Fibrillin-1 and fibrillin-2 are essential for formation of thick oxytalan fibers in human nonpigmented ciliary epithelial cells in vitro

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ABSTRACT

The ciliary zonule, also known as Zinn's zonule, is composed of oxytalan fibers. However, the mechanism by which epithelial cells in the ciliary body form these fibers is not fully understood. We examined human non-pigmented ciliary epithelial cells to determine the appearance and amount of oxytalan fibers in terms of positivity for their major components, fibrillin-1 and fibrillin-2. Examination of fibrillin-1 and fibrillin-2 expression by immunofluorescence revealed that thin fibers positive for fibrillin-1 on Day 2 changed to thick fibers by Day 8. The fibers positive for fibrillin-2 appeared on the thick fibrillin-1-positive fibers after Day 4. Northern blot analysis revealed that the level of fibrillin-1 did not change markedly, while induction of fibrillin-2 gene was evident at Day 5. Western blot analysis showed that fibrillin-1 deposition increased gradually, while that of fibrillin-2 increased markedly from Day 5 to Day 8. Fibrillin-1 suppression did not lead to formation of fibrillin-2-positive thick fibers, whereas fibrillin-2 suppression led to the formation of fibrillin-1-positive thin fibers, but not thick fibers. These results suggest that both fibrillin-1 and fibrillin-2 are essential for the formation of thick oxytalan fibers in the ciliary zonule and are informative for clarifying the mechanism of homeostasis of the ocular matrix.

INTRODUCTION

The elastic system fibers that give tissue resilience and flexibility are of three types – oxytalan, elaunin, and elastic fibers – differing in their relative proportions of microfibrils and elastin [1]. Oxytalan fibers are composed of pure microfibrils. Among the microfibrillar molecules, fibrillin-1 and fibrillin-2 are the best characterized [2], and are known to interact to form homotypic and heterotypic dimers [3, 4]. Fibrillins are assembled pericellularly into microfibril arrays that appear to undergo maturation into transglutaminase-cross-linked microfibrils [5]. Oxytalan fibers were first described in periodontal ligaments (PDL) by Fullmer and Lillie [6]. Oxytalan fibers are distributed mainly in the ciliary zonule as well as in PDL [7].

The ciliary zonule, also known as Zinn's zonule, is a ligament that links the lens to the ciliary body in the eye. The role of the ciliary zonule is to convey retraction of the ciliary muscles effectively to the lens, thereby controlling the thickness of the lens during focusing. The ciliary zonule is composed of fibrillin-rich oxytalan fibers [8]. A study using guinea pigs has shown that nonpigmented ciliary epithelial cells in the ciliary

body express fibrillin-1 [9]. Therefore, it is thought that nonpigmented ciliary epithelial cells produce the oxytalan fibers of the ciliary zonule. However, very few data on the mechanism of oxytalan fiber formation in the ciliary zonule are currently available.

We have previously investigated the formation and degradation of oxytalan fibers in PDL using a PDL cell culture system [10, 11]. Also, using fibroblast culture, we have clearly demonstrated that oxytalan fibers, which consist of fibrillin-1 and fibrillin-2, are formed in cell/matrix layers. Mutations of the fibrillin-1 and fibrillin-2 genes result in the severe heritable connective tissue diseases Marfan syndrome and congenital contractural arachnodactyly (CCA), respectively [12-14]. In Marfan syndrome, the ciliary zonule becomes loose and forms disorganized bundles [15, 16]. The phenotype of CCA is known to overlap that of Marfan syndrome. Both syndromes are characterized by the presence of an ectopic lens, due to abnormalities of oxytalan fibers in the ciliary zonule supporting the lens. However, the roles of fibrillin-1 and fibrillin-2 in the metabolism of the ciliary zonule have not been analyzed in detail. Therefore, this study, we used a human nonpigmented ciliary epithelial cell (HNPCEC) culture model of pure oxytalan fibers to investigate the role of fibrillin-1 and fibrillin-2 in the development of oxytalan fibers in the ciliary zonule, and found that both fibrillin-1 and fibrillin-2 are essential for the formation of a thick oxytalan fiber bundle, but have different roles in the development of the fibers.

MATERIALS and METHODS

Cells and culture

HNPCEC were purchased from ScienceCell Research Laboratories (Carlsbad, CA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% newborn calf serum (NCS; Invitrogen) and 100 units/ml penicillin and 100 μ g/ml streptomycin (Roche Diagnostics, Mannheim, Germany) at 37oC in humidified air containing 5% CO2. When outgrowth of the cells reached confluence, they were harvested with 0.025% trypsin (Invitrogen) in phosphate-buffered saline (PBS) and transferred to plastic culture dishes at a 1:4 split ratio. For experiments, the cells were trypsinized and seeded at 1 x 10^6 cells/ml per 35 mm culture dish (Corning Incorporated, Corning, NY, USA). The HNPCEC were found to be confluent after 72 hr (this day being set as Day 0). HNPCEC were from three different donors and were used from third to sixth passages in this study.

Immunofluorescence

At 2, 4, 6 and 8 days of culture, HNPCEC were fixed in ice-cold 4% paraformaldehyde for 15 min, followed by washing with PBS. Nonspecific immunoreactivity was blocked with 1% goat serum (Sigma, Saint Louis, MO, USA) in PBS for 1 hr at room temperature. The cell/matrix layers were then incubated for 2 hr at room temperature with the appropriate primary antibodies [clone 11C1.3, the monoclonal antibody against human fibrillin-1 diluted 1:1000 (Themo Fisher Scientific Anatomical Pathology, Fremont, CA, USA); rabbit antibody against human fibrillin-2 diluted 1:1000 (Elastin Products Co., Owensville, MO, USA)]. Controls included the use of preimmune normal mouse and rabbit IgG for incubation with the primary antibody. After being rinsed in PBS, the cells were incubated with Alexa Fluor®488-labeled goat antimouse IgG antibody or Alexa Fluor® 568-labeled goat antirabbit IgG antibody (Molecular Probes, Eugene, OR, USA), diluted 1:2000 with blocking buffer, for 1 hr at room temperature. After a final wash, the cells were stained with DAPI (4´,6-diamidino-2-phenylindole,dihydrochloride) and viewed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

Northern blot analysis

Total RNA was prepared from the cultured HNPCEC at 2, 5, and 8 days using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was subjected to Northern blot analysis, as described previously [17]. The probes for recognition of human fibrillin-1 and fibrillin-2 were also generated as described [18]. Briefly, templates were obtained using the reverse transcription-polymerase chain reaction (RT-PCR) with RNA extracted from human gingival fibroblasts, an approach that has previously been shown to produce significant amounts of both fibrillins [17]. The polymerase chain reaction products were ligated into the pT7/T3-α18 vector (Life Technologies, Grand Island, NY, USA), and then the plasmid was linearized with Hind III and then T7 RNA polymerase mixed with digoxigenin (DIG)-labeled nucleotides (Roche Molecular Biochemicals, Mannheim, Germany) to generate the digoxigenin-labeled 698 bp fibrillin-1 and 583 bp fibrillin-2 RNA probes. The RNA probe for β-actin was from Roche Molecular Biochemicals. Densitometric analysis of the signals was performed using the Image J program (National Institutes of Health,

Bethesda, MD, USA) after finding the linear portion by sequential dilution of the proteins. Small variations in protein loading were corrected by normalization relative to the intensity of the corresponding band of β-actin. Each value presented is expressed as the mean ±SD, and all quantitative results represent at least four independent analyses. The Student's *t*-test was used for analyzing differences between experimental groups.

Western blot analysis

At 2, 5, and 8 days of culture, cell/matrix samples were prepared as described previously [19]. The proteins (5 μg) were subjected to electrophoresis on 4-12% NuPAGE Bis-Tris gel (Invitrogen) for Western blot analysis, as described previously [20]. The primary antibodies used were those against human fibrillin-1 (mouse monoclonal antibody, clone 11C1.3; Themo Fisher Scientific Anatomical Pathology) and fibrillin-2 (rabbit polyclonal antibody; Elastin Products) and β-actin (Sigma) at 1:5000 dilution. Prestained molecular weight markers (Invitrogen) were also run on each blot. Densitometric analysis of the signals was performed in the same way as that for Northern blot analysis.

Small interfering RNA (siRNA) design and transient transfection

siRNAs for human fibrillin-1 (accession # NM_000138) and fibrillin-2 (accession # NM_001999) were designed and synthesized by Sigma-Aldrich Corp. The synthesized siRNA for fibrillin-1 corresponded to bases 9392-9414 in mRNA of the 3'-untranslated region. The siRNA sequence was sense 5'-CAAGUACUAGGUUGUCCAUUU-3', antisense 3'-AUGGACAACCUAGUACUUGUA-5'. The negative control (scrambled order) was sense 5'-UCAUUUAUCGUCGGAAUAGCU -3', antisense 3'-CUAUUCCGACGAUAAAUGAAU-5'. The sequence of the negative control was designed as a randomized version of bases 9392-9414 of fibrillin-1. Basic Local Alignment Serch Tool (NIH, Bethesda MD, USA) searches indicated that this siRNA was specific for fibrillin-1 and had no homology with other proteins.

The synthesized siRNA for fibrillin-2 corresponded to bases 5091-5113 in the coding region. The siRNA sequence was sense 5'-GGUCGCUAUGAGUGUAACUGC-3', antisense 3'- AGUUACACUCAUAGCGACCAG -5'. The negative control (scrambled order) was: sense 5'-UUUAGCGGAAAUCGCGCUUGU-3', antisense 3'-AAGCGCGAUUUCCGCUAAAGC-5'. The sequence of the negative control was

designed as a randomized version of bases 5091-5113 in the coding region of fibrillin-2. Basic Local Alignment Serch Tool searches indicated that this siRNA was specific for fibrillin-2, and had no homology with other proteins.

Transfection was performed continuously on days 1, 4, and 7 of culture. The siRNA was transfected into HNPCEC using HiPerFect Transfection Reagent (Qiagen). First, 237.5 μl of OptiMEM medium/dish (Invitrogen) and 12.5 μl of the transfection reagent were preincubated for 10 min at room temperature. During this time, 748 μl of OptiMEM medium was mixed with 2 μl of 100 μM 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 siRNAs. After 12 hr of incubation, the transfection medium was replaced with fresh complete medium (Dulbecco's modified Eagle medium with 10% FCS). Mock transfection of cultures with the transfection reagent alone was used as a control. HNPCEC were transfected 3 times with the siRNA duplex (0, 200 nM), with a 72-hr interval between, and analyzed on Day 8.

RESULTS

Production of an oxytalan fiber network by HNPCEC

We first examined whether HNPCEC produce oxytalan fibers (Figure. 1). On Day 2 of culture, positive staining for fibrillin-1 was observed on oxytalan fibers, which appeared as networks of thin fibers. At this time, fibrillin-2-positive fibers were not clearly detectable. On day 4 of culture, thicker oxytalan fibers positive for fibrillin-1 appeared randomly, and their formation proceed to gather a former observed thin diameter of fibers. Moreover, fibrillin-2-positive fibers appeared on the thicker fibers where fibrillin-1 is positive (arrows).

On Day 6 of culture, the thick oxytalan fibers positive for fibrillin-1 that had appeared on Day 4 appeared to have become wider and were associated with thinner fibers. Fibers positive for fibrillin-2 were detected on the thick fibrillin-1-positive fibers. On Day 8 of culture, the thick fibrillin-1-positive fibers occupied most of the area in which positivity for fibrillin-2 was detected.

Gene expression levels of fibrillin-1 and fibrillin-2 in HNPCEC culture

Total RNA was extracted from the HNPCEC after 2, 5, and 8 days of culture. One microgram of each RNA sample was blotted and hybridized with the fibrillin-1 and fibrillin-2 RNA probes (Figure. 2A). Changes in the intensities of the signals were compared with β-actin mRNA signals as an internal control. Densitometric analysis showed that fibrillin-1 levels in the HNPCEC did not change significantly during the experimental period. In contrast, fibrillin-2 levels increased significantly three-fold from Day 2 to Day 5, and thereafter decreased, reaching a level two-thirds of that on Day 5 by Day 8.

Fibrillin-1 and fibrillin-2 deposition in cell/matrix layers of HNPCEC culture

Deposition of fibrillin-1 and fibrillin-2 in HNPCEC cultures was investigated on days 2, 5, and 8 by Western blotting of cell/matrix layer lysates (Figure. 2B).

Fibrillin-1 deposition increased 1.7-fold from Day 2 to Day 5 and then remained at almost the same level until Day 8. On the other hand, the density of fibrillin-2 remained almost constant from Day 2 to Day 5 and then increased significantly by about twofold from Day 5 to Day 8. This increase was consistent with the results of Northern blotting analysis, which indicated that the gene expression of fibrillin-2 increased threefold from Day 2 to Day 5.

Effect of fibrillin-1 or fibrillin-2 knockdown on oxytalan fiber formation

The effect of suppression of each of the genes for fibrillin-1 and fibrillin-2 on oxytalan fiber formation in HNPCEC culture was investigated. siRNA effectively reduced the level of fibrillin-1 and fibrillin-2 mRNA to <90% of that of the (mock-transfected) control (Figure. 3). In contrast, scrambled siRNA had no effect on the expression of fibrillin-1 or fibrillin-2 gene, providing evidence that this siRNA was specific.

The immunohistochemical assay on Day 8 revealed that fibers positive for fibrillin-1 and fibrillin-2 in mocktransfected control cells were similar to those seen at 8th day, as shown in Figure. 1 (Figure. 4). That is, the thick fibrillin-1-positive fibers occupied most of the area in which fibrillin-2 positivity was observed.

Cells transfected with fibrillin-1 siRNA produced thin fibers positive for fibrillin-2, and did not form thick fibers. Fibrillin-1-positive fibers were not detectable, as expected.

Cells transfected with scrambled fibrillin-1 siRNA yielded results similar to those for the control (mock-transfected) cells, as expected (data not shown).

Cells transfected with fibrillin-2 siRNA produced fibrillin-1-positive networks of thin

fibers, resembling those of the control at Day 2, as shown in Figure 1. As expected, fibrillin-2-positive fibers were not detectable. Cells transfected with scrambled fibrillin-2 siRNA showed features similar to those of the control (mock-transfected) cells (data not shown).

DISCUSSION

In this study, for the first time we have demonstrated that HNPCEC express fibrillin-1 and fibrillin-2 and form a network of oxytalan fibers. Oxytalan fibers give tissue flexibility and extensibility [21]. Ciliary zonules, composed of oxytalan fibers, are transparent fibers that link the lens to the ciliary body in the eye [7]. This structure is similar as the PDL, where oxytalan fibers link the cementum of the tooth to the alveolar bone [22]. We have reported previously that PDL fibroblasts express fibrillin-1 and fibrillin-2, forming oxytalan fibers in vitro [11, 17]. However, the individual roles of fibrillin-1 and fibrillin-2 in the formation of oxytalan fibers have not been clarified. The two molecules interact to form homotypic and heterotypic dimers [3, 4]. In the context of elastic fiber formation, the functions of fibrillin-1 and fibrillin-2 overlap, and either of the two molecules is required for this purpose [23]. However, it has remained unclear how they contribute to the formation of oxytalan fibers, which are elastin-free fibers.

In this study, the expression pattern of fibrillin-1 was found to be ubiquitous in terms of its gene expression and fiber distribution. Fibrillin-1-positive fibers appeared earlier than fibrillin-2-positive fibers, as shown in Figure 1, apparently supplying basal fibers that matured and thickened to become functional. During the formation of oxytalan fibers, relatively thinner fibers that were positive for fibrillin-1 appeared on Day 2 of culture and gradually became gathered together by Day 8. Thinner fibers were never detectable around the thick fibers. Moreover, after Day 4, fibrillin-2 positivity was evident in the area where the thinner fibers had gathered. This appeared to be coincidental with the induction of fibrillin-2 gene expression by Day 5, and the markedly increased deposition of the protein detected by Northern and Western blot assays by Day 8 (Figure 2). This suggests that fibrillin-2 is related to the accumulation of thin fibers. To test this hypothesis, we used siRNA to specifically knock down fibrillin-1 and fibrillin-2 (Figure 3). Knockdown of fibrillin-2 resulted in fibrillin-1-positive fibers remaining up to Day 8 (Figure 4), thus appearing to support our hypothesis. On the other hand, fibrillin-2 was unable to form thick fibers without fibrillin-1. The gene expression of fibrillin-2 was not affected by fibrillin-1 knockdown.

Therefore, fibrillin-1-positive fibers may act for the scaffold of fibrillin-2 depositions. These results suggest that both fibrillins are necessary for the formation of thick fibers. Fibrillin-2 is not observed in whole zonule by mass spectrometry analysis [24]. Moreover, fibrillin-2 is normally expressed only during the developing tissues. In this study, fibrillin-2 is thought to be related with the thickness of oxytalan fibers during the development of oxytalan fibers. After fibrillin-2 contributes to form thick oxytalan fibers during development,it may be catabolized at the stage of functional adult oxytalan fibers in culinary zonule. We are now analyzing the metabolism of fibrillin-2 in the long culture system.

Mutations of fibrillin-1 and fibrillin-2 cause Marfan syndrome and CCA, respectively [12, 13]. Marfan syndrome and CCA are associated with both ocular and cardiovascular defects [25]. The ciliary zonule in Marfan syndrome shows a disorganized bundle [16]. The ocular defect results from an ectopic lens, caused by abnormalities of the ciliary zonule anchoring the lens to the ciliary body. These facts are supported by our present findings that both fibrillin-1 and fibrillin-2 are necessary for the formation of thick oxytalan fibers.

The formation of oxytalan fibers is controlled by other fibrillin-associated molecules, such as EMILIN-1 and fibulin-5 [26, 27]. At the ultrastructural level, EMILIN-1 was first reported to be localized in oxytalan fibers of the ciliary zonule, as well as in elastic fibers [26]. Therefore, EMILIN-1 may play a role in homeostasis of the ciliary zonule. EMILIN-1 is known to bind with fibulin-5 [28]. Using PDL cell culture, we have previously demonstrated that both EMILIN-1 and fibulin-5 control the amounts of oxytalan fibers [20, 29]. Moreover, we have found that fibulin-5 is upregulated in response to tension strain, thus controlling the formation of oxytalan fiber bundles in PDL culture. Oxytalan fibers in human ciliary zonules are 20~30 μm in diameter and bear the strain of ciliary muscle contraction that occurs when focusing the lens [30].

We speculate that either fibrillin-1 or fibrillin-2 may form a complex with EMILIN-1and/or fibulin-5 to regulate the thickness of oxytalan fibers in ciliary zonule. Therefore, to examine the functional characteristics of oxytalan fibers, we are now analyzing the response of oxytalan fiber bundles in the ciliary zonule to mechanical strain.

In conclusion, our present results suggest that both fibrillin-1 and fibrillin-2 are necessary for the formation of thick oxytalan fibers. Our findings may help to explain the mechanism of homeostasis of oxytalan fibers in the ciliary zonule.

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Figure 1

Oxytalan fibers positive for fibrillin-1 and fibrillin-2 in ciliary nonpigmented epithelial cells.

Double immunofluorescence for fibrillin-1 (A) and fibrillin-2 (B) in cultures of human nonpigmented ciliary epithelial cells (HNPCEC). HNPCEC were cultured, then

simultaneously labeled for fibrillin-1 (A), fibrillin-2 (B), and superimposition of both labels (C) on days 2, 4, 6, and 8. DAPI was used for nuclear staining. Bar: 20 μ m.

Figure 2

Gene expression and protein deposition of fibrillin-1 and fibrillin-2 in HNPCEC.

(A) Northern blot analysis: Total cell RNA was extracted and 1 μ g was subjected to Northern blot analysis, as described in the "Materials and Methods". Densitometric analysis of the time course of changes in the levels of fibrillin-1 and fibrillin-2 mRNA was measured by Northern blotting. Expression levels of all mRNAs were analyzed using Image J software(National Institutes of Health) and normalized relative to the expression of β-actin mRNA. The levels of expression in culture samples at Day 2 were arbitrarily assigned a value of 1. The results are represented as mean ± SD for four independent experiments. Student's *t*-test was used for analyzing differences between experimental groups. (B) Western blot analysis: Total cell proteins were extracted and 5 μ g was subjected to Western blot analysis, as described in the "Materials and Methods". Densitometric analysis of the time course of changes in the levels of fibrillin-1 and fibrillin-2 was conducted by Western blotting. All expression levels were analyzed using Image J software and normalized relative to the expression of β-actin protein. The levels of expression in culture samples obtained on Day 2 were arbitrarily assigned a value of 1. The results represent as mean ± SD for four independent experiments (*$p<0.05$ versus value at 2 weeks).

Figure 3

Specific suppression of the fibrillin-1 or fibrillin-2 gene in HNPCEC by siRNAs.

(A)HNPCEC were mock transfected (left column) or transfected with scramble siRNA duplex for fibrillin-1 (middle column) and fibrillin-1 (right column). Equal amounts of RNA (1 μ g) at Day 2 was subjected to Northern blot analysis. (B)HNPCEC were mock transfected (left column) or transfected with scramble siRNA duplex for fibrillin-2 (middle column) and fibrillin-2 (right column). Equal amounts of RNA (1 μ g) was subjected to Northern blot analysis.

Figure 4

Effect of fibrillin-1 or fibrillin-2 suppression on oxytalan fiber formation.

HNPCEC were transfected with 200 nM siRNA for fibrillin-1 (middle column), or with 200 nM siRNA for fibrillin-2 (right column). Mock transfected cells are shown in the left column. Immunofluorescence staining was performed with antibodies against fibrillin-1 (A) and fibrillin-2 (B), and superimposition of both labels (C) on Day 8. DAPI was used for nuclear staining. Bar: 20 μ m.

Figure 1

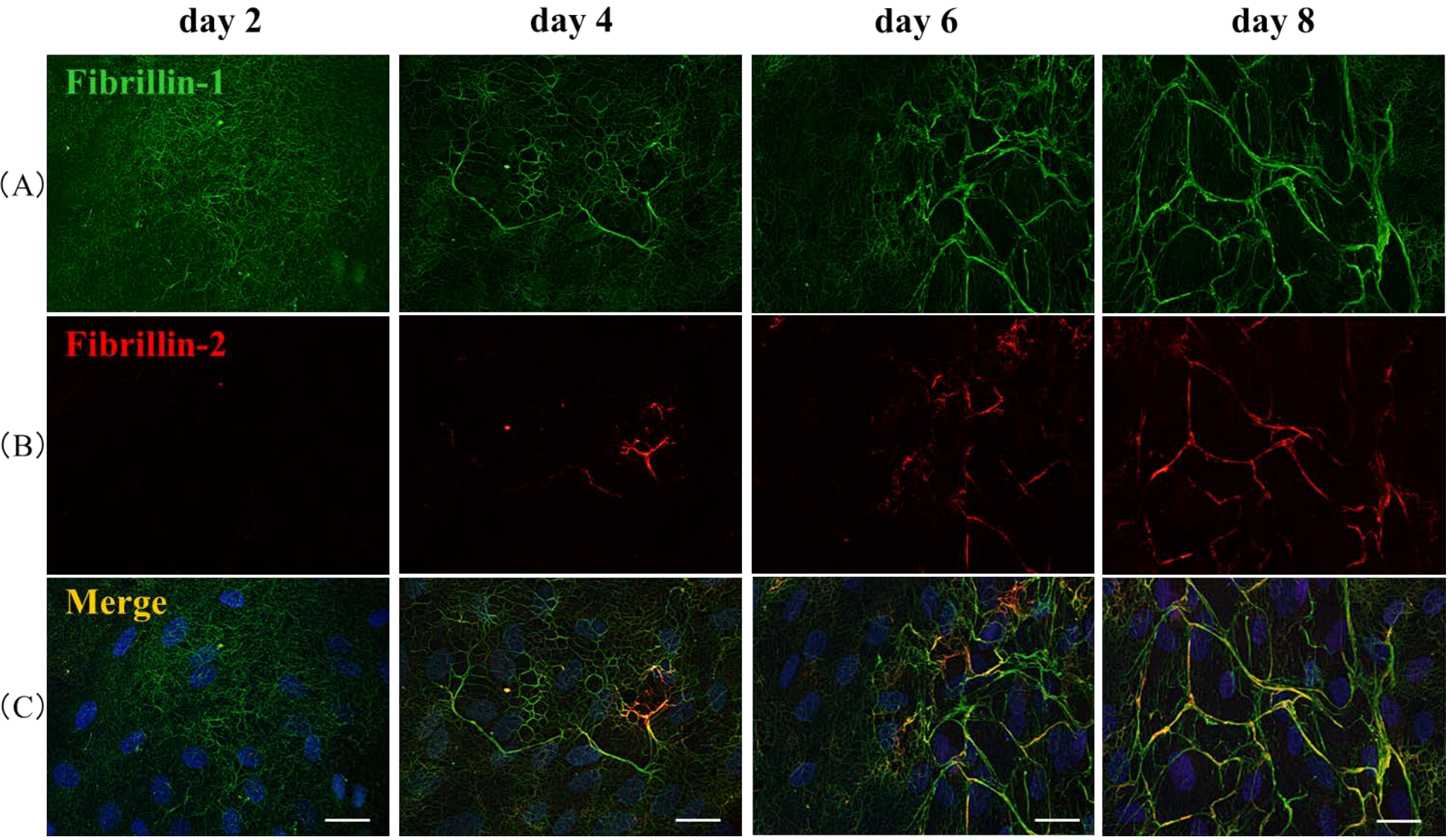


Figure 2

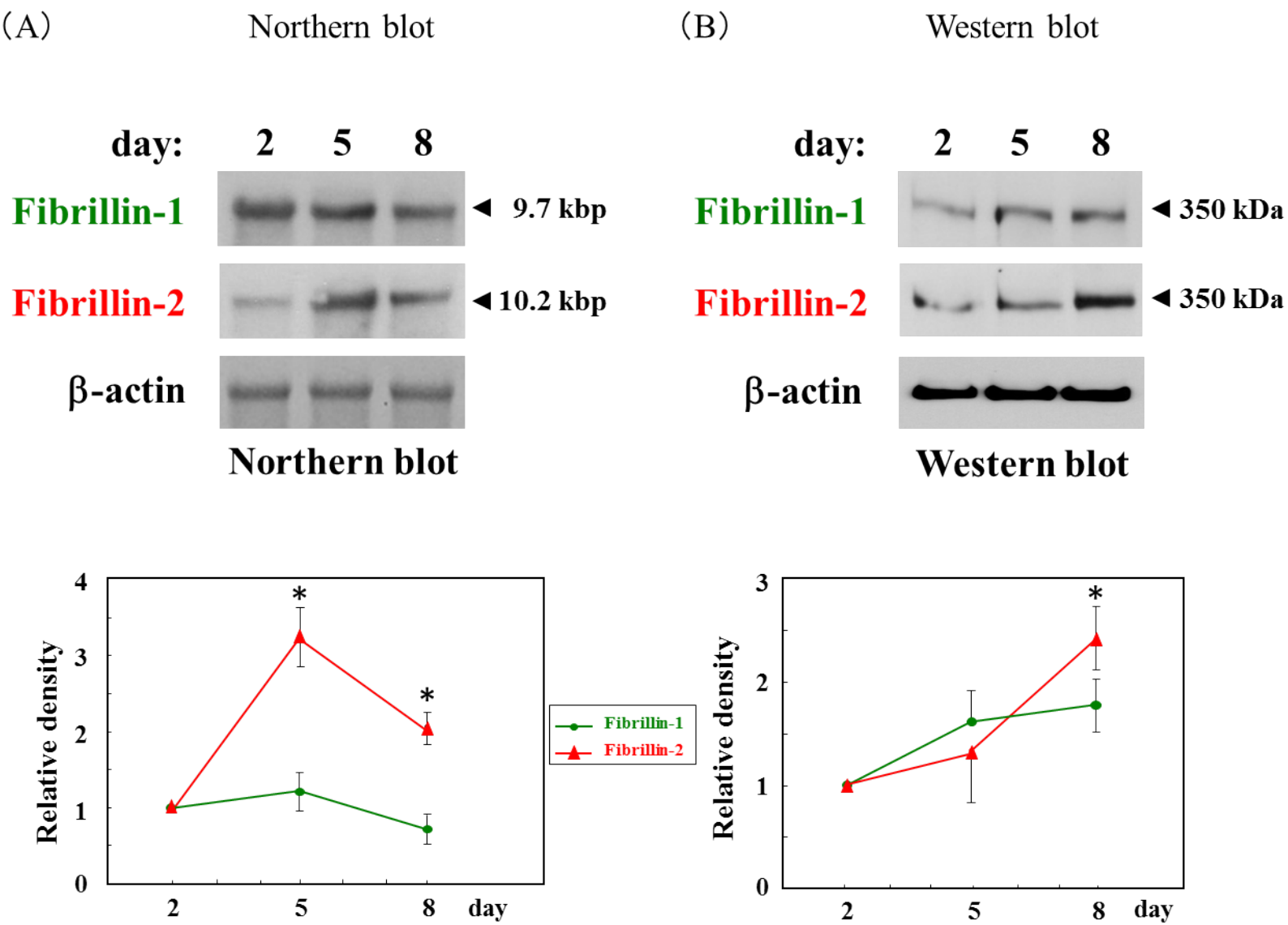

(A)
Northern blot
day: 2 5 8
Fibrillin-1
9.7 kbp
Fibrillin-2
10.2 kbp
β-actin
Northern blot
(B)
Western blot
day: 2 5 8
Fibrillin-1
350 kDa
Fibrillin-2
350 kDa
β-actin
Western blot
Relative density
day
Fibrillin-1
Fibrillin-2
*

Figure 3

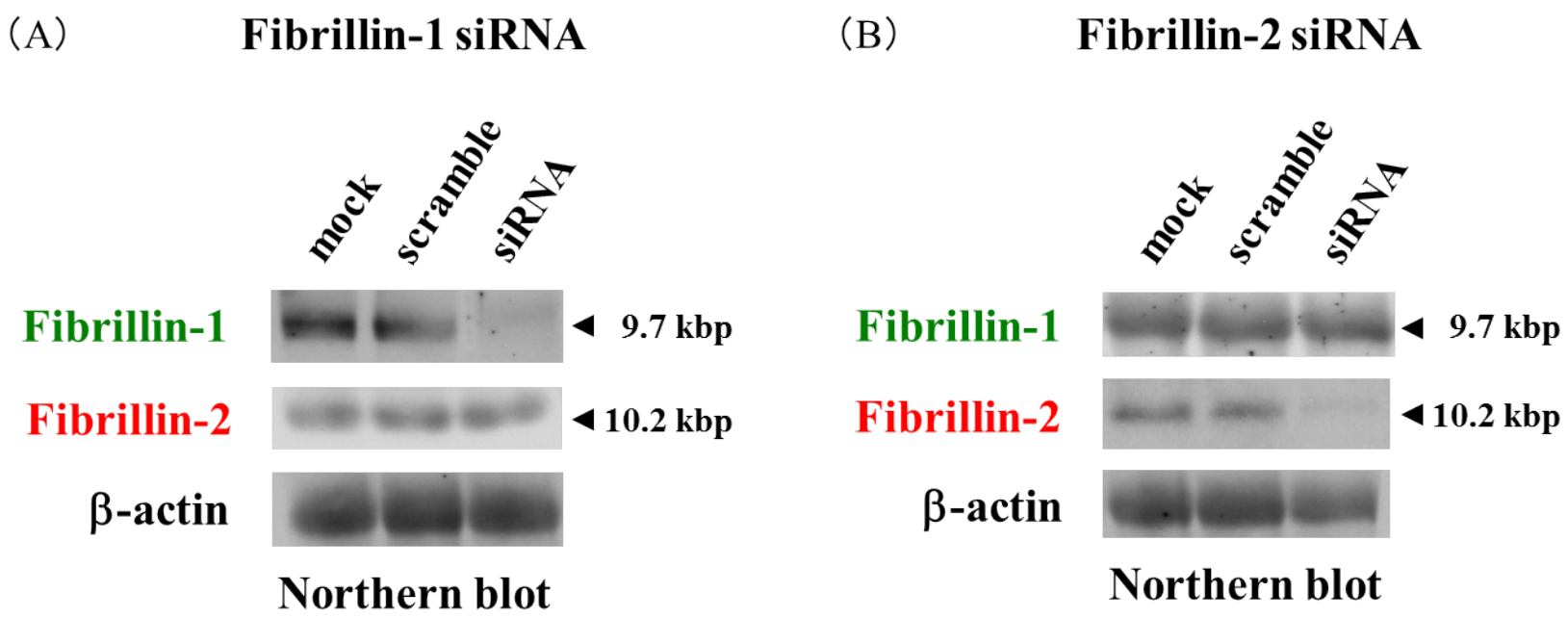
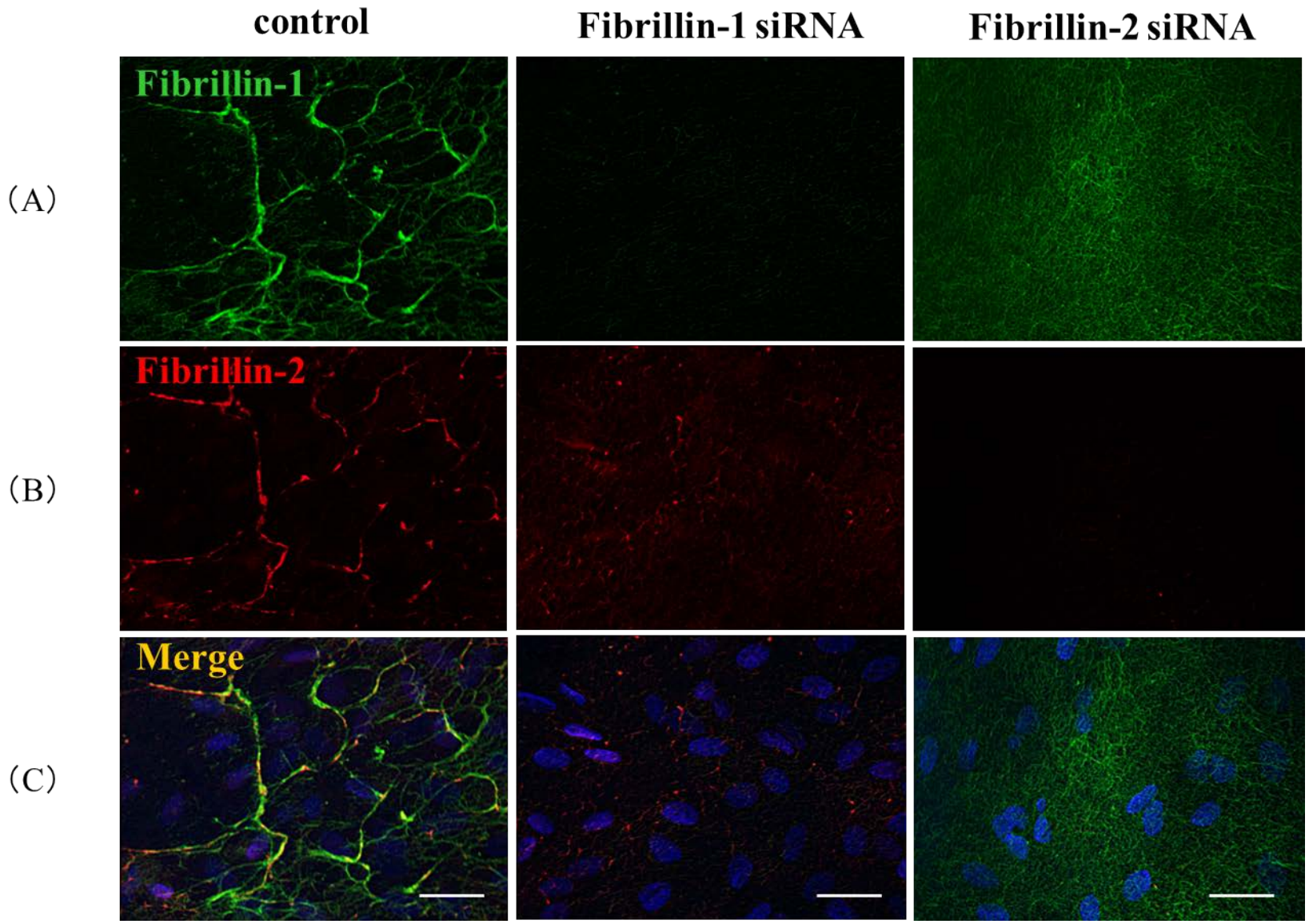

(A)
Fibrillin-1 siRNA
mock
scramble
siRNA
Fibrillin-1
9.7 kbp
Fibrillin-2
10.2 kbp
β-actin
Northern blot
(B)
Fibrillin-2 siRNA
mock
scramble
siRNA
Fibrillin-1
9.7 kbp
Fibrillin-2
10.2 kbp
β-actin
Northern blot

Figure 4


control
Fibrillin-1 siRNA
Fibrillin-2 siRNA
(A)
Fibrillin-1
(B)
Fibrillin-2
(C)
Merge