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Fibulin-4 and -5, but not Fibulin-2, are Associated with Tropoelastin Deposition in Elastin-Producing Cell Culture

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Elastic system fibers consist of microfibrils and tropoelastin. During development, microfibrils act as a template on which tropoelastin is deposited. Fibrillin-1 is the major component of microfibrils. It is not clear whether elastic fiber-associated molecules, such as fibulins, contribute to tropoelastin deposition. Among the fibulin family, fibulin-2, -4 and -5 are capable of binding to tropoelastin and fibrillin-1. In the present study, we used the RNA interference (RNAi) technique to establish individual gene-specific knockdown of fibulin-2, -4 and -5 in elastin-producing cells (human gingival fibroblasts; HGF). We then examined the extracellular deposition of tropoelastin using immunofluorescence. RNAi-mediated down-regulation of fibulin-4 and -5 was responsible for the diminution of tropoelastin deposition. Suppression of fibulin-5 appeared to inhibit the formation of fibrillin-1 microfibrils, while that of fibulin-4 did not. Similar results to those for HGF were obtained with human dermal fibroblasts. These results suggest that fibulin-4 and -5 may be associated in different ways with the extracellular deposition of tropoelastin during elastic fiber formation in elastin-producing cells in culture.

Key words: elastic fiber, fibrillin, Fibulin, microfibrils, tropoelastin

I. Introduction

Elastic fibers consist of cross-linked elastin and fibrillin microfibrils [8]. It has been proposed that elastogenesis requires the deposition of tropoelastin, a soluble precursor of cross-linked elastin, on microfibrils, which are a pre-formed scaffold. Elastogenesis is also regulated by several elastin/microfibril-associated molecules such as fibulins and microfibril-associated glycoproteins (MAGPs).

We have biochemically investigated the metabolism of elastic system fibers by using both elastin-producing and elastin-non-producing cells [19, 20]. Our previous studies showed that MAGP-1 is required for tropoelastin deposition in cultured human gingival fibroblasts (HGF), as representative elastin-producing cells [21]. This culture model has been considered useful for investigating elastin deposition at the cellular level. However, it remains unclear how the fibulin family contributes to elastin deposition.

Fibulins are extracellular glycoproteins that are associated with elastic fibers [1, 3, 17]. The fibulin family consists of glycoproteins that share a common structure, including a C-terminal fibulin-type globular domain preceded by calcium-binding epidermal growth factor (cbEGF)-like modules. Fibulins 1-5 bind to tropoelastin with different affinities [9]. Among the seven types of fibulins, fibulin-5, known as DANCE (developmental arteries and neural crest epidermal growth factor like), was the first to be investigated in relation to elastogenesis [12, 22]. Fibulin-5 can bind to tropoelastin [22] and fibrillin-1 [5]. The existence of molecules that can bind to fibrillin-1 suggests that the molecules on microfibrils have some role in elastogenesis. Fibulin-5 is thought to control the proper orientation of elastic fibers by

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acting as a bridge molecule on elastin and microfibrils [11]. We previously demonstrated that expression of the fibulin-5 gene is regulated by the tropoelastin gene, and correlated with tropoelastin deposition in cultured HGF [21]. Seven fibulins have now been identified, and many researchers have focused on their relationship with elastic fibers. Among the fibulins, fibulin-2 and -4 are known to be located at the interface between elastin and fibrillin microfibrils [9, 14]. However, only fibulin-2, -4 and -5 can interact with fibrillin-1, suggesting that the binding of these fibulins to fibrillin microfibrils may affect the deposition of tropoelastin.

To gain a clearer grasp of the role of fibulins, we generated cells in which fibulin-2, -4 and -5 were individually knocked down using the RNA interference (RNAi) technique to carry out immunohistochemical analysis of tropoelastin deposition in cultures of HGF. We found that siRNA-mediated gene silencing of human fibulin-4 and -5 reduced the extracellular deposition of tropoelastin.

II. Materials and Methods

Cells and culture

The protocol for these experiments was reviewed and approved by the Fukuoka Dental College Research Ethics Committee, and informed consent was obtained from the tissue donors.

HGF were isolated from three different donors, and cultured as described previously [18]. Briefly, the gingival tissues were obtained surgically from molar teeth extracted for orthodontic reasons. After washing in phosphate-buffered saline (PBS) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin (Roche Diagnostics, Mannheim, Germany), the gingival samples were cut into small pieces, plated in petri dishes, and incubated in Minimum Essential Medium (MEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% newborn calf serum (NCS; Invitrogen) at 37°C in humidified air containing 5% CO_2. When the outgrowth of cells reached confluence, they were harvested with 0.025% trypsin (Invitrogen) in PBS, and transferred to plastic culture dishes at a 1:4 split ratio. For experiments, the cells were trypsinized and seeded at 1×10^6 cells/ml per 35-mm culture dish (Corning Inc., Corning, NY, USA) in MEM supplemented with 10% NCS, 100 units/ml penicillin and 100 mg/ml streptomycin. HGF was used from the 3rd to 6th passages in this study.

Human dermal fibroblasts were purchased from Cell Applications Inc. (San Diego, CA), and cultured under the same conditions as those for HGF.

Small interfering RNA (siRNA) design and transient transfection

siRNAs for human fibulin-2 (accession # BC051690) and fibulin-4 (accession # CR541934) were designed and synthesized by Sigma Aldrich Corp. (Tokyo, Japan).

The synthesized siRNA for fibulin-2 corresponded to bases 3917–3939 in the 3'-untranslated coding region. The siRNA sequence was: sense 5'-CAGAGACACGCGACCAUGUUG-3', antisense 3'-ACAUGGUCGCGUGUCUCUGGU-5. The negative control (scrambled order) was: sense 5'-CGCGAAGCCUAGACUACGAUC-3', antisense 3'-UCGUAGUCUAGGCUUCGCGCU-5'. The sequence of the negative control was designed as a randomized version of bases 3917–3939 in the 3'-untranslated region of fibulin-2. BLAST searches indicated that this siRNA was specific for fibulin-2, and had no homology with other proteins.

The synthesized siRNA for fibulin-4 corresponded to bases 1129–1151 in the coding region. The siRNA sequence was: sense 5'-CAGAUCCGUGCUGGAAACUCG-3', antisense 3'-AGUUUCCAGCACGGAUCUGAA-5. The negative control (scrambled order) was: sense 5'-AUCGACGAUCCCUAUUGGCGU-3', antisense 3'-GCCAAUAGGGAUCGUCGAUCU-5'. The sequence of the negative control was designed as a randomized version of bases 1129–1151 in the coding region of fibulin-4. BLAST searches indicated that this siRNA was specific for fibulin-4, and had no homology with other proteins.

The siRNA and negative control for human fibulin-5 (accession # NM_006329) used for this study have been described previously [13]. This siRNA corresponded to bases 1165–1190 in the coding region of Fibulin-5. The siRNA sequence was: sense 5'-GGCAGAGAAUUUUACAUGCGGCAAAAG-3', antisense 3'-UACCGUCUCUUAAAAUGUACGCCGUUU-5. The negative control (scrambled order) was: sense 5'-CGGUCUAGACUAGCGAGAUAAUAGAAG-3', antisense 3'-UAGCCAGAUCUGAUCGCUCUAUUAUCU-5'.

Transfection was performed on days 1 and 4 of culture continuously. The siRNA was transfected into HGF using X-treme GENE siRNA transfection reagent (Roche, Mannheim, Germany). First, 237.5 ml of OptiMEM medium/dish (Invitrogen, Grand Island, NY, USA) and 12.5 ml of the transfection reagent were preincubated for 10 min at room temperature. During this time, 748 ml of OptiMEM medium was mixed with 2 ml of 100 mM siRNA. The two mixtures were then combined and incubated for 20 min at room temperature to allow formation of their complex. The entire mixture was added to the cells in one dish, resulting in a final concentration of 200 nM for the siRNAs. After 12 hr of incubation, the transfection medium was replaced with fresh complete medium (MEM with 10% FCS). Mock transfection of cultures with the transfection reagent alone was used as a control. HGF was transfected twice with the siRNA duplex (0, 200 nM), with a 72-hr interval in between, and analyzed at 7 days.

Immunofluorescence

At 7 days of culture, HGF and human dermal fibroblasts were fixed in ice-cold 4% paraformaldehyde for 15 min, followed by washing with PBS. Nonspecific immunoreactivity was blocked with 1% bovine serum albumin in PBS for 1 hr at room temperature. The cell layers were then incubated for 2 hr at room temperature with the appropriate primary antibodies (monoclonal antibody against human fibrillin-1 diluted 1:1000; Thermo Fisher Scientific, Fre-

mont, CA, USA: rabbit antibody against bovine tropoelastin diluted 1:1000; Elastin Products Co., Owensville, MO, USA: rabbit antibody against human fibulin-2 diluted 1:1000; Sigma Chemical Co., St. Louis, MO, USA: rabbit antibody against human fibulin-4 diluted 1:1000; Sigma Chemical Co., St. Louis, MO, USA: goat antibody against human fibulin-5 diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Controls included the use of preimmune normal goat or rabbit IgG for incubation with the primary antibody. After rinsing in PBS, the cells were incubated with Alexa Fluor® 568-labeled donkey anti-goat or rabbit IgG antibody or Alexa Fluor® 488-labeled donkey anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA), diluted 1:2000 with blocking buffer, for 1 hr at room temperature. After the final washing, the cells were stained with DAPI and viewed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK). The images were scanned and densitometric semi-quantitative analysis of the signals was performed using the Image J program (National Institutes of Health, Bethesda, MD, USA) as described previously [6]. Data represent the mean±S.D. (standard deviation) of three independent experimental determinations. Student's *t* test was used to determine the statistical significance of differences at $p<0.05$.

III. Results

Localization of fibulin-2, -4 and -5 on microfibrils

We examined whether fibulin-2, -4 and -5 are localized on microfibrils (Fig. 1). Positive staining for fibrillin-1 was observed on microfibrils, appearing as networks of fiber patterns. Fibulin-2, -4 and -5 were labeled on fibrillin-1-immunolabeled microfibrils of HGF cultured for 7 days. Control immune serum produced no labeling (not shown). These results showed that fibulin-2, -4 and -5 were co-localized with microfibrils.

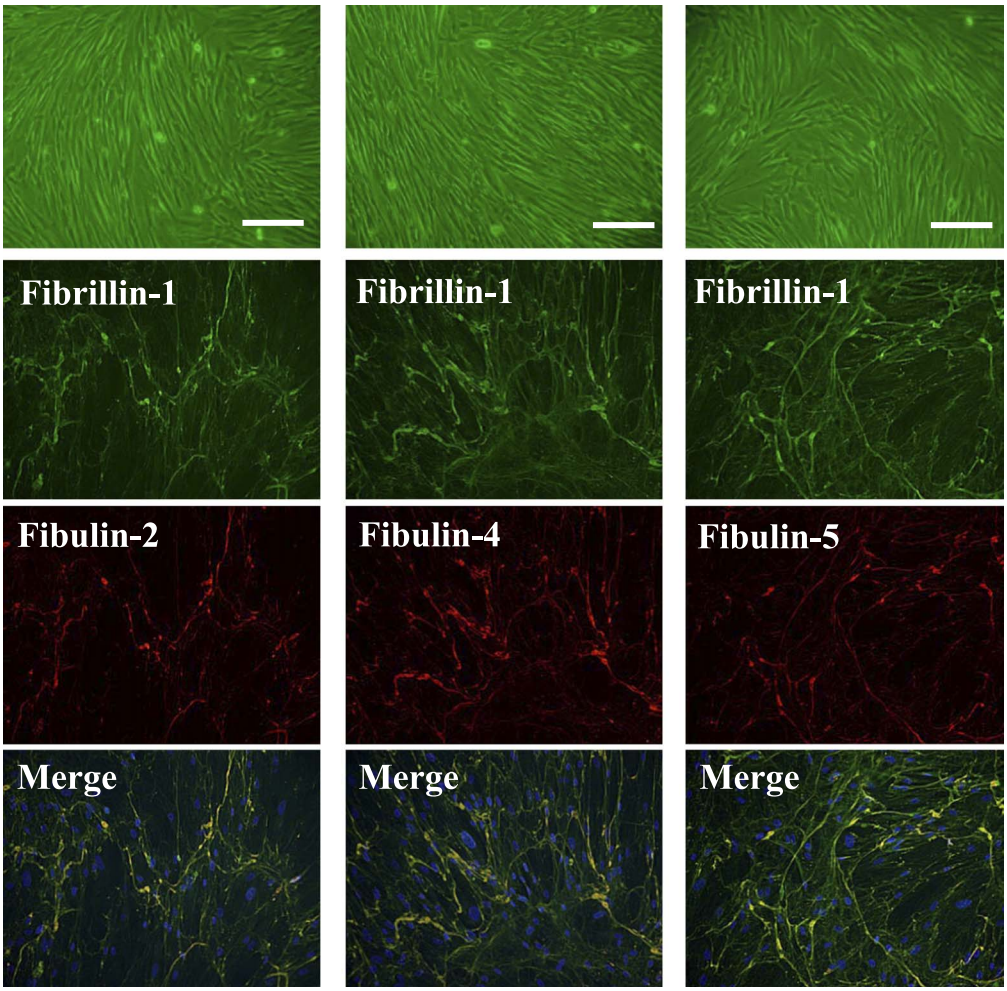

Fig. 1. Immunolocalization of fibulin-2, -4 and -5 to microfibrils. Double immunofluorescence for fibrillin-1/fibulin-2 (left lane), fibrillin-1/fibulin-4 (middle lane) and fibrillin-1/fibulin-5 (right lane) in cultures of human gingival fibroblasts (HGF). HGF were cultured for 7 days, and then simultaneously labeled for fibrillin-1 (green) (upper panels), fibulins (red) (middle panels), and superimposition of both labels (lower panels). Each of the images of a phase contrast is shown in the upper lane. DAPI was used for nuclear staining (blue). Bar=200 nm.

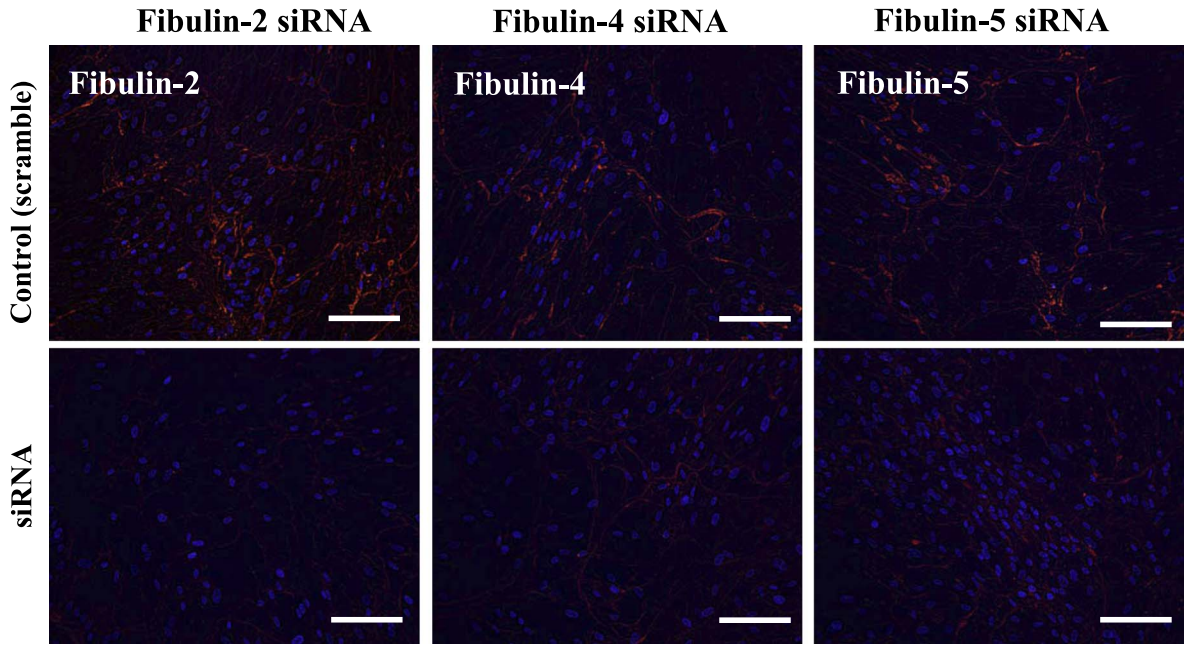

Fig. 2. siRNA for each of fibulin-2, -4 and -5 suppresses deposition of its protein. HGF were cultured for 7 days, and then transiently transfected with 200 nM siRNA for 7 days (lower lane). Scrambled siRNA-transfected culture was used as a control (upper lane). HGF were immunolabeled with antibodies against fibulin-2 (left lane), fibulin-4 (middle lane) and fibulin-5 (right lane). Each fibulin appears in red. DAPI was used for nuclear staining (blue). Bar=200 nm.

siRNA for each of fibulin-2, -4 and -5 suppresses deposition of its protein

Next, in order to investigate the function of fibulin-2, -4 and -5, we used siRNA to suppress the expression of each. The immunolabeling data showed that when 200 nM siRNA was used for transfection, the siRNA effectively diminished the level of fibulin-2, -4 and -5 staining in comparison with that of the control (scrambled siRNA) in the HGF cell/matrix layers at 7 days (Fig. 2). In contrast, vehicle only (without siRNA) had no effect on fibulin-2, -4 and -5 deposition, and no difference from the control (scrambled siRNA) was evident (data not shown), proving that each siRNA was specific for each fibulin.

Deposition of tropoelastin on fibulin-2, -4 and -5 siRNA-transfected cell layers

The effect of suppression of each of the genes for fibulin-2, -4 and -5 on tropoelastin deposition in HGF cell layers was investigated by immunohistochemical assay.

For fibulin-2 siRNA-transfected cells, tropoelastin staining on microfibrils appeared the same as that for the control (scrambled siRNA-transfected cells) (Fig. 3). On the other hand, fibulin-4 suppression reduced the level of tropoelastin deposition to 10% of the control (scrambled siRNA-transfected cells) (Fig. 4). Similarly, for fibulin-5 siRNA-transfected cells, tropoelastin staining was diminished to 20% of the control (scrambled siRNA-transfected cells) (Fig. 5). Although fibrillin-1 staining was observed as microfibrils in cells transfected with each of the siRNAs (Figs. 3–5), each appearance of the microfibrils was differ-

Fig. 3. Effect of fibulin-2 suppression on tropoelastin deposition. (**A**) HGF were transiently transfected with 200 nM scrambled siRNA for fibulin-2 (left lane), or transiently transfected with 200 nM siRNA for fibulin-2 (right lane). Immunofluorescence staining was performed with antibodies against fibrillin-1 (upper panels) and tropoelastin (middle lane), and superimposition of both labels (lower panels). DAPI was used for nuclear staining (blue). Bar=200 nm. (**B**) The intensity of fluorescence was obtained by Image J for control set as 100. Data represent the mean Data represent the mean±S.D. (standard deviation) of three independent experimental determinations. Student's *t* test was used to determine the statistical significance of differences at $p<0.05$.

Fig. 4. Effect of fibulin-4 suppression on tropoelastin deposition. (**A**) HGF were transiently transfected with 200 nM scrambled siRNA for fibulin-4 (left lane), or transiently transfected with 200 nM siRNA for fibulin-4 (right lane). Immunofluorescence staining was performed with antibodies against fibrillin-1 (upper panels) and tropoelastin (middle lane) and superimposition of both labels (lower panels). DAPI was used for nuclear staining (blue). Bar=200 nm. (**B**) The intensity of fluorescence was obtained by Image J for control set as 100. Data represent the mean Data represent the mean±S.D. (standard deviation) of three independent experimental determinations. Student's *t* test was used to determine the statistical significance of differences at $p<0.05$.

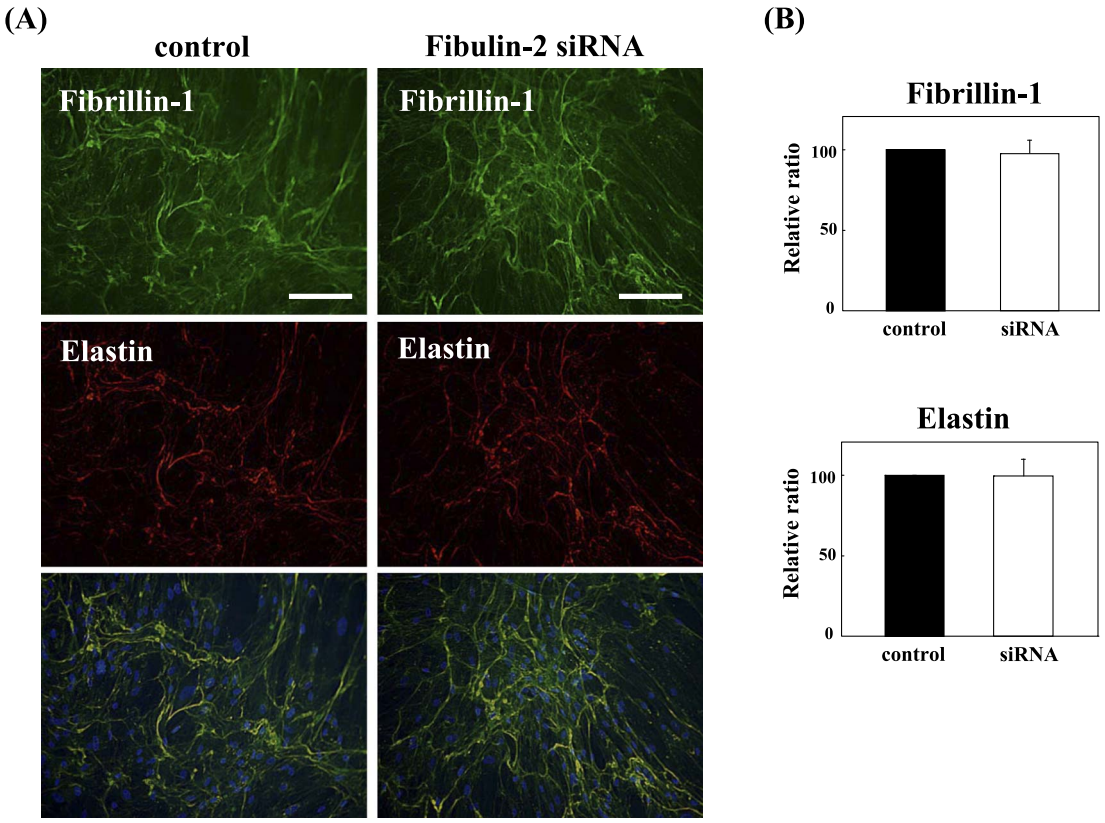
(A)
control
Fibulin-2 siRNA
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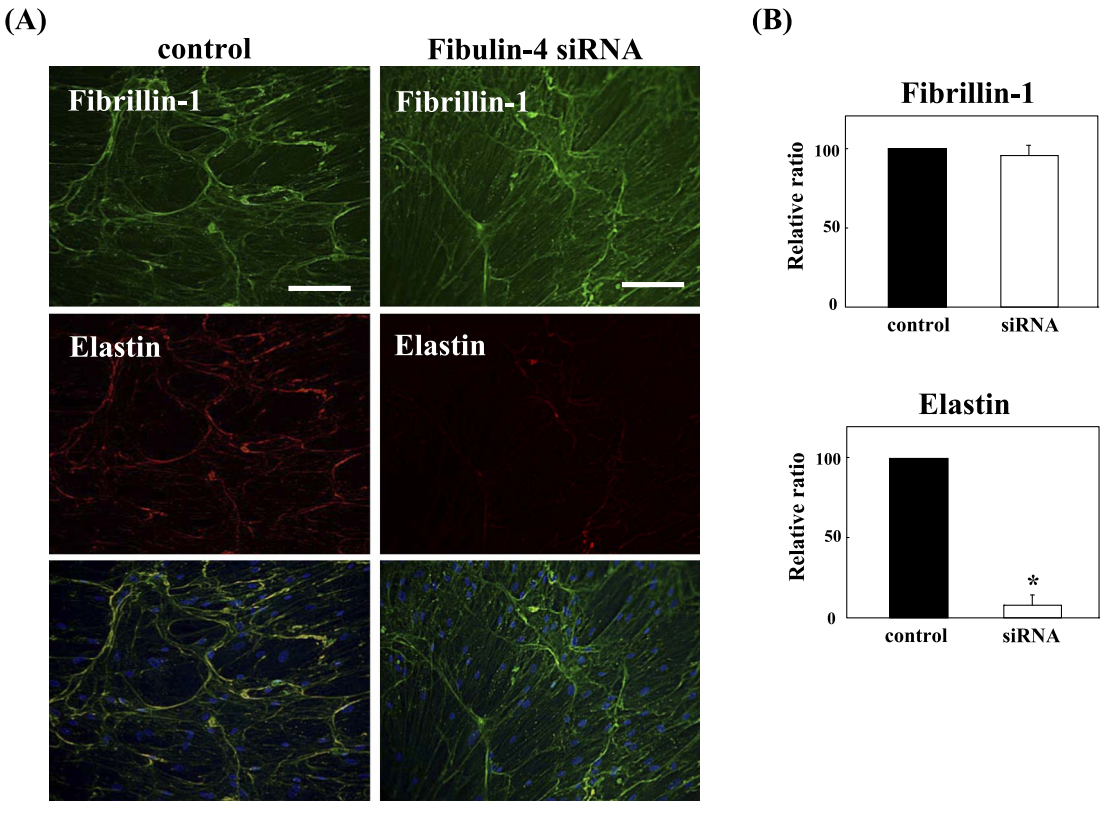
Fig. 3


(A)
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Fibulin-4 siRNA
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Fig. 4

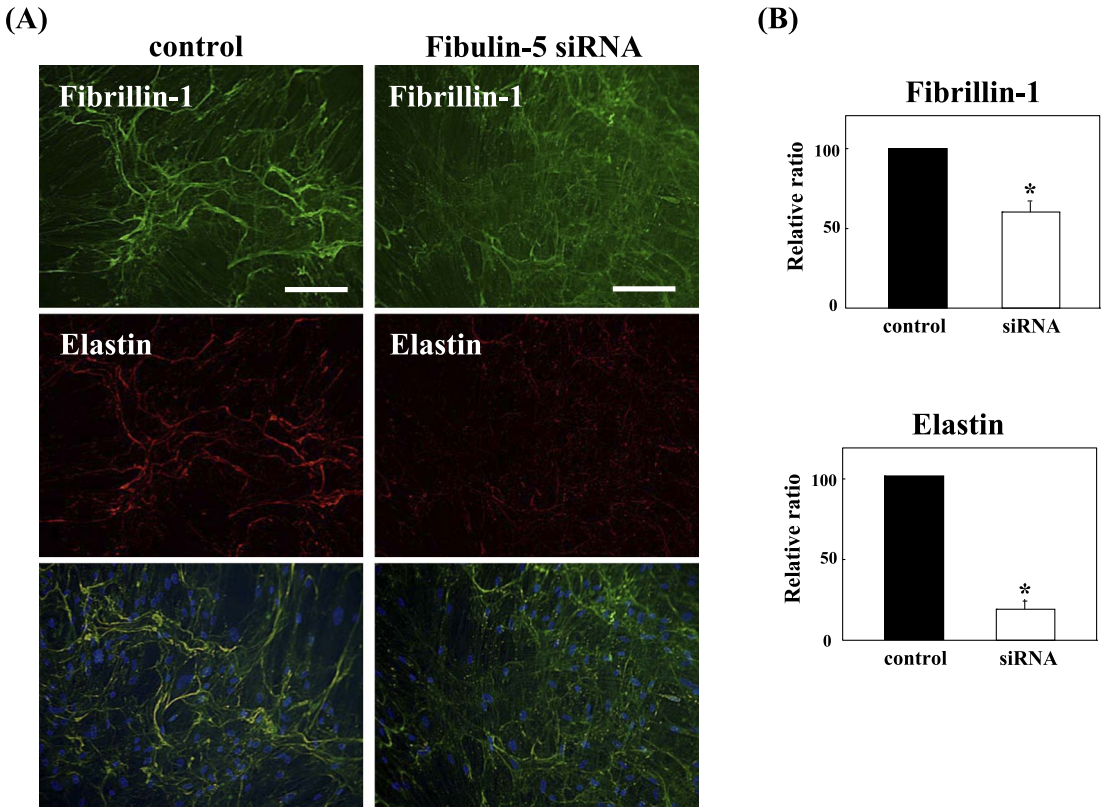

Fig. 5. Effect of fibulin-5 suppression on tropoelastin deposition. (**A**) HGF was transiently transfected with 200 nM scrambled siRNA for fibulin-5 (left lane), or transiently transfected with 200 nM siRNA for fibulin-5 (right lane). Immunofluorescence staining was performed with antibody against fibrillin-1 (upper panels) and tropoelastin (middle lane) and superimposition of both labels (lower panels). DAPI was used for nuclear staining (blue). Bar=200 nm. (**B**) The intensity of fluorescence was obtained by Image J for control set as 100. Data represent the mean±S.D. (standard deviation) of three independent experimental determinations. Student's *t* test was used to determine the statistical significance of differences at $p<0.05$.

ent. The intensity of microfibrils had the same degree as those of the control fibulin-2 or fibulin-4 siRNA transfected cells (Figs. 3, 4). However, the intensity of fibrillin-1 staining was decreased to about 60% of the control, reflecting the decrease of tropoelastin deposition, in comparison with the scrambled siRNA-transfected cells (Fig. 5).

In order to further generalize these results, we used human dermal fibroblasts, which are also known to produce elastin and deposit tropoelastin on fibrillin microfibril networks [7]. The results showed the same tendency, i.e. that suppression of each of fibulin-4 or -5 inhibited tropoelastin deposition, whereas suppression of fibulin-2 did not (Fig. 6). Moreover, formation of microfibrils was hindered by fibulin-5 knockdown, but not by fibulin-4 knockdown.

IV. Discussion

In the present study using the RNA interference technique, we have demonstrated for the first time that fibulin-4 and -5 affect the extracellular deposition of tropoelastin by human elastin-producing cells in culture. In this study, suppression of fibulin-4 or fibulin-5, but not fibulin-2, reduced tropoelastin deposition in an *in vitro* culture system. It is generally thought that tropoelastin is laid down on microfibrils as a template. Figure 5 shows that fibulin-5 suppression inhibited not only tropoelastin deposition but also fibrillin-1 microfibril formation. This is in agreement with our recent demonstration that suppression of fibulin-5 by siRNA decreased oxytalan fiber formation in cultured human periodontal ligament cells [6]. Therefore, the decrease of tropoelastin can be explained in terms of its deposition on the template. On the other hand, fibulin-4 suppression did not affect the appearance of microfibrils, as shown in Figure 4, despite a decrease of tropoelastin deposition. Fibulin-4-deficient mice show abnormalities of elastic fibers, which result in severe lung and vascular defects [10]. It is known that the ternary complex of fibulin-4, lysyl-oxidase and tropoelastin promotes elastin cross-linkage [2]. Due to the lack of such a complex, despite formation of a normal microfibril network, fibulin-4 suppression may inhibit the deposition of tropoelastin. Fibulin-4 is thought to play a different but essential role from that of fibulin-5 in the process of elastogenesis. Therefore, analysis of lysyl-oxidase family is currently under way. Fibulin-2 also binds to tropoelastin [9, 15] and fibrillin-1[4], but was shown not to be important for tropoelastin deposition in the present study. Our culture

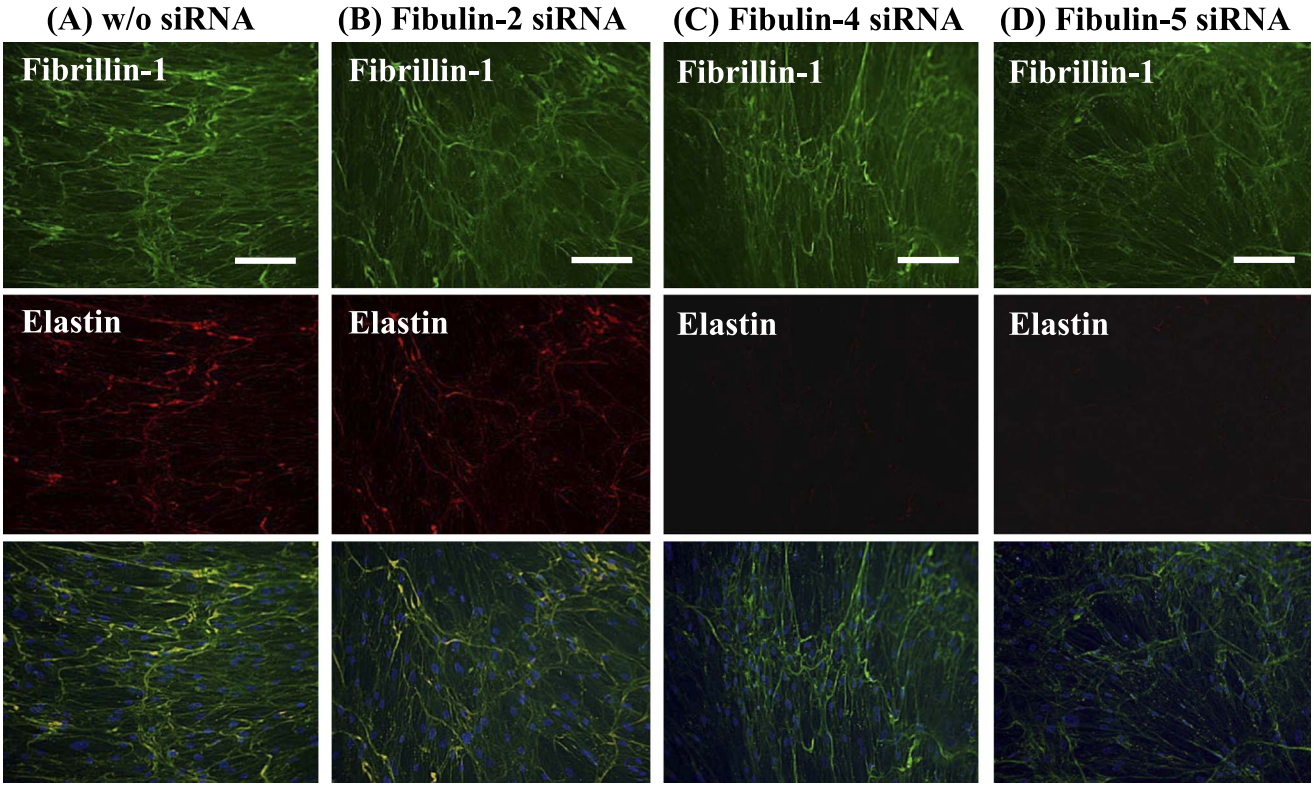

Fig. 6. Effect of fibulin suppression on tropoelastin deposition in human dermal fibroblasts. Human dermal fibroblasts were transiently transfected with 200 nM siRNA for fibulin-2 (**B**), fibulin-4 (**C**) and fibulin-5 (**D**). Mock-transfected (vehicle only) culture was used as a control (**A**). Immunofluorescence staining was performed with antibodies against fibrillin-1 (upper panels) and tropoelastin (middle lane) and superimposition of both labels (lower panels). DAPI was used for nuclear staining (blue). Bar=200 nm.

results are in accord with those of gene targeting for fibulin-2 in mice, whose elastic fibers appear grossly and anatomically normal [16]. Fibulin-2 may work for the maintenance of elastic fibers after their formation has been completed. Further analysis will be necessary to clarify the function of fibulin-2 in elastogenesis.

In conclusion, we have obtained direct evidence to support the involvement of fibulin-4 and -5 in the extracellular deposition of tropoelastin in elastin-producing cells such as HGF and human dermal fibroblasts cultures. These results suggest that this culture system would be a useful experimental tool for investigating the mechanism of elastogenesis in detail.

V. Acknowledgments

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VI. References

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