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Regeneration of elastic fibers by three-dimensional culture on a collagen scaffold and the addition of latent TGF-β binding protein 4 to improve elastic matrix deposition

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ABSTRACT

The objective of this study was to investigate the effects of latent TGF-β binding protein 4 (LTBP-4) on elastic fiber regeneration in three-dimensional cultures of human dermal fibroblasts (HDFs). Appropriate collagen scaffold for elastic fiber regeneration was also examined. Collagen sponges cross-linked at 120 °C and composed of small pores (25 μm on average) was favorable for elastic fiber regeneration by HDFs. Addition of LTBP-4, followed by culture for 21 days, accelerated elastic fiber accumulation within the scaffolds. Conditioned scaffolds containing either HDFs or LTBP-4-built mature elastic fibers were implanted between the dermis and the cutaneous muscle of mice. The combined use of HDFs and LTBP-4 resulted in thicker tissues containing elastic fibers. These results indicate that weakly cross-linked collagen sponges can be used as scaffolds for regenerating elastic fibers both *in vitro* and *in vivo*, and that the addition of LTBP-4 accelerates the deposition of both elastin and fibrillin-1, and increases cell proliferation. These techniques may be useful for generating cutaneous or cardiovascular tissue equivalents; furthermore, they may serve as a useful method for the three-dimensional analyses of drugs used to treat skin diseases or to examine the microstructure of elastin networks.

1. Introduction

Elastic fibers are a major component of the extracellular matrix (ECM) and provide mechanical elasticity to tissues, including the skin, arteries, and lungs, etc. [1,2]. After their initial synthesis, these fibers are gradually degraded as a person ages and are not replaced. Degradation of elastic fibers resulted in many age-related phenotypes, such as wrinkled skin, emphysema, and arteriosclerosis [3,4]. In addition, elastic fibers are lost (or their numbers markedly reduced) in pathological scars such as keloids [5–7]. Because the turnover rate of elastic fibers is very low, they do not fully regenerate when damaged. To date, there is no established method of regenerating thick elastic fibers.

The assembly of elastic fibers involves a complex stepwise process that includes the synthesis and secretion of tropoelastin by cells. This is followed by the formation of tropoelastin microaggregates, initial cross-linking of elastin molecules, and the deposition of microaggregated elastin on microfibrillar templates; finally, the elastin is cross-linked by lysyl oxidase [8].

We previously reported that a microfibril-associating molecule, latent TGF-β binding protein 4 (LTBP-4), plays an essential role in elastic fiber assembly [9]. LTBP-4 induces the formation of elastic fibers by interacting with fibulin-5, a tropoelastin-binding protein

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required for elastogenesis [10–14]. The molecular mechanism underlying LTBP-4-induced elastic fiber organization is thought to involve recruitment of the tropoelastin/fibulin-5 complex onto the microfibrils. In the absence of LTBP-4, the tropoelastin/fibulin-5 complex is not deposited on microfibrils in a linear fashion. Knocking down LTBP-4 in human dermal fibroblasts (HDFs) prevents the deposition of elastin; however, the addition of high concentrations of LTBP-4 induces elastic fiber assembly in a dose-dependent manner, with levels exceeding those observed in monolayer cultures of control cells [9].

Prior studies of elastogenesis were based almost exclusively on monolayer cell cultures. Although several studies report elastogenesis in three-dimensional cell cultures constructed using tissue-engineered scaffolds, none have developed a method of selectively inducing elastic fiber development on three-dimensional scaffolds. A previous study showed that dermal fibroblasts generated elastic fibers in fibrin and collagen gels treated with TGF-β, insulin, and plasmin for 4 weeks [15]. Other studies report that a skin substitute based on co-cultured dermal fibroblasts and keratinocytes underwent elastogenesis; however, this was not observed when fibroblasts were cultured alone [16,17]. For elastic implants to be useful internally (e.g., for the replacement of cardiovascular or dermal tissue), elastic fibers of a certain thickness must be generated in a short period of time in the absence of keratinocytes. Here, we examined the effects of LTBP-4 on elastic fiber regeneration by HDFs cultured in three-dimensional collagen scaffolds.

2. Materials and methods

2.1. Scaffold preparation

The collagen scaffold used in this study comprised materials modified from the collagen sponges used in currently available dermal equivalents (PELNAC®, GUNZE, Tokyo, Japan), which were developed previously by our group [18–20]; however, the cross-linking method and pore size of the sponges used herein were different.

The following five types of collagen sponges were prepared: 1) 25-μm pore size, cross-linked at 110 °C (110-small); 2) 25-μm pore size, cross-linked at 120 °C (120-small); 3) 25-μm pore size, cross-linked at 140 °C (140-small); 4) 100-μm pore size, cross-linked at 140 °C (140-large); and 5) 100-μm pore size, cross-linked at 140 °C, followed by 0.2% glutaraldehyde (140G-large; the same as PELNAC®)[20].

Briefly, a hydrochloric acid solution (pH 3.0) containing 0.3% atelocollagen derived from porcine skin (Nitta Gelatin Inc., Osaka, Japan) was stirred at 1800–2000 rpm for 5 min (small pore sponges) or 60 min (large pore sponges) using a refrigerated homogenizer. The resulting solution was poured into a mold and frozen rapidly at −40 °C before being freeze-dried for 48 h. The sheet was cross-linked by heating in a vacuum oven for 24 h at 110 °C (110-small), 120 °C (120-small), or 140 °C (140-small, 140-large, 140G-large). The 140G-large sheet was further cross-linked by immersion in 0.05 M acetic acid solution containing 0.2 wt% glutaraldehyde at 4 °C for 24 h, followed by rapid freezing at −135 °C and freeze drying for 48 h. The resulting sponge sheets comprised multiple pores with an average diameter of 25 μm (small-pore sponges) or 100 μm (large-pore sponges). The sponges were then cut into round disks with a diameter of 11 mm and attached to silicone rings.

2.2. Cell culture

HDFs were isolated from tissue excised from three patients (all 3 months-of-age) during cleft lip repair surgery. The study was approved by the Institutional Review Board at Kyoto University Faculty of Medicine, and informed consent was obtained in accordance with the ethical standards formulated in the Helsinki Declaration. Skin tissues were cut into 1–2 mm^3 pieces, placed into plastic tissue culture dishes, and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 100 units/100 μg/0.25 μg ml^{-1} penicillin/streptomycin/amphotericin B and 10% fetal bovine serum (FBS; Sigma–Aldrich). Cells were propagated at 37°C/5% CO_2, and semi-confluent cultures of fibroblasts were passaged by trypsinization. Cells were used at passages 5–8.

2.3. Recombinant human FLAG-tagged LTBP-4 preparation

Recombinant human FLAG-tagged LTBP-4 was prepared as previously reported [9]. Briefly, human full-length *LTBP4S* cDNA was amplified by PCR, and subcloned into pEF6/FLAG which was modified from pEF6/V5 (Life Technologies, Carlsbad, CA, USA) with incorporation of C-terminal FLAG-tag. Recombinant human FLAG-tagged LTBP-4 (rLTBP-4S with an FLAG tag) was purified using TALON affinity resin (Takara, Kyoto, Japan) from serum-free conditioned medium of 293T cells stably transfected with pEF6/FLAG-LTBP4S.

2.4. Three-dimensional culture of HDFs

The collagen sponges were placed on a thermoplastic hydrogel (Mebiol Gel; Mebiol Inc., Kanagawa, Japan). Next, 1×10^6 fibroblasts were seeded on the circular collagen scaffolds, which were then maintained at 37°C/5% CO_2 in DMEM/F12 (Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 100 units/100 μg/0.25 μg ml^{-1} penicillin/streptomycin/amphotericin B and 10% FBS (n = 3). The medium was replaced every 7 days for a period of 21 days. For experiments conducted to verify the effects of LTBP-4, recombinant FLAG-tagged LTBP-4 was added to the medium every 7 days at a concentration of 5 μg/ml (n = 3 per group).

2.5. Immunohistochemical analysis of elastin regeneration

Frozen sections of discs were fixed in ice-cold acetone (−30 °C) for 5 min, followed by treatment with 25% Block Ace (Dainippon, Tokyo, Japan) for 30 min. The sections were then incubated for 1 h with the following primary antibodies: a monoclonal mouse anti-elastin antibody (Merck Millipore, Darmstadt, Germany), a polyclonal rabbit anti-LTBP-4 antibody (made by immunizing rabbits with recombinant full-length human LTBP-4, as previously reported [9]), or a mouse anti-fibrillin-1 monoclonal antibody (Merck Millipore). All antibodies were diluted 1:200 in Antibody Diluent (Dako Co., Glostrup, Denmark). The sections were then incubated for 30 min with the following secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) diluted 1:200 in Antibody Diluent. Nuclei were stained with Hoechst 33258. Sections were imaged using a C1si confocal microscope (Nikon Instruments, Tokyo, Japan).

Overlapping elastin and LTBP-4 signals were quantified using NIS-Elements AR Analysis 4.00.06 software (Nikon Instruments). The number of HDFs in each of the three samples stained by Hoechst 33258 was counted. Five high-power (×400) fields (HPF) per section were then selected at random, and the total numbers of HDFs was counted.

2.6. Immunohistochemical analysis of exogenous recombinant FLAG-tagged LTBP-4 and endogenous (secreted) LTBP-4

Frozen sections of discs were fixed in 4% paraformaldehyde for 10 min. After treatment with 25% Block Ace (Dainippon, Tokyo, Japan) for 30 min, the sections were incubated with primary antibodies (a mouse anti-FLAG M2 monoclonal antibody (Sigma–Aldrich) and a rabbit anti-LTBP-4 polyclonal antibody), both diluted 1:200 in Antibody Diluent (Dako Co.), for 1 h. The sections were then incubated for 30 min with Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 546 goat anti-rabbit IgG antibodies (Life Technologies) diluted 1:200 in Antibody Diluent. Nuclei were stained with Hoechst 33258. Sections were imaged using a C1si confocal microscope (Nikon Instruments).

2.7. Animals and transplantation experiments

The collagen sponges (120-small) were divided into four groups ($n = 3$ per group) and incubated in DMEM/F12 medium at 37 °C in 5% CO_2 in the presence or absence of 1×10^6 HDFs and in the presence or absence of recombinant LTBP-4 (5 μg/ml). After 4 h of incubation, the sponges were implanted between the dermis and cutaneous muscle on the backs of 5-week-old male athymic nude BALB/c mice. The number of animals used in the study was kept to a minimum, and all possible efforts were made to reduce suffering. All experiments were conducted according to the protocols established by the Animal Research Committee of Kyoto University.

2.8. Histological analysis of tissue excised from the animal models

Mice were sacrificed 21 days after grafting, and the grafted tissues were harvested together with the surrounding tissues. The tissue specimens were fixed with 4% paraformaldehyde at 4 °C for 24 h and embedded in paraffin. Histological sections were prepared (4 μm thick) and stained with Elastica van Gieson (EVG). The sections were then observed under a light microscope, and the areas containing elastic fibers (stained black) were measured. Three fields within each specimen were measured (magnification, ×400).

2.9. Statistical analysis

Differences between two groups were assessed using the Mann–Whitney U test. Differences between three or more groups were examined using Tukey's test. A P value < 0.05 was considered significant.

3. Results

3.1. Appropriate collagen sponge conditions for elastic fiber regeneration

To examine differences in elastic fiber formation under different scaffold conditions, samples were immunostained for fibrillin-1, a major component of microfibrils and elastin. Immunofluorescence analysis of each conditioned collagen sponge after culture for 21 days revealed differences both in the distribution of HDFs and in the deposition of elastin and fibrillin-1 (Fig. 1(A)). Hoechst staining revealed that the HDFs were densely packed in layers within sponges composed of small pores (110-small, 120-small, 140-small), but were more diffuse in sponges composed of large pores (140-large, 140G-large). Fibrillin-1 deposition in the small-pore sponges was both linear and thick; however, deposition was restricted to the surface of the large-pore sponges. Linear deposition of elastin was observed in the 120-small and 140-small sponges, but not in 110-small, 140-large and 140G-large sponges. Analysis of elastin labeling on Day 21 revealed that the 120-small sponges contained statistically more elastin than the 140-small sponges (Fig. 1B). Based on these results, we decided that the 120-small sponges were most appropriate for supporting elastic fiber regeneration; therefore, this scaffold was used for the following experiments.

3.2. Effects of LTBP-4 on elastic fiber regeneration

To examine the effect of LTBP-4, a microfibril-associated molecule essential for elastic fiber assembly, on three-dimensional elastic fiber regeneration *in vitro*, 120-small collagen sponges were seeded with HDFs and cultured in the presence or absence of LTBP-4 (5 μg/ml) prior to immunostaining (Fig. 2(A)). LTBP-4 was not detected in samples cultured in the absence of LTBP-4 on Day 7; however, LTBP-4 was detected on Days 14 and 21, with the level increasing in a time-dependent manner. By contrast, LTBP-4 was detected on Day 7 in samples treated with LTBP-4. Elastin was observed after 7 days under all culture conditions; however, the levels of elastin were higher in samples cultured in the presence of LTBP-4. Merged images were then generated, which showed the superimposition of LTBP-4, elastin, and cell nuclei. The linearly deposited elastin colocalized with LTBP-4. Linear deposition of fibrillin-1 was detected in all sponges after 7 days of incubation, and increased with culture time. However, levels were higher in sponges cultured in the presence of LTBP-4 than in those cultured in the absence of LTBP-4. Next, we measured the amount of elastin deposition induced by LTBP-4 by examining the colocalization of elastin and LTBP-4 on Day 21 (Fig. 2(B)). There were 1.7–3.7 times as many areas of colocalization in LTBP-4-treated samples than in non-treated samples ($P < 0.0005$). The number of cells/HPF was then counted on Day 21 as a measure of cell proliferation within the scaffolds. There were significantly more cells in samples cultured in the presence of LTBP-4 ($P < 0.025$; Fig. 2(C)). These results suggest that LTBP-4 accelerated both elastic fiber regeneration and cell proliferation in three-dimensional cultures of HDFs.

3.3. Localization of exogenous recombinant FLAG-tagged LTBP-4 and endogenous (secreted) LTBP-4

Although HDFs secreted endogenous LTBP-4 as the culture time elapsed, the addition of LTBP-4 clearly accelerated elastic fiber regeneration. Therefore, we next examined whether the source of LTBP-4 had any effect by immunostaining for recombinant LTBP-4 and total LTBP-4 (Fig. 3). Recombinant FLAG-tagged LTBP-4 was detected in samples cultured in the presence of LTBP-4 on Day 7, and the levels increased over time. Labeling of LTBP-4 detects both added and secreted LTBP-4. This antibody detected both exogenous and endogenous (secreted) LTBP-4. Although the amount of LTBP-4 was greater in samples cultured in the presence of LTBP-4 at all time points, LTBP-4 was still detected in samples cultured in the absence of LTBP-4 on Days 14 and 21. The crucial difference between these two samples seemed to be whether LTBP-4 was present during the initial culture period.

3.4. Effect of number of LTBP-4 addition on elastic fiber regeneration

To determine whether the presence of LTBP-4 is required throughout the entire culture period or it is sufficient to be present during the initial 7 days of culture, we prepared two groups of cultured HDFs: for one group, a single dose of LTBP-4 was added only at the beginning, while the other group received a dose of LTBP-4 every 7 days, i.e. three times in total. Immunostaining revealed that the areas of elastin and LTBP-4 labeling were very similar throughout the culture period, regardless of the number of

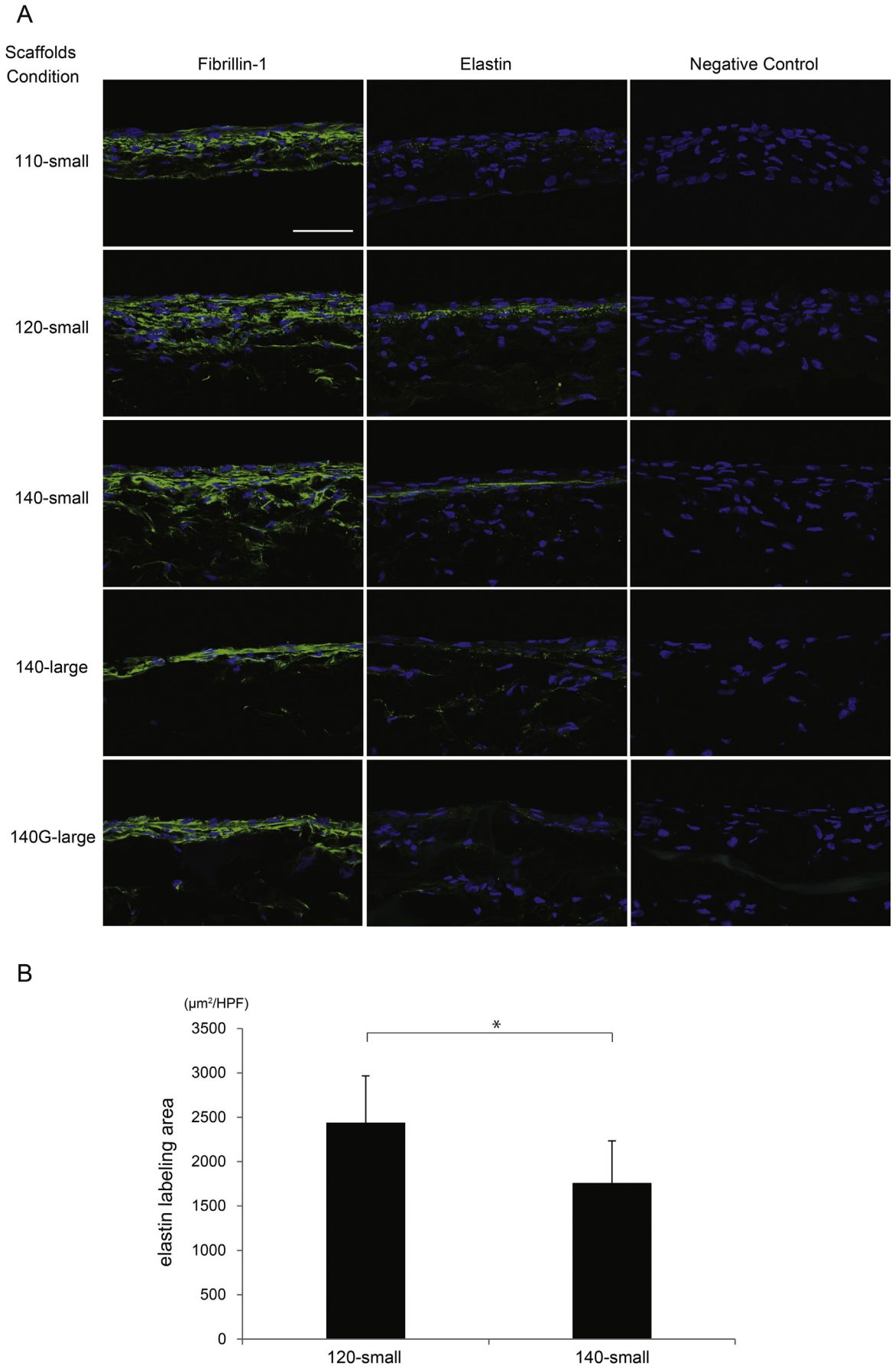

Fig. 1. (A) Immunofluorescence analysis of different collagen sponges seeded with HDFs and cultured for 21 days. Scale bar = 50 μm. (B) Quantitative analysis of elastin labeling of the 120-small and 140-small sponges. Data are expressed as the mean ± SE. *P < 0.005.

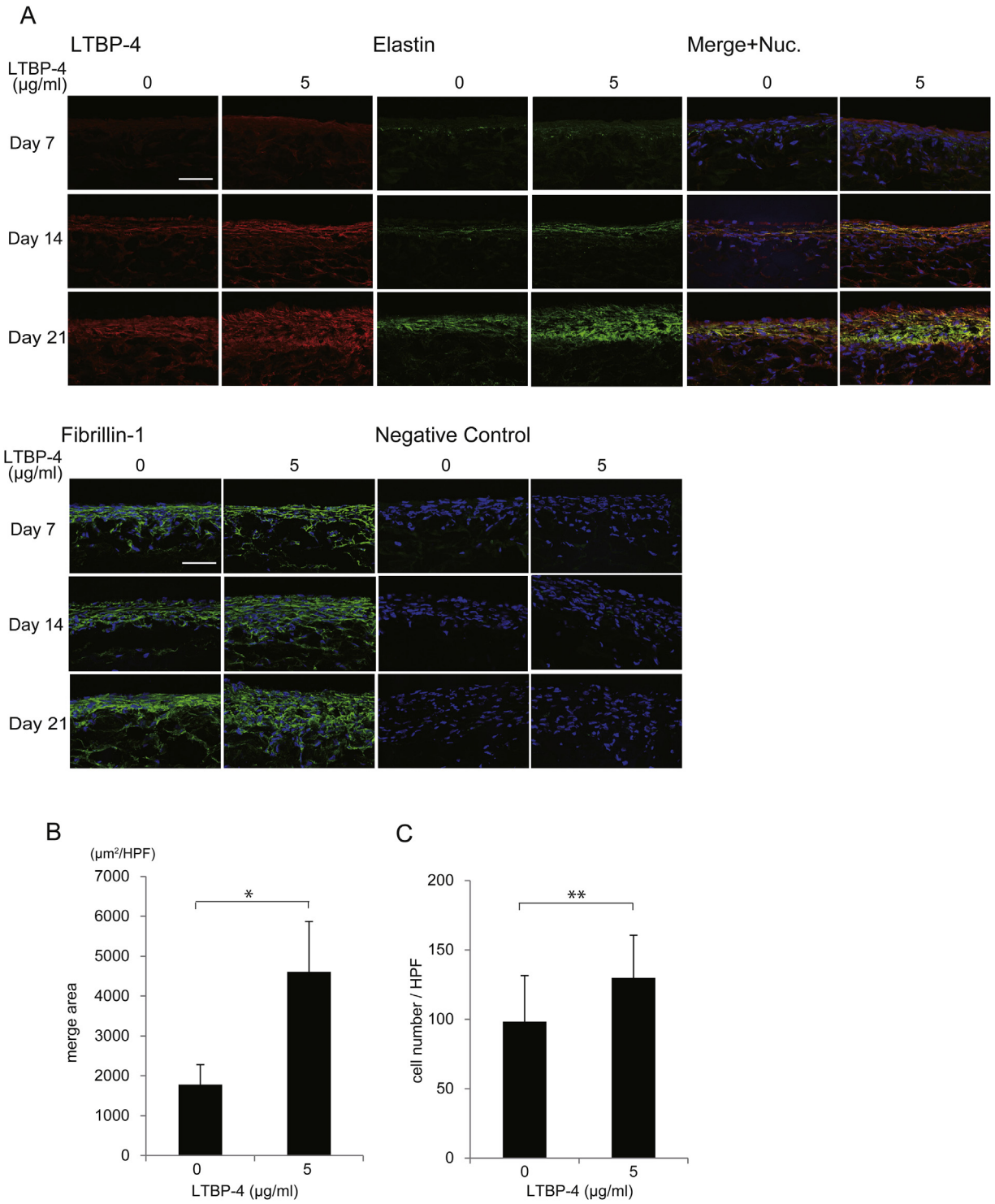

Fig. 2. (A) Immunofluorescence of the 120-small collagen sponges seeded with HDFs and cultured in the presence or absence of LTBP-4 (5 μg/ml). LTBP-4 was added to the medium every 7 days. Scale bar = 50 μm. (B) Quantitative analysis of the merged areas of elastin and LTBP-4 on Day 21. Data are expressed as the mean ± SE. *P < 0.0005. (C) Number of cells per high-power field on Day 21. Data are expressed as the mean ± SE. **P < 0.025.

LTBP-4 doses (a single initial dose or one dose every 7 days) (Fig. 4(A)). In all cases, elastogenesis was greater than that observed in the absence of LTBP-4 (Fig. 4(A)). Quantitative analysis of elastin/LTBP4 colocalization on Day 21 revealed that the areas of colocalization in samples cultured in the presence of LTBP-4 were significantly larger than those in samples cultured in the absence of LTBP-4; however, there were no statistically significant differences between samples according to the number of LTBP-4 doses (Fig. 4(B)). The number of cells/HPF on Day 21 was significantly higher in samples cultured in the presence of LTBP-4 than in samples cultured in the absence of LTBP-4, although again, the number of LTBP-4 doses had no significant effect (Fig. 4(C)) ($P < 0.01$; addition of LTBP-4 every 7 days *vs.* no LTBP-4. $P < 0.001$; a single dose of LTBP4 *vs.* no LTBP-4) Taken together, these findings

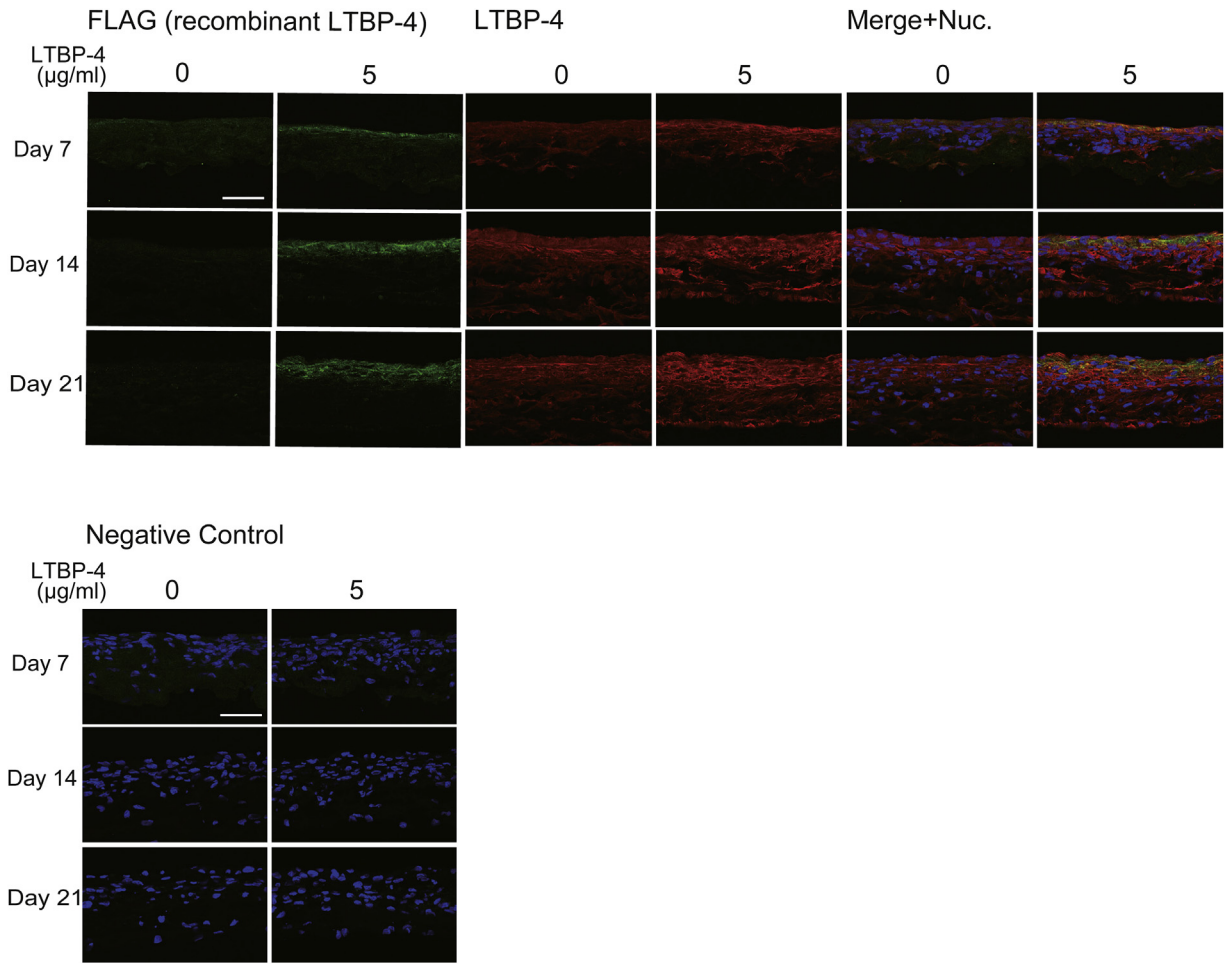

Fig. 3. Immunostaining of recombinant LTBP-4 and total LTBP-4. Sections were stained with an anti-Flag antibody to detect exogenous recombinant Flag-LTBP-4 or with an anti-LTBP-4 antibody to detect total LTBP-4. Total LTBP-4 included both exogenous and endogenous (secreted) LTBP-4. Scale bar = 50 μm.

suggest that LTBP-4 is only required during the initial culture period.

3.5. *Effect of LTBP-4 addition on elastic fiber regeneration in vivo*

Finally, we examined elastic fiber regeneration in three-dimensional scaffolds *in vivo*. Collagen sponges containing (or not containing) HDFs were cultured in the presence or absence of recombinant LTBP-4 and implanted between the dermis and the cutaneous muscle in mice, and EVG staining was then performed 21 days later. Histological analyses revealed elastic fibers (stained black) were regenerated in scaffolds containing HDFs, in scaffolds treated with LTBP-4, and in scaffolds containing HDFs treated with LTBP-4 (Fig. 5(A)). Quantitative analysis revealed that the elastic fiber-containing area within graft tissue from mice implanted with sponges containing HDFs and treated with LTBP-4 was significantly higher than that in mice implanted with sponges containing untreated HDFs or sponges treated with LTBP-4 alone (Fig. 5(B)).

4. Discussion

Here, we report the development of a three-dimensional culture system based on collagen sponges, which were cross-linked at 120 °C; these sponges contained small pores in which HDFs proliferate and generate elastic fibers in the absence of keratinocytes or cytokines.

Recent studies show that the phenotype and matrix synthesizing properties of fibroblasts are affected by cell-ECM and cell–cell signaling [21–25]. Zhao et al. demonstrated that fibroblasts are affected by surface chemistry and topology. Each cell within a three-dimensional porous composite scaffold with a honeycomb-patterned surface is attached via the ECM secreted by the cell itself; ECM secretion, cell adhesion, and proliferation within such scaffolds are better than in scaffolds comprising large open pores [26]. Here, we showed that, small-pore scaffolds, which provide a surface that enables cells to attach at a higher density, promote cell proliferation and ECM production to a greater extent than large-pore scaffolds that do not provide a sufficient surface area for cell attachment. Furthermore, sponges cross-linked at 110 °C decomposed before the cells had a chance to secrete ECM components.

Although studies report elastic fiber formation in full-thickness skin equivalents comprising dermal fibroblasts and epidermis [16,17,27], none have demonstrated elastin expression in dermal equivalents containing fibroblasts alone. These findings that fibroblasts could not induce elastin expression without the presence of keratinocytes in their systems suggest that dysregulation of the elastin synthesis pathway. Long and Tranquillo showed that HDFs trapped within fibrin gels produce mature elastic fibers in the presence of TGF-β1, insulin, and plasmin (all of which upregulate elastin gene expression) [15]. Here, we took a collagen sponge that is already in clinical use (PELNAC®) and modified the cross-linking conditions and pore size. This modified material could easily be

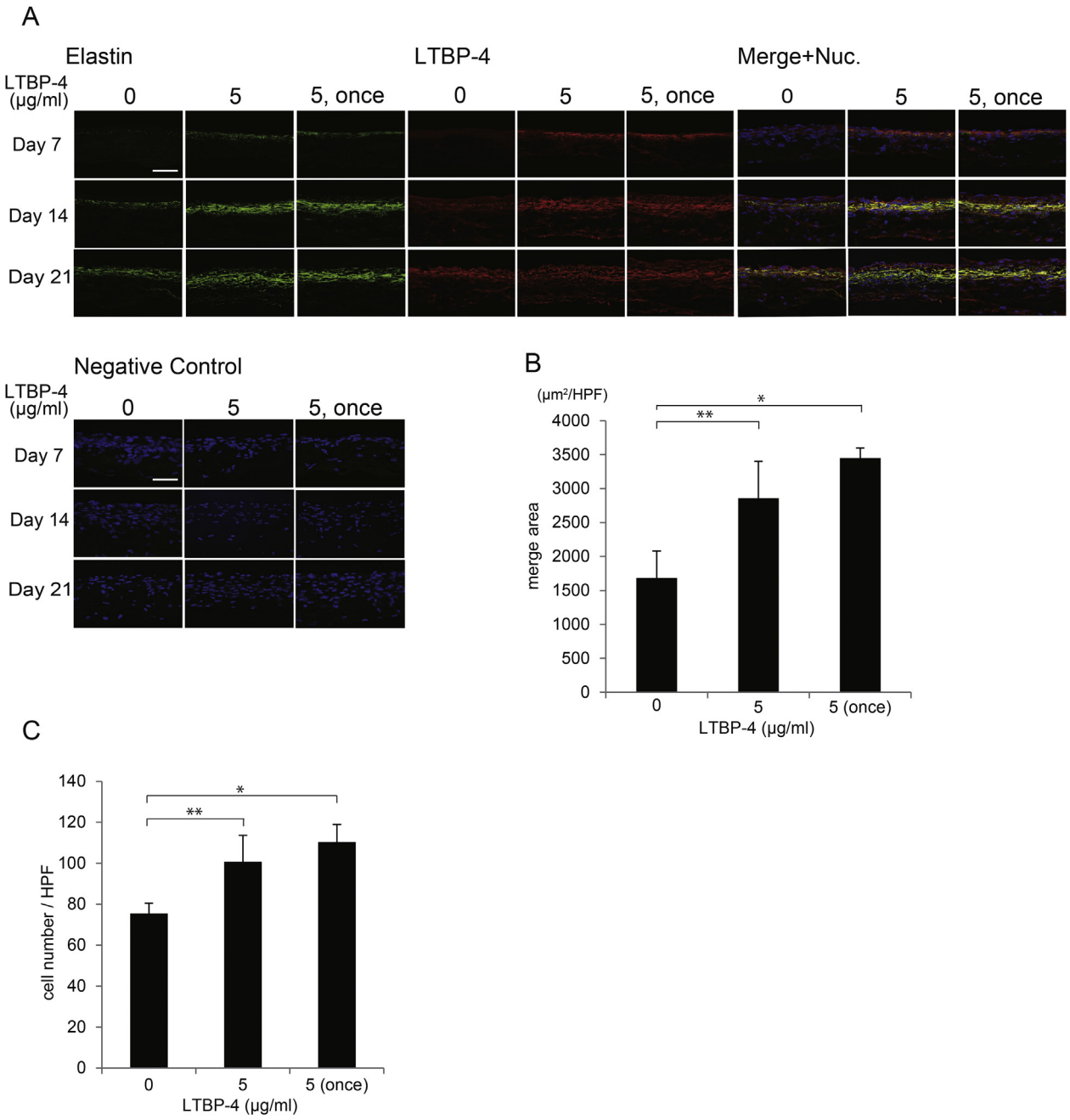

Fig. 4. (A) Samples seeded with HDFs and cultured without LTBP-4, in the presence of a single dose of LTBP-4, or in the presence of a weekly dose of LTBP-4 were immunostained on Day 21. LTBP-4 = 5; LTBP-4 added every 7 days (i.e., a weekly dose of L TBP-4). LTBP-4 = 5, once; LTBP-4 added during the first 7 days of culture (i.e., a single dose of LTBP-4). Scale bar = 50 μm. (B) Quantitative analysis of the merged areas of elastin and LTBP-4 on Day 21. Data are expressed as the mean ± SE. *P < 0.001, **P < 0.01. (C) Number of cells per high-power field on Day 21. Data are expressed as the mean ± SE. *p < 0.001 and **p < 0.01.

applied in clinical practice.

We observed increased elastic fiber regeneration and greater cell numbers in three-dimensional HDF cultures in the presence of recombinant LTBP-4. Previous studies show that the stiffness of the material surrounding the cells affects crucial cellular functions, including proliferation and differentiation [28,29]. Kantola et al. reported that the C-terminal domain of LTBP-4 supports fibroblast adhesion [30]. They also used fibroblast cultures to show that LTBP-4 colocalized first with fibronectin and then with fibrillin-1; therefore, LTBP-4 may stimulate cell growth by increasing cell adhesion and assembling the extracellular matrix. Increasing the amount of LTBP-4 from 5 μg/ml to 10 μg/ml did not lead to further increases in elastin regeneration (data not shown), suggesting that the amount of elastin secreted by cells is limited.

Analysis of exogenous recombinant FLAG-tagged LTBP-4 and endogenous (secreted) LTBP-4 revealed that cells secreted a significant amount of LTBP-4, even those in scaffolds that were treated with a single dose of recombinant LTBP-4 and cultured for 14 days. Treating scaffolds with three doses of LTBP-4 over 21 days did not result in increased elastin production, suggesting that LTBP-4 need only be present during the early stages to promote elastic fiber regeneration. A previous study reported strong expression of the tropoelastin gene at Day 7–10 in cultures of full-thickness skin equivalents [17]. Therefore, LTBP-4 present during the early stages

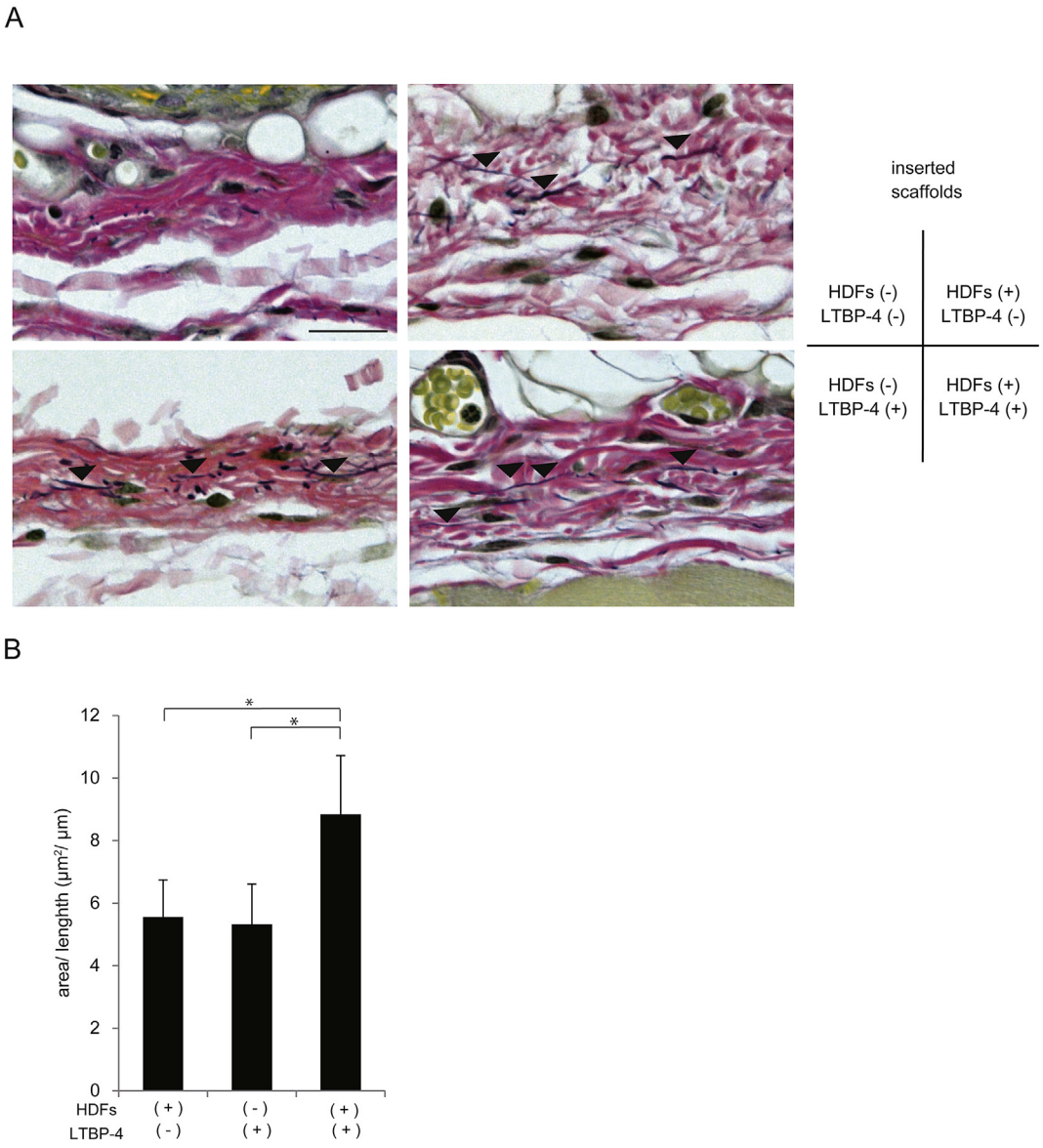

Fig. 5. (A) Elastica van Gieson (EVG) staining of the collagen scaffold 21 days after grafting between the dermis and the cutaneous muscle of mice. Scale bar = 20 μm. (B) Quantitative analysis of the tissue areas containing elastic fibers. *$p < 0.001$.

of culture (during which the levels of elastin mRNA increases) may increase the efficiency of tropoelastin deposition on microfibrils.

The results of the mouse models demonstrated that elastic fiber regenerated in scaffolds incubated with either HDFs or LTBP-4 or both. A single treatment with LTBP-4 had a clear effect; HDFs in scaffolds treated with LTBP-4 built thick tissue containing mature elastic fibers.

The collagen sponges developed herein were capable of regenerating elastic fibers and, when exposed to LTBP-4, regenerated abundant elastic fibers in a short period of time both *in vitro* and *in vivo*; thus these systems have potential for use as skin or cardiovascular tissue equivalents. This culture method may also be useful for examining the microstructure of elastin networks in three dimensions.

We must point out that the cells used in this study were derived from infants. However, adult HDFs exhibit levels of elastin production comparable with those of neonatal HDFs [15]. Therefore, it is likely that adult HDFs will also generate elastic fibers in three-dimensional culture in the presence of LTBP-4, although further studies are needed to confirm this.

5. Conclusions

The findings presented herein show that HDFs generate elastic fibers when cultured in a three-dimensional collagen scaffold based on a readily available dermal equivalent (PELNAC®). The scaffold used herein was subjected to minor modifications (crosslinking temperature, 120 °C; pore size, 25 μm). Exposing HDFs cultured within this scaffold to LTBP-4, which is essential for elastic fiber assembly, led to the regeneration of thick tissues containing abundant elastic fibers (both *in vitro* and *in vivo*). This method has potential application as a tissue-equivalent and may be useful for the analyses of the elastic microstructure in three dimensions.

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