Interaction between Periostin and BMP-1 Promotes Proteolytic Activation of Lysyl Oxidase*

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Intra- and intermolecular covalent cross-linking between collagen fibrils, catalyzed by lysyl oxidase (LOX), determines the mechanical properties of connective tissues; however, mechanisms that regulate the collagen cross-linking according to tissue specificity are not well understood. Here we show that periostin, a secretory protein in the dense connective tissues, promotes the activation of LOX. Previous studies showed that periostin null mice exhibit reduced collagen cross-linking in their femurs, periosteum, infarcted myocardium, and tendons. Presently, we showed that active LOX protein, formed by cleavage of its propeptide by bone morphogenetic protein-1 (BMP-1), was decreased in calvarial osteoblast cells derived from periostin null mice. Overexpression of periostin promoted the proteolytic cleavage of the propeptide, which increased the amount of active LOX protein. The results of co-immunoprecipitation and solid phase binding assays revealed that periostin interacted with BMP-1. Furthermore, this interaction probably resulted in enhanced deposition of BMP-1 on the extracellular matrix, suggesting that this enhanced deposition would lead to cleavage of the propeptide of LOX. Thus, we demonstrated that periostin supported BMP-1-mediated proteolytic activation of LOX on the extracellular matrix, which promoted collagen cross-linking.

Collagen fibrils underlie the mechanical strength of connective tissues, such as bone, tendon, and skin. Post-translational modifications of collagen fibrils are important for both structural and mechanical properties. Collagen fibrillogenesis consists of multiple processes (1). Synthesized pro-collagen chains in the endoplasmic reticulum are folded into the triple helix structure and transported into the Golgi. In the Golgi, pro-collagens are processed by ADAMTS (pro-collagen N-proteinase) and tolloid (pro-collagen C-proteinase) family enzymes and secreted into the extracellular milieu. Collagen assembling into fibrils, which are then stabilized by the formation of intramolecular cross-linking that is catalyzed by the enzyme lysyl oxidase (LOX).2 The strength of connective tissues is determined by the amount of total collagen cross-linking, as well as by the total collagen content (2–4). The crucial role of collagen cross-linking in connective tissues has been demonstrated in terms of some genetically inherited diseases: Ehlers-Danlos syndrome, homocystinuria, Menkes disease, and occipital horn syndrome. These diseases are attributed to reduced LOX activity, followed by diminished collagen cross-linking, resulting in connective tissue dysfunctions (5–7).

LOX catalyzes the oxidative deamination of peptidyl lysine residues in collagen molecules to α-aminoacidic-δ-semialdehyde. Spontaneous condensation reactions of the resultant aldehydes lead to the formation of covalent cross-linking in fibrillar collagens (8). This amine oxidase activity of LOX is regulated by proteolytic cleavage of the LOX propeptide. LOX is synthesized as an inactive precursor, pro-LOX, and then activated by pro-collagen C-proteinase (bone morphogenetic protein-1: BMP-1)-mediated processing of the N-terminal propeptide (9). Although the activation mechanism of LOX has been demonstrated partly as described above, the mechanism that regulates the activity of LOX according to tissue specificity is not well understood.

To investigate this regulatory mechanism, we focused on periostin. Periostin is a secretory protein expressed in collagen-rich fibrous connective tissues, such as periosteum, periodontal ligament, aorta, and heart valve (10, 11). Previous studies showed reduced collagen cross-linking in femurs, periosteum, infarcted myocardium, and tendons from periostin−/− mice (12–14). Overexpression of periostin by adenoviral infection in cardiac valvulogenic tissue increases the overall viscosity, which is a measure of collagen cross-linking (13). Thus, these data suggest that periostin plays a role in the formation of collagen cross-links. However, the mechanism by which periostin promotes collagen cross-linking remains to be addressed.

In this study, we demonstrate that periostin supported BMP-1-mediated LOX activation. Overexpression of periostin enhanced proteolytic cleavage of pro-LOX in C3H10T1/2 cells. Co-immunoprecipitation and solid phase binding assays revealed that periostin directly bound to BMP-1. LOX has been reported to interact with an extracellular matrix protein, fibronectin (15), and our previous study showed that periostin

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2 The abbreviations used are: LOX, lysyl oxidase; BMP, bone morphogenetic protein; HA, hemagglutinin; EGF, epidermal growth factor; COB, calvarial osteoblasts; RT, reverse transcription; WT, wild type; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; CTR, C-terminal region.
interacts with fibronectin as well (16), thus suggesting periostin-mediated scaffolding for the interaction between pro-LOX and BMP-1 on the fibronectin matrix.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit anti-mouse periostin antibodies (anti-RDI) were described previously (14). Rabbit polyclonal anti-LOX antibodies (Abcam Inc., Cambridge, MA), rabbit polyclonal anti-BMP-1 antibodies (Thermo Scientific, Yokohama, Japan), goat polyclonal anti-BMP-1 antibodies (R & D Systems, Inc., Minneapolis, MN), mouse monoclonal anti-β-actin antibody AC-15 (Sigma-Aldrich), mouse monoclonal anti-HA antibody (Nacalai Tesque, Kyoto, Japan), rabbit polyclonal anti-HA antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich), and rabbit polyclonal anti-DDDK-tag antibodies (Medical & Biological Laboratories Co., Ltd., Woburn, MA) were purchased from the sources indicated. Alexa Fluor 568-labeled goat anti-mouse antibodies, Alexa Fluor 488-labeled donkey anti-rabbit antibodies, and Alexa Fluor 488-labeled donkey anti-rabbit antibodies were obtained from Molecular Probes (Invitrogen). Alexa Fluor 647-labeled mouse anti-GM130 antibody (Nacalai Tesque, Kyoto, Japan), goat polyclonal anti-BMP-1 antibodies (R & D Systems, Inc., Minneapolis, MN), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich), and rabbit polyclonal anti-DDDK-tag antibodies (Medical & Biological Laboratories Co., Ltd., Woburn, MA) were purchased from the sources indicated. Alexa Fluor 568-labeled goat anti-mouse antibodies, Alexa Fluor 488-labeled donkey anti-rabbit antibodies, and Alexa Fluor 488-labeled donkey anti-rabbit antibodies were obtained from Molecular Probes (Invitrogen). Alexa Fluor 647-labeled mouse anti-GM130 antibody was purchased from BD Biosciences (San Jose, CA), and Cy3-labeled donkey anti-rabbit antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Animals**—The care and experiments with animals were in accordance with the guidelines of the animal care and use committees at Tokyo Institute of Technology. The generation of periostin−/− mice was described previously (14).

**Plasmid Constructions**—Expression vectors for periostin, its deletion forms, and the W65A mutant form were described previously (12). To generate the EMI domain conjugated with an HA tag at its C-terminal end, we first phosphorylated the 5′ end of the following oligonucleotide: 5′-ATGGCAGCTGTA-CCCATACGACTCCAGCTGTCAGCCGAGC-3′ and 5′-GGCCGCTCAAGCTGACGCTGCTGCTGATATACG-GACGCTGCCATTAAAC-3′. To replace the RD1–4-CTR coding region with the HA tag, we annealed the phosphorylated oligonucleotides in the thermal cycler and then inserted them into the BstXI/NotI site of pcDNA3 (Invitrogen). Alexa Fluor 647-labeled mouse anti-GM130 antibody was purchased from BD Biosciences (San Jose, CA), and Cy3-labeled donkey anti-rabbit antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**RT-PCR Analysis**—The periosteum of tibia and femur was obtained from 12-week-old WT and periostin−/− mice, which were prepared as previously described. Total mRNA was extracted by using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer’s protocol. One microgram of total mRNA was used for reverse transcription, and the cDNA was synthesized by using a cDNA synthesis kit first strand with avian myeloblastosis virus reverse transcriptase (TaKaRa Bio Inc., Siga, Japan). Specific primer sets for each LOX family gene, periostin, BMP-1, and glyceraldehyde-3-phosphate dehydrogenase were designed. RT-PCR products were transferred with plasmid DNAs by using polyethyleneimine (Sigma-Aldrich). Stably transfected cells were cultured in the presence of 4 or 8 μg/mL of puromycin (Nacalai Tesque).

**Cell Culture and Transfection**—Primary cultures of mouse calvarial osteoblasts (COBs) were prepared according to the standard collagenase method. COB cells were maintained in α-minimum essential medium (Nacalai Tesque), supplemented with 10% fetal bovine serum (Thermo Scientific, Yokohama, Japan) according to the manufacturer’s protocol. One microgram of total mRNA was used for reverse transcription, and the cDNA was synthesized by using a cDNA synthesis kit first strand with avian myeloblastosis virus reverse transcriptase (TaKaRa Bio Inc., Siga, Japan). Specific primer sets for each LOX family gene, periostin, BMP-1, and glyceraldehyde-3-phosphate dehydrogenase were designed. RT-PCR products were transferred with plasmid DNAs by using polyethyleneimine (Sigma-Aldrich). Stably transfected cells were cultured in the presence of 4 or 8 μg/mL of puromycin (Nacalai Tesque).

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CCACATACGTCGACAG-3' (sense) and 5'-GGTCGTAGTG-CCGGTAAGCTCG-3' (antisense) (annealing temperature, 63°C; number of cycles, 29); lysyl oxidase-like 2 protein (LOXL2), 5'-ACCTGACCTGTTGCTTTAATG-3' (sense) and 5'-TGAAGAAGCTTCCATGCCTG-3' (antisense) (annealing temperature, 63°C; number of cycles, 29); glycosyl oxidase-3 protein (GLOX-3), 5'-GGTCGACTCAAATTTGTGTC-3' (sense) and 5'-CGCCTGTGCT-3' (antisense) (annealing temperature, 65°C; number of cycles, 29); periostin, 5'-GGTAACATCGACTCCCTAGTAC-3' (antisense) and antisense 5'-GCTCAATCACCCACCTAGTAC-3' (antisense) (annealing temperature, 65°C; number of cycles, 29); BMP-1, 5'-GGAATTCGGCATTGTGGGAGCCGTCAACATGC-3' (antisense) and 5'-TGGTGGCAATTTGTGTCAGGACTCCTGGTCT-3' (sense) (annealing temperature, 60°C; number of cycles, 19 for periosteum, 22 for COB cells, and 22 for 10T1/2 transfectants). The amplification profile was 95°C for 1 min for hot start, followed by denaturation at 95°C for 30 s, annealing for 30 s at the temperature indicated, extension at 72°C for 30 s for the indicated number of cycles, with a final extension at 72°C for 3 min. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized under UV light.

Western Blot Analysis—The cells were grown to 2 days' post-confluence, washed with PBS, and scraped into SDS sample buffer containing 50 mM dithiothreitol. For cell culture supernatants, the cells were grown to 2 days' post-confluence, washed with PBS, and incubated with serum-free Dulbecco's modified Eagle's medium. After an additional 48 h, the cell culture supernatants were collected. After concentration of total proteins, the purified BMP-1-FLAG was dissolved in PBS over-night at 4°C. The plates were blocked for 1 h at room temperature with PBS containing 1% bovine serum albumin. Next, the plates were incubated with various concentrations (0–100 nM) of purified ΔEM/DCTR-α-HA in PBS overnight at 4°C and washed with PBS. Bound ΔEM/DCTR-α-HA was detected by using anti-HA antibody followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody. The plates were washed and incubated with peroxidase substrate O-phenylenediamine (0.4 mg/ml) in 50 mM citrate buffer, pH 4.6, containing 0.003% H2O2 for 30 min. Absorbance was read at 495 nm.

Immunofluorescence Microscopy—The cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After a wash with PBS, they were permeabilized with PBS containing 0.5% Triton X-100 for 5 min at room temperature. After blocking with 0.1% bovine serum albumin in TBS (preheated at 56°C for 30 min) for 1 h at room temperature, the cells were incubated with the primary antibodies in 1% bovine serum albumin in TBS overnight at 4°C. Further incubation with an optimal dilution of fluorescence-tagged secondary antibodies was performed for 1 h, followed by washing twice with TBS. The nuclei were stained with TOPRO3 (Molecular Probes, Invitrogen). The fluorescent images were collected by using a laser-scanning confocal microscope (FV1000-BX61; Olympus, Japan) with 40× UPlanApo NA 0.85 objective lenses and imaging software (FLUOVIEW Ver. 1.6a; Olympus). The images were imported into Photoshop (Ver. CS2; Adobe) for cropping and linear contrast adjustment.

Quantification of Collagen Cross-linking—C3H10T1/2 stable transfectants were grown to 2 days' post-confluence. The pyridinoline and deoxypyridinoline contents were determined by a high performance liquid chromatography method (18).

Statistics—The data were summarized as the means ± S.E. Statistical significance was assessed by use of the unpaired t test (p values < 0.05 were considered significant).

RESULTS

Decreased Amount of Active LOX Protein in Periostin-/- COB Cells—To examine whether the reduced cross-linking of collagens in the periostin-/- femur and periosteum is due to a...
reduced level of LOX mRNA expression, we performed semi-quantitative RT-PCR analysis. We prepared total RNAs from the periosteum of WT and periostin\(^{-/-}\) tibia and femur that were then subjected to reverse transcription. To detect the expression of LOX family genes, we generated specific primer sets for each gene (details under “Experimental Procedures”). RT-PCR analysis showed no significant difference in the expression of LOX family genes between the WT and periostin\(^{-/-}\) periosteum (Fig. 1A), indicating that the reduced cross-linking of collagens in the periostin\(^{-/-}\) mice might possibly be caused by decreased activity of LOX proteins.

LOX activity is regulated by proteolytic cleavage of the LOX propeptide. To examine an active LOX protein in periostin\(^{-/-}\) osteoblasts, we prepared total cell lysates from WT and periostin\(^{-/-}\) COB cells grown to 2 days’ post-confluence, subjected the lysates to SDS-PAGE, and then blotted the separated proteins with anti-LOX antibody. The sizes of N-glycosylated pro-LOX and active LOX have been regarded as ~50 (19) and 32 kDa (20). The amount of active LOX protein (32 kDa) was less in cell lysates from periostin\(^{-/-}\) COB cells than in those from WT COB cells (Fig. 1B, arrow). Moreover, the amount of N-glycosylated pro-LOX protein (50 kDa) was also decreased in cell lysates from the periostin\(^{-/-}\) COB cells (Fig. 1B, arrowhead). In addition, we observed a 35-kDa band (Fig. 1B, asterisk). This 35-kDa band, which was reported to correspond to an intermediate processed form of LOX (21), was slightly weaker in the case of the periostin\(^{-/-}\) COB cells. Quantification of the total and active LOX proteins using WT and periostin\(^{-/-}\) COB cells is shown in Fig. 1C, indicating a significant difference in the total and active LOX proteins between the WT and periostin\(^{-/-}\) COB cells.

To examine LOX mRNA expression levels in WT and periostin\(^{-/-}\) COB cells, we prepared total RNAs from WT and periostin\(^{-/-}\) COB cells that were then subjected to reverse transcription. Semi-quantitative RT-PCR analysis showed no significant difference in the expression of the LOX gene between WT and periostin\(^{-/-}\) COB cells (Fig. 1D), which is consistent with the result of RT-PCR analysis of the WT and periostin\(^{-/-}\) periosteum (Fig. 1A). These results indicate that the amount of active LOX protein was decreased in the periostin\(^{-/-}\) COB cells, suggesting that the LOX activity would be attenuated in periostin\(^{-/-}\) osteoblasts.

**Overexpression of Periostin Increases the Amount of Active LOX Protein in the C3H10T1/2 Cells**—We examined whether overexpression of periostin would increase the amount of active LOX protein. We established fibroblastic C3H10T1/2 stable transfectants with an expression vector for periostin that had been conjugated with an HA tag at the C-terminal end (10T1/2-periostin-HA) or with the empty vector (10T1/2-control). We prepared cell lysates from the 10T1/2-control and -periostin-HA stable transfectants grown to 2 days’ post-confluence that were then subjected to SDS-PAGE. To confirm the overexpression of periostin, we performed Western blot analysis with anti-periostin or anti-HA antibodies. 10T1/2-periostin-HA expressed higher levels of periostin than the 10T1/2-control, which slightly expressed endogenous periostin (Fig. 2A). To determine whether overexpression of periostin would increase the amount of active LOX protein, we performed Western blot analysis with the anti-LOX antibody. The amount of active LOX protein (32 kDa) was increased in cell lysates from 10T1/2-periostin-HA compared with that of the 10T1/2-control (Fig. 2B, arrow). The ratio of the active LOX protein in
the total one was significantly increased in the 10T1/2-periostin-HA, compared with that in the 10T1/2-control (Fig. 2C). To examine LOX mRNA expression levels in 10T1/2-control and 10T1/2-periostin-HA, we performed semi-quantitative RT-PCR analysis and found no significant difference in the expression of LOX gene between 10T1/2-control and 10T1/2-periostin-HA cells (Fig. 2D). These results indicate that overexpression of periostin increased the proteolytically cleaved form of pro-LOX.

**Co-localization of Periostin and BMP-1 in the Golgi**—To study the mechanism by which periostin enhanced the proteolytic cleavage of pro-LOX, we focused on BMP-1. We first examined BMP-1 mRNA expression levels in periosteum and COB cells from WT and periostin−/− mice. Semi-quantitative RT-PCR analysis showed no significant difference in the expression of BMP-1 gene between WT and periostin−/− mice. We also observed no significant difference in the expression of BMP-1 gene between WT and periostin−/− periosteum or COB cells (Fig. 3A). Previous studies demonstrated that periostin is localized in the Golgi (22) and that BMP-1 is localized in the trans-Golgi network and plasma membrane (23). To examine the co-localization of periostin-HA and BMP-1-FLAG and then fluorescently stained with anti-HA, anti-FLAG, and anti-GM130 antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIGURE 3. Co-localization of periostin and BMP-1 in the Golgi.** A, expression of BMP-1 gene in periosteum and COB cells from WT and periostin−/− mice. Semi-quantitative RT-PCR analysis shows no significant difference in the expression of the BMP-1 gene in either periosteum or COB cells between the two groups. B, expression of BMP-1 gene in 10T1/2-control and -periostin-HA cells. Semi-quantitative RT-PCR analysis shows no significant difference in the expression of the BMP-1 gene between the two groups. C, co-localization of periostin and BMP-1 in the Golgi. 293 cells were transfected with the expression vectors for periostin-HA and BMP-1-FLAG and then fluorescently stained with anti-HA, anti-FLAG, and anti-GM130 antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ostin and BMP-1 in the Golgi, we performed an immunofluorescence analysis using 293 cells that had been stably transfected with the expression vector for BMP-1 that had been conjugated with a FLAG-tag at its C-terminal end (BMP-1-FLAG). The stable transfecant was then transiently transfected with the expression vector for periostin-HA and then fluorescently stained with anti-HA antibody, anti-FLAG antibody, and antibody against GM130, which is a Golgi marker. Fluorescent signals for both periostin-HA and BMP-1-FLAG were detected in the Golgi, indicating that periostin-HA was co-localized with BMP-1-FLAG in the Golgi (Fig. 3C).

Periostin Binds to BMP-1—The co-localization of periostin and BMP-1 possibly indicates that periostin interacted with BMP-1. To assess this interaction, we performed a co-immunoprecipitation assay. We established 293 cells stably transfected with the expression vector for periostin-HA (Fig. 4A). Co-immunoprecipitation was performed with cell lysates from the above 293 cell stable transfectants that had also been transiently transfected with the expression vector for BMP-1-FLAG. Periostin-HA interacted with BMP-1-FLAG in cell lysates (Fig. 4B). This result indicates that periostin bound to BMP-1. Periostin consists of six domains: the N-terminal EMI domain, four tandem repeats of the fas1 domain, and a C-terminal region (CTR) (10, 11). To identify the binding domain, we used expression vectors of deletion forms of periostin that had been conjugated with an HA tag at their C-terminal end, as described previously (12). Additionally, we generated the expression vector of the EMI domain conjugated with an HA tag at its C-terminal end (EMI-HA). The structures of these representative constructs are shown in Fig. 4A. We established 293 cell stable transfectants with each of these expression vectors. Co-immunoprecipitation was performed with cell lysates from the 293 cell stable transfectants that had been also transiently transfected with the expression vector for BMP-1-FLAG. The deletion forms for the EMI domain (ΔEMI-HA) and CTR (ΔCTR-HA) interacted with BMP-1-FLAG (Fig. 4B). The deletion form for both the EMI domain and the CTR (ΔEMIΔCTR-HA) also interacted with BMP-1-FLAG, whereas the EMI-HA did not (Fig. 4B). These results indicate that the four tandem repeats of the fas1 domain were involved in interaction with BMP-1.

Metalloproteinase Domain of BMP-1 Binds to Periostin—BMP-1 consists of a signal peptide, a prodomain, a metalloproteinase domain, two CUB domains, an EGF-like domain, an additional CUB domain, and a short specific sequence at the C terminus (24). To identify the binding domain of BMP-1 for periostin, we generated expression vectors of the intact and deletion forms of BMP-1-FLAG. The structures of these representative constructs are shown in Fig. 4C. Co-immunoprecipitation was performed with cell lysates from the transfectants stably expressing periostin-HA that had also been transiently transfected with each of the expression vectors. BMP-1-FLAG interacted with periostin-HA (Fig. 4D). The deletion forms for the CUB3 domain (ΔC3-FLAG) and for the CUB1, 2, and 3 domains and the EGF-like domain (ΔCUBΔEGF-FLAG) also interacted with periostin-HA (Fig. 4D), suggesting the prodomain and the metalloproteinase domain to be involved in the interaction with periostin. To further identify the binding domain, we generated expression vectors for the prodomain of BMP-1-FLAG (PD1-FLAG and PD2-FLAG), as shown in Fig. 4C. PD2-FLAG contains the furin recognition motif, whereas the PD1-FLAG does not. Neither PD1-FLAG nor PD2-FLAG interacted with periostin-HA (Fig. 4D). These results indicate that the metalloproteinase domain interacted with periostin.

To confirm the direct binding between periostin and BMP-1, we purified the four tandem repeats of the fas1 domain (ΔEMIΔCTR-HA) of periostin and BMP-1-FLAG from the cell culture supernatant of the 293T cells stably expressing ΔEMIΔCTR-HA and the total cell lysate of the 293T cells stably expressing BMP-1-FLAG, respectively. The purified BMP-1-FLAG proteins were coated on a microtiter plate and subjected to a solid phase binding assay. The purified ΔEMIΔCTR-HA proteins bound to BMP-1-FLAG coated on a microtiter plate in a dose-dependent manner (Fig. 4E), indicating the direct binding between the four tandem repeats of the fas1 domain and BMP-1.

Periostin-enhanced Incorporation of BMP-1 into the Fibronectin Matrix—Although we showed the interaction between periostin and BMP-1, it is unclear how the interaction affected the function of BMP-1. Previous studies demonstrated that periostin (16) or BMP-1 directly binds to fibronectin and that BMP-1 is localized on the fibronectin matrix (25). We then examined the localization of BMP-1 on the fibronectin matrix in the 10T1/2-control and -periostin-HA stable transfectant cultures by immunofluorescence analysis. Strong fluorescent signals for BMP-1 were detected on the fibronectin matrix in the 10T1/2-periostin-HA cell cultures (Fig. 5A, arrowheads); in contrast, strong signals for BMP-1 were rarely detected there in the 10T1/2-control cell cultures (Fig. 5A). On the other hand, BMP-1 on the fibronectin matrix was decreased in the periostin(W65A)-COB cell cultures, compared with that in the WT COB cell cultures (supplemental Fig. S1). Thus, these results indicate that periostin promoted the deposition of BMP-1 on the fibronectin matrix. A recent study of ours demonstrated that the tryptophan residue at position 65 in the EMI domain of periostin is essential for the interaction between periostin and fibronectin and that the mutant with the tryptophan residue substituted to alanine (W65A) does not bind to fibronectin (12). On the other hand, this W65A mutant of periostin bound to BMP-1 (data not shown). We then established C3H10T1/2 stable transfectants with the expression vector for the W65A mutant form that had been conjugated with an HA tag at its C-terminal end, 10T1/2-periostin(W65A)-HA, and performed immunofluorescence analysis. In the 10T1/2-periostin(W65A)-HA cell cultures, strong fluorescent signals for BMP-1 on the fibronectin matrix were rarely detected as well as those in the 10T1/2-control ones (Fig. 5A), indicating that the W65A mutant form did not promote the ECM deposition of BMP-1. To evaluate the incorporation of secreted BMP-1 into the fibronectin matrix, we performed Western blot analysis using total cell lysates and cell culture supernatants of the 10T1/2 stable transfectants. BMP-1 was significantly increased in the total cell lysate of the 10T1/2-periostin-HA, compared with those of the 10T1/2-control and -periostin(W65A)-HA (Fig. 5B). Consequently, BMP-1 was decreased in the cell culture supernatant of the 10T1/2-periostin-HA (Fig. 5B). These results are...
FIGURE 4. Interaction between the fas1 domain of periostin and the metalloproteinase domain of BMP-1. A, schematic view of domain structures of the intact and domain-deletion forms of periostin. These intact and deletion forms were expressed as C-terminal HA-tagged proteins. B, the four tandem repeats of the fas1 domain bind to BMP-1-FLAG. 293 cells were transfected with the expression vectors for periostin-HA or its deletion forms and BMP-1-FLAG. Total cell lysates of the 293 transfectants were subjected to a co-immunoprecipitation (IP) assay with anti-HA antibody-conjugated agarose. Immunoprecipitated complexes were separated by SDS-PAGE and then blotted with anti-HA and anti-FLAG antibodies, respectively. C, schematic view of domain structures of the intact and domain deletion forms of BMP-1. These intact and deletion forms were expressed as C-terminal FLAG-tagged proteins. S, signal sequence; P, prodomain; M, metalloproteinase domain; C1–3, CUB1–3 domains; E, EGF-like domain; Sp, specific region. D, the metalloproteinase domain of BMP-1-FLAG binds to periostin-HA. Co-immunoprecipitation assays between periostin-HA and BMP-1-FLAG or its deletion forms were performed with cell lysates of the 293 transfectants. E, the direct interaction between ΔEMIΔCTR-HA and BMP-1-FLAG in a solid phase. The purified BMP-1-FLAG (open circle) or bovine serum albumin (closed circle) was coated onto wells of the microtiter plate at 5 μg/ml. Binding of ΔEMIΔCTR-HA (0–100 nM) was performed at 4 °C overnight. The bound ΔEMIΔCTR-HA was detected with anti-HA antibody, followed by horseradish peroxidase-conjugated secondary antibody. The error bars represent the means ± S.E.
Periostin increases the deposition of BMP-1 on the fibronectin matrix. A, localization of BMP-1 and fibronectin in the C3H10T1/2 transfectant cell cultures. The C3H10T1/2 transfectants harboring the empty vector (control), the periostin-HA expression vector (periostin-HA), or the periostin(W65A)-HA expression vector (periostin(W65A)-HA) were grown to 2 days' post-confluence and fluorescently stained with anti-BMP-1 and anti-fibronectin antibodies without prior detergent treatment. The nuclei were stained with TOPRO3. The arrowheads indicate strong fluorescent signals for BMP-1 on the fibronectin matrix. B, overexpression of periostin-HA increases the amount of BMP-1 protein in the total cell lysate and decreases that in the cell culture supernatant (sup.) of the C3H10T1/2 transfectants. The total cell lysates and cell culture supernatants of the C3H10T1/2 transfectants were separated by SDS-PAGE and blotted with anti-BMP-1 and anti-β-actin antibodies, respectively. C, C3H10T1/2 transfectants harboring the empty vector (control), the periostin-HA expression vector (periostin-HA), or the periostin(W65A)-HA expression vector (periostin(W65A)-HA) were grown to 2 days' post-confluence and quantitatively analyzed for the total amount of LOX-mediated pyridinium cross-linking (n = 5). PYD, pyridinoline; DPD, deoxypyridinoline. The error bars represent the means ± S.E. The asterisk indicates a significant difference (p < 0.0001).
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Consistent with those of the immunofluorescence analysis (Fig. 5A). These results suggest that the interaction between periostin and fibronectin was involved in the incorporation of BMP-1 into the fibronectin matrix.

To examine whether the interaction between periostin and fibronectin played an essential role in the promotion of collagen cross-linking, we performed quantitative analysis of collagen cross-linking in the C3H10T1/2 stable transfectants. The total amount of LOX-mediated pyridinium cross-linking, which was an increase of pyridinoline and desoxypyridinoline content per mol of collagen, was increased 3.3-fold in the 10T1/2-periostin-HA compared with that for the 10T1/2-control (Fig. 5C). In the 10T1/2-periostin(W65A)-HA, we found no increase in the content of pyridinoline and desoxypyridinoline (Fig. 5C), suggesting that the interaction between periostin and fibronectin was involved in the promotion of collagen cross-linking. Consequently, we propose that periostin acts as a scaffold for the interaction between BMP-1 and fibronectin to enhance the incorporation of BMP-1 into the fibronectin matrix, which results in the promotion of collagen cross-linking.

DISCUSSION

In this study, we demonstrate that periostin bound to BMP-1 and promoted the proteolytic activation of pro-LOX. Periostin is mainly expressed in collagen-rich fibrous connective tissues that are constantly subjected to mechanical stress (10, 11). Previous studies showed that periostin−/− mice exhibit aberrant collagen fibrillogenesis, such as reduced collagen cross-linking in their femurs, infarcted myocardium, and tendons (12–14). The covalent cross-linking of collagen is catalyzed by LOX, which is an essential step in collagen fibrillogenesis (1). Our findings provide a novel molecular mechanism by which periostin supports BMP-1-mediated proteolytic activation of pro-LOX and thereby promotes collagen cross-linking. The cross-linking determines mechanical properties of the ECM, suggesting that periostin plays a role in the development and maintenance of mechanical properties of the connective tissues.

Periostin Interacts with Both BMP-1 and Fibronectin—The present study reveals that periostin interacted with BMP-1. The results of the co-immunoprecipitation and solid phase binding assays showed that the four tandem repeats of the fas1 domain directly interacts with the metalloproteinase domain of BMP-1. The N-terminal EMI domain of periostin has been demonstrated to directly interact with fibronectin and type I collagen (13, 16). A recent study revealed that fibronectin directly interacts with BMP-1 (25) and enhances the activity of BMP-1 (25). Our data revealed the increased deposition of BMP-1 on the fibronectin matrix in the 10T1/2-periostin-HA cell cultures. The W65A mutant form of periostin, however, did not increase the deposition of BMP-1, indicating that the interaction between periostin and fibronectin is important for the incorporation of BMP-1 into the fibronectin matrix. These results suggest that periostin acts as a scaffold for the interaction between BMP-1 and fibronectin to promote incorporation of BMP-1 into the fibronectin matrix. Furthermore, a previous study showed that the four tandem repeats of the fas1 domain directly interact with tenascin-C and that periostin promotes the incorporation of tenascin-C into the ECM (12). Those results also suggest that periostin acts as a scaffold for the assembly of tenascin-C and the ECM.

The co-localization of periostin and BMP-1 was detected in the Golgi. Periostin was earlier indicated to be localized in the Golgi (22) and in the matricellular space (26). BMP-1 has been reported to be localized in the Golgi (23), as well as on the fibronectin matrix (25). These results possibly indicate that periostin assists the interaction between fibronectin and BMP-1.

On the other hand, the direct binding between the four tandem repeats of the fas1 domain and the metalloproteinase domain of BMP-1 indicates a possibility that the four tandem repeats have an effect on the folding and activity of the metalloproteinase domain. Further study is necessary to clear this possibility.

Periostin Plays a Role in Proteolytic Activation of Pro-LOX—Periostin-mediated incorporation of BMP-1 into the fibronectin matrix prompts us to envisage that the increased deposition of BMP-1 on the fibronectin matrix promotes proteolytic activation of pro-LOX. It has been demonstrated that the binding of pro-LOX to fibronectin is critical for proteolytic activation of LOX (15). The present study showed that overexpression of periostin enhanced proteolytic activation of pro-LOX in C3H10T1/2 cells. It has also been shown that overexpression of periostin in cardiac valvulogenic tissue increased the overall viscosity, which is a measure of collagen cross-linking (13). Thus, these results suggest that periostin brings BMP-1 into close proximity of pro-LOX deposited on the fibronectin matrix, thereby promoting collagen cross-linking.

Periostin−/− mice exhibited tibial periostitis that is one of inflammatory-like stress reactions (12), as well as the reduced amount of collagen cross-linking in their femur and periostea. The expression of LOX mRNA is highly responsive to various physiological conditions, such as growth, wound repair, and aging, as well as to disease states (27, 28). Our RT-PCR analysis, however, showed no significant difference in the expression of LOX and LOX family genes between WT and periostin−/− periostea. These data suggest that reduced cross-linking of collagen fibrils does not result from the altered expression of LOX and LOX family genes.

The amount of active LOX protein was decreased in periostin−/− COB cells, suggesting that reduced cross-linking of collagen fibrils was due to the decreased amount of active LOX protein. Our data showed that N-glycosylated pro-LOX proteins were also decreased in periostin−/− COB cells, possibly indicating that periostin plays a role in the proteolytic cleavage of pro-LOX and/or in the stabilization of LOX proteins. It has been demonstrated that periostin directly binds to fibronectin (16) and that the fibronectin ECM architecture is severely disrupted in periostin−/− COB cell cultures (12). Cellular fibronectin has been reported to bind to LOX with high affinity (15). In addition, overexpression of fibronectin increased LOX proteins in total cell lysates.3 These results imply that the decreased amount of active and N-glycosylated pro-LOX pro-
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