western blotting. (*A*) Domain structure of the full-length fibulin-5 and the fibulin-5 deletion/truncation mutants. The deletion mutants were expressed as C-terminal FLAG-tagged proteins. The truncation mutants were expressed as N-terminal FLAG-tagged proteins flanked by preprotrypsin signal sequence and C-terminally fused to the Fc region of murine IgG. (*B*) Interaction of LTBP-4S with full-length fibulin-5 and with the deletion mutants lacking the N-terminal domain or the central calcium binding EGF-like repeat domains, but not with the deletion mutant lacking the C-terminal domain. (*C*) Interaction of LTBP-4S with the fibulin-5 truncation mutant containing the C-terminal domain, but not with other domains.

Fig. S3. Solid-phase binding assays. (*A*) Fibulin-5 directly interacts with LTBP-4. Solid-phase binding assays on recombinant LTBP-4S or BSA were performed using variable concentrations of recombinant FLAG-tagged fibulin-5 as soluble ligands. After washing, FLAG-tagged proteins bound to LTBP-4S or BSA were detected using anti-FLAG antibody. (*B*) Fibulin-5 strongly interacts with tropoelastin, whereas LTBP-4 does not directly interact with tropoelastin. Solid-phase binding assays on recombinant tropoelastin or BSA were performed using variable concentrations of recombinant FLAG-tagged LTBP-4S or FLAG-tagged fibulin-5 as soluble ligands. After washing, FLAG-tagged proteins bound to tropoelastin or BSA were detected using anti-FLAG antibody. Signals detected for binding on BSA were subtracted as nonspecific background from signals detected for binding on tropoelastin. All measurements were performed in triplicate, and values shown are means ± SD.
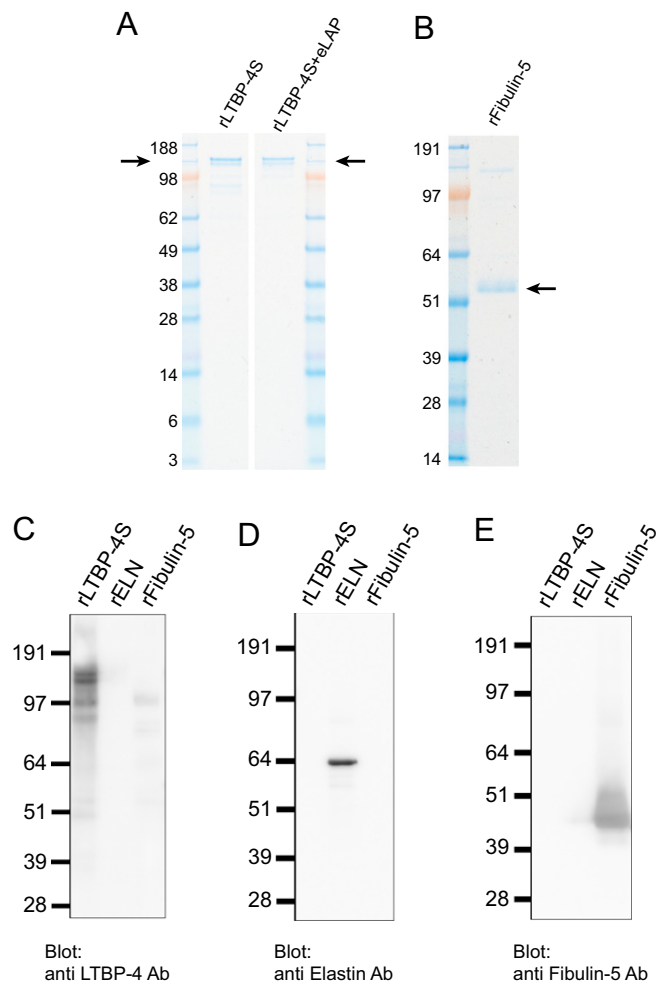
Fig. S4. Purity of the recombinant proteins and specificity of the antibodies used in this study. 6× His-tagged rLTBP-4S, rLTBP-4S + eLAP (*A*) and rFibulin-5 (*B*) were purified by chelating chromatography from culture media of 293T clones stably overexpressing the respective proteins. A Coomassie blue–stained SDS/PAGE gel at a reduced condition of a representative preparation is shown. Arrows indicate the recombinant proteins, whereas most of minor bands in the rLTBP-4S and rLTBP-4S + eLAP lane are considered to be degradation products of the recombinant protein, as indicated by the Western blot with anti–LTBP-4 antibody (*C*). (*C–E*) Antibodies for LTBP-4, elastin and fibulin-5 did not cross-react; 100 ng of each recombinant proteins were subjected to SDS/PAGE under nonreduced conditions, followed by Western blotting with anti–LTBP-4 polyclonal (1:1,000), anti-elastin monoclonal (1:500), and anti–fibulin-5 monoclonal (1:1,000) antibodies.

Fig. S5. qPCR analysis of gene knockdown in HDFs. (*A* and *B*) Total RNA from siRNA-transfected HDFs was extracted 7 and 14 d after transfection. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *LTBP4*, *FBLN5*, and *GAPDH*. The mRNA levels relative to untransfected HDFs are shown. Data are presented as means ±SE of four independent experiments. Both *LTBP4* siRNA (*A*) and *FBLN5* siRNA (*B*) effectively suppressed the expression of target genes. Ctrl, control. (*C*) qPCR analysis of elastic fiber–related genes in HDFs transfected with control siRNA or *LTBP4* siRNA, cultured with or without rLTBP-4S (30 nM). Total RNA from siRNA-transfected HDFs was extracted 4 d after transfection. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *ELN*, *FBLN4*, *FBLN5*, *LOX*, *LOXL1*, *FBN1*, and *GAPDH*. The mRNA levels relative to nontransfected HDFs are shown. Data are presented as means ±SE of four independent experiments, each of which were performed in duplicate. (*D*) qPCR analysis of elastic fiber–related genes in HDFs cultured with or without TGF-β1 (1 nM). HDFs were cultured without serum for 48 h and then were added with TGF-β1. Total RNA of HDFs was extracted 12 h after addition of TGF-β1. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *ELN*, *FBLN4*, *FBLN5*, *LOX*, *LOXL1*, *FBN1*, and *GAPDH*. The mRNA levels of HDFs without TGF-β1 were set at 1. Data are presented as means ±SE of three independent experiments.
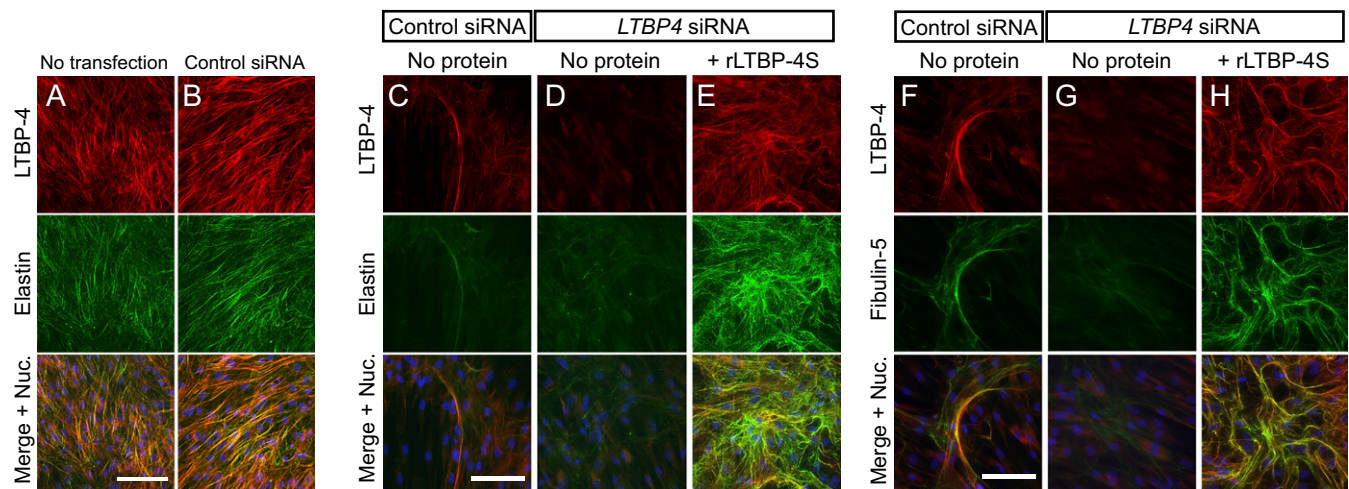
Fig. S6. Elastogenesis in cultures of neonatal and adult HDFs. (*A* and *B*) Control siRNA transfection does not affect elastic fiber assembly. HDFs were transfected with or without control siRNA and cultured in 10% serum-containing media. The cells were fixed 13 d after transfection and stained with anti–LTBP-4 and anti-elastin antibodies. (*C–H*) Normal adult HDFs (106–05 a) were transfected with control siRNA or *LTBP4* siRNA and cultured in 10% serum-containing media supplemented with or without rLTBP-4S (15 nM). The cells were fixed 13 d after transfection and stained with anti–LTBP-4 polyclonal, anti-elastin monoclonal, and anti–fibulin-5 monoclonal antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)
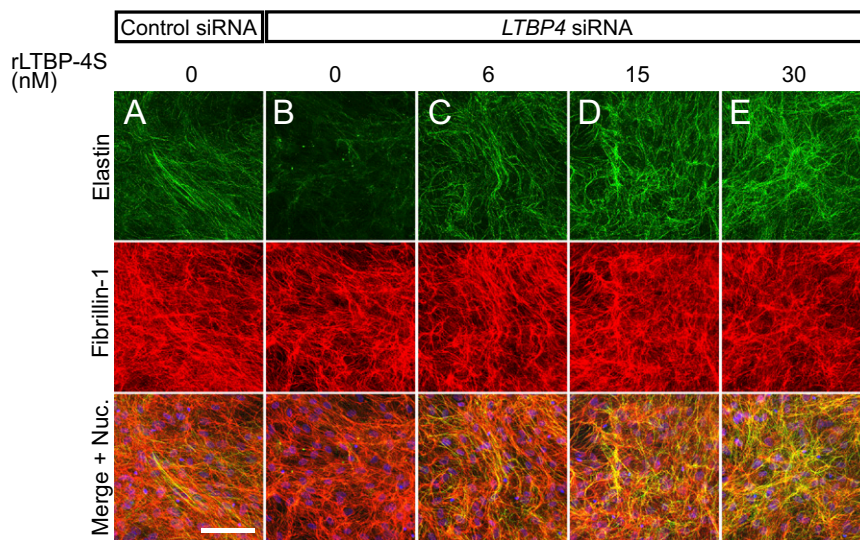
Fig. S7. *LTBP4* knockdown reduced elastin deposition and rLTBP-4S addition augmented elastin deposition, without changing the configuration of fibrillin microfibrils. HDFs were transfected with control siRNA (*A*) or *LTBP4* siRNA (*B–E*) and cultured in 10% serum containing media supplemented with rLTBP-4S as indicated. The cells were fixed 13 d after transfection and stained with anti-elastin and anti–fibrillin-1 antibodies as indicated. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)

Fig. S8. Elastogenesis in culture of MEFs. (*A–F*) WT MEFs developed an elastic fiber meshwork, whereas *Ltbp4S*^{−/−} MEFs did not. MEFs were cultured in 10% serum containing media on glass coverslips coated with collagen (Cellmatrix; Nitta Gelatin), with or without rLTBP-4S protein (15 nM). The cells were fixed 13 d after plating and stained with goat anti-mouse LTBP-4 polyclonal (R&D Systems), anti-mouse elastin polyclonal (PR385; EPC), and anti–fibulin-5 monoclonal antibodies. (*G–I*) LTBP-4 colocalizes with fibrillin-1. MEFs from WT and *Ltbp4S*^{−/−} embryos were cultured with or without rLTBP-4S protein (15 nM) and were fixed 7 d after plating, followed by immunostaining with rabbit anti–fibrillin-1 polyclonal and goat anti-mouse LTBP-4 polyclonal antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)
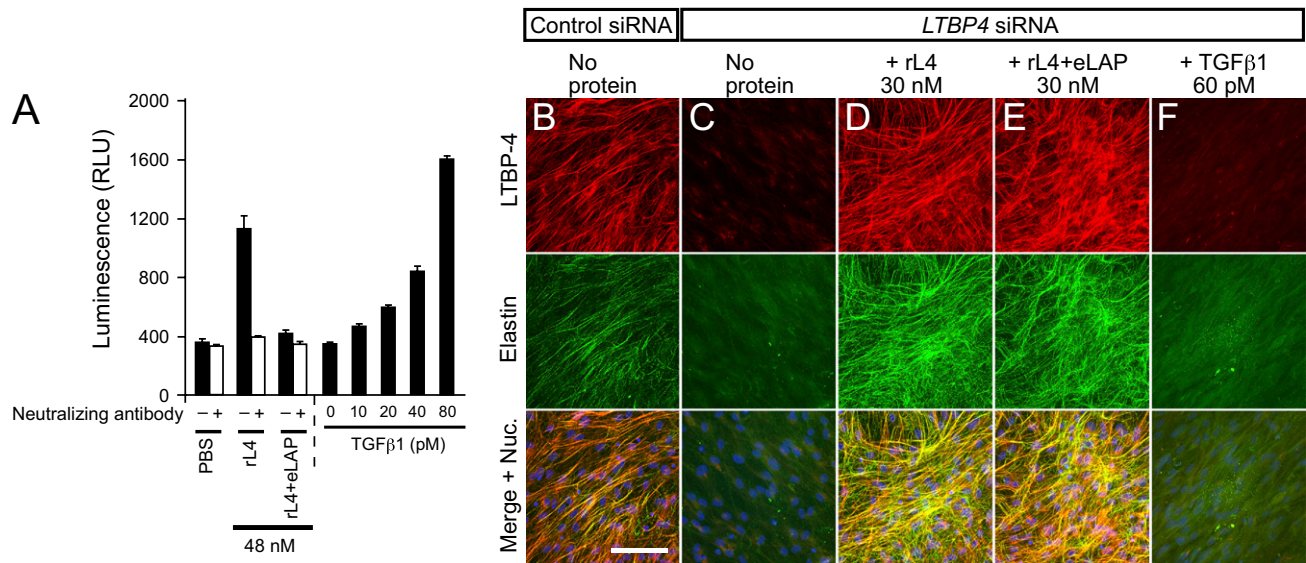
Fig. S9. Elastogenic property of LTBP-4 is not dependent on bound TGF-β. (*A*) TGF-β activity of rLTBP4-S (rL4) was measured using a cell-based TGF-β bioassay system. rL4 was purified from the medium of 293T cells expressing N-terminal 6×histidine (His)-tagged LTBP-4S. rL4 + eLAP was purified from the medium of 293T cells overexpressing His-tagged LTBP-4S and untagged LAP without TGF-β sequence. The fresh media containing each recombinant protein were heat-treated to release active TGF-β and subjected to TGF-β activity assay with or without anti–TGF-β neutralizing antibody (means ±SD, *n* = 3). (*B*–*F*) HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured for 13 d in 10% serum containing media with or without recombinant protein as indicated. Cells were immunostained with anti–LTBP-4 or anti-elastin antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)
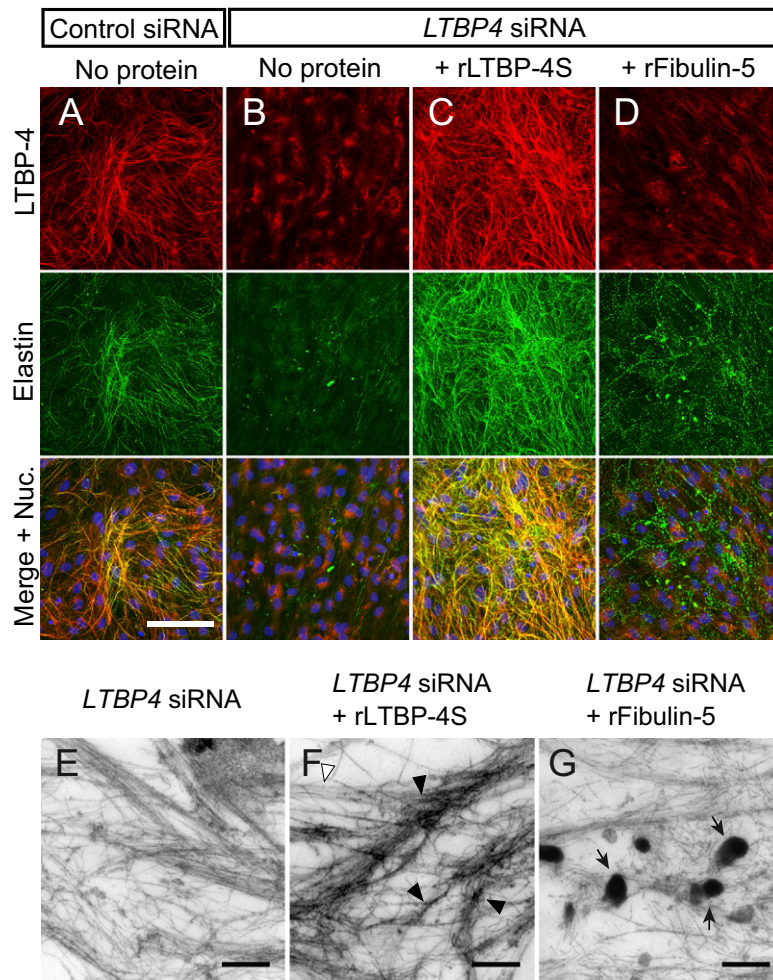
Fig. S10. Aggregated elastin deposition in a punctate pattern in the absence of LTBP-4. HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured for 13 d in 10% serum containing media with or without recombinant proteins as indicated (rLTBP-4S 30 nM or rFibulin-5 60 nM), followed by immunostaining with anti–LTBP-4 and anti-elastin antibodies (*A–D*) or by transmission electron micrography (*E–G*). (*A–D*) *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. Addition of rFibulin-5 to *LTBP4* KD cell culture caused increased elastin deposition, but only in a punctate pattern. (Scale bar, 100 μm.) (*E–G*) Elastin was stained by tannic acid. In rLTBP-4S-supplemented cell culture, both elastin-negative microfibrils (open arrowhead) and microfibrils with fine elastin particles (closed arrowheads) were observed. In rFibulin-5-supplemented culture, large granules of aggregated elastin (arrows) were observed. (Scale bars, 500 nm.)