Silk fibroin sponges with cell growth-promoting activity induced by genetically fused basic fibroblast growth factor

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Abstract: Transgenic silkworm technology has enabled the biological properties of silk fibroin protein to be altered by fusion to recombinant bioactive proteins. However, few studies have reported the fabrication of genetically modified fibroin proteins into three-dimensional spongy structures to serve as scaffolds for tissue engineering. We generated a transgenic silkworm strain that produces fibroin fused to basic fibroblast growth factor (bFGF) and processed the fibroin into a spongy structure using a simple freeze/thaw method. NIH3T3 mouse embryonic fibroblasts grown on bFGF-fused fibroin sponges proliferated and spread out well, showing half the population doubling time of cells cultured on wild-type fibroin sponges. Furthermore, the number of primary rabbit articular chondrocytes growing on bFGF-fused fibroin sponges was around five-times higher than that of the wild-type control at 3-days post cell-seeding. As the physical properties of wild-type and bFGF-fused fibroin sponges were almost identical, it is suggested that bFGF fused to fibroin retained its biological activity, even after the bFGF-fused fibroin was fabricated into the spongy structure. The bFGF-fused fibroin sponge has the potential for widespread application in the field of tissue engineering, and the method of fabricating this structure could be applicable to other recombinant bioactive fibroin proteins. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 82–93, 2016.

Key Words: basic fibroblast growth factor, silk fibroin, transgenic technology, scaffold, cell proliferation


INTRODUCTION

Bombyx mori silkworm silk has been used in medicine for centuries as a surgical suture and, more recently, degummed B. mori silk protein (fibroin) has been FDA-approved for clinical use.1 After being dissolved, fibroin can be processed into films, meshes, gels, and sponges for biomedical applications, including as scaffolds for tissue engineering.2–4 In addition, recent advances in transgenic silkworm technology5 have enabled alterations of the biological properties of fibroin. Recombinant bioactive proteins can be produced in the posterior silk glands (PSGs), fused to fibroin protein, for example, basic fibroblast growth factor (bFGF) fused to fibroin, which stimulated endothelial cell proliferation,6 and fibroin containing collagen or the RGD peptide, which promoted fibroblast and chondrocyte adhesion.7–9 These studies suggest that the recombinant bioactive peptide/protein, with its accurately folded or refolded structure, is able to show its biological function even when expressed fused to fibroin protein. However, the modified fibroin proteins reported previously were not fabricated into three-dimensional (3D) forms, but simply coated a cell culture plate,6,7,9 except in our previous report on RGD-fused fibroin sponges for use in cartilage regeneration.8 Therefore, it remains unclear whether recombinant bioactive proteins (not peptides such as RGD) expressed fused to fibroin can exert their bioactivity even after being processed into the fibroin 3D structure.

The single-chain heparin-binding protein, bFGF, was originally isolated and identified from bovine brain and pituitary, based on its stimulatory effect on fibroblast proliferation.10,11 This growth factor is a notoriously unstable protein.12 It is inactivated by treatment with a variety of solvents, such as diluted acid, organic solvents, and solutions of guanidinium chloride.13 Additionally, the growth factor exhibits instability when stored at room temperature, exposed to alkaline pH, or incubated with catalytic amounts of Cu2+ ion.14 Therefore, it is challenging and meaningful to...
fabricate a fibroin 3D structure with the bioactivity of recombinant bFGF fused to the fibroin.

Various methods of fabricating fibroin spongy structures have been reported. A fibroin solution was lyophilized in the presence of a chemical cross-linker to form a spongy structure. Repeated freeze/thawing was reported as an effective method of forming a fibroin 3D porous structure. Nazarov et al. used three fabrication techniques (freeze-drying, salt-leaching, and gas-foaming) to form spongy structures of fibroin. These studies successfully prepared fibroin sponges with a porosity, pore size, and mechanical strength suitable for cell culture. However, it is well established that chemical cross-linkers and repeated freeze/thawing should be avoided to maintain the bioactivity of proteins. In addition, most of the previous studies used methanol treatment of fibroin to induce water insolubility, but alcohol would influence the tertiary structure of proteins. In addition, most of the previous studies used methanol treatment of fibroin to induce water insolubility, but alcohol would influence the tertiary structure of proteins. In addition, most of the previous studies used methanol treatment of fibroin to induce water insolubility, but alcohol would influence the tertiary structure of proteins.

In this study, we generated a transgenic silkworm strain that produces silk fibroin protein fused to bFGF and, for the first time, prepared a fibroin sponge that retains the bioactivity of the growth factor. The bFGF-fused fibroin molecule was designed so that bFGF would be linked to the fibroin light chain (L-chain) protein via a collagenase cleavage site, aiming at continuous effects of the growth factor. The cleavage site is composed of the amino acid sequence, PLGIAG, which is cleaved between the Gly and Ile residues by collagenase. We first evaluated the proliferation of fibroblasts on the fibroin sponge, and then cultured primary chondrocytes on the sponge, as these cells are known to show poor growth in 3-D culture [e.g., in a collagen gel].

**MATERIALS AND METHODS**

**Vector carrying complementary DNA (cDNA) encoding bFGF-fused L-chain**

An expression plasmid for modified fibroin L-chain was constructed as follows, and the sequences of the primers are shown in Supporting Information Table SI. The fibroin L-chain promoter along with a part of the open reading frame of the fibroin L-chain (FibLpro-FibL) and the terminator of the fibroin L-chain (FibLPra) were amplified by polymerase chain reaction (PCR) of the previously reported plasmid, pBac[3×P3-DsRed2afm]-LLL-EGFP-His, with primer sets (FibLpro-FibL: pLC-pro5 and pLC-L_ORF-3_BHI; FibLPra: pLCP(A)−5_SalI and pLCP(A)−3_FseI). The amplified fragments, FibLpro-FibL and FibLPra, were treated with Ascl and BamHI and with FseI and SalI, respectively, and cloned between the Ascl and FseI sites of the pH-C-EGFP along with an annealed primer set (MCS-5 and MCS-3). The resulting plasmid was designated as pLC-mcs.

Two oligonucleotides (bFGF_col2link-5Bgl2 and bFGF_col2link-3) were mixed, and an oligonucleotide cassette, bFGF_col2link-5Bgl2long, was obtained by PCR-amplification. Then, using two oligonucleotides (bFGF_col2link-5Bgl2long and bFGF_ORF-3SalI), cDNA encoding 155-amino acid residue-long human bFGF (accession number, J04513) was amplified by PCR from a cloned cDNA encoding human bFGF, which was kindly provided by Dr. Tomita. This amplified fragment was treated with BglII and SalI and cloned between BamHI and SalI sites of the pLC-mcs. The resulting plasmid was designated as pLC-col2bFGF.

To obtain a transfer plasmid for the bFGF-fused fibroin L-chain, the expression cassette in the pLC-col2bFGF was digested with FseI and Ascl, and, finally, the resulting fragment was cloned between the FseI and blunt-ended Ascl sites of the pBac[3×P3-DsRed2afm]E1. This transfer plasmid was designated as pBac[3×P3-DsRed2afm]E1_pLC-col2bFGF (Fig. 1).

**Transgenesis and screening**

Silkworm transgenesis was performed as described by Tamura et al. with minor modifications. Briefly, the plasmid pBac[3×P3-DsRed2afm]E1_pLC-col2bFGF was injected into eggs of wild-type B. mori silkworms along with a helper plasmid pHA3PIG coding for piggyBac transposase at 3–6 h post-oviposition. Hatched larvae (G0) were reared and permitted to mate with each other. The resulting embryos (G1) were screened using a fluorescent microscope (MZ16 FA; Leica Microsystems, Germany) for transgenic individuals with DsRed2 expression 6–7 days after oviposition. The transgenic silkworms were reared together and sib-mated for at least three generations, with sequential screening by the strong excitation of DsRed2 fluorescence in the adult eye. The resulting strain was designated NK34, which carries the transgene.
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cocoon shells of wild-type or NK34 silkworms were degummed in 8 M urea, dissolved in 9 M LiBr aqueous solution, and dialyzed against Dulbecco’s phosphate buffered saline (D-PBS; Wako Pure Chemical Industries, Japan). After centrifugation to remove impurities, the protein concentration was adjusted using the absorbance of 280-nm ultraviolet light. Different concentration collagenase solutions (collagenase L; Nitta Gelatin, Japan) were added to the fibroin solution (final concentrations, 4 mg mL\(^{-1}\) fibroin and 0, 0.4, 4, or 40 \(\mu\)g mL\(^{-1}\) collagenase). Following incubation at 37°C for 24 h, the protein composition of the solutions was analyzed by SDS-PAGE on Mini-PROTEAN® TGX gels (Any kD™; Bio-Rad Laboratories, USA), under reducing conditions. Separated proteins were fixed and visualized by immersing the gel in EzStain AQia solution (Atto, Japan) containing Coomassie Brilliant Blue (CBB). For western blotting, separated proteins on another gel were transferred to a polyvinylidine fluoride membrane using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories) and assayed with a Chemi-Lumi One Super kit (Nacalai Tesque, Japan). Signals were detected with a chemiluminescence imaging system (LAS-3000 mini; Fujifilm, Japan). A rabbit anti-human bFGF polyclonal antibody (ab10420; Abcam plc, UK) and horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (ab6721; Abcam plc) were used as the primary and secondary antibody, respectively. Novex® Sharp Protein Standard and MagicMark™ XP Western Standard were used as markers for CBB staining and western blotting, respectively (Invitrogen, USA).

Bioactivity of bFGF-fused fibroin added to culture medium

Because bFGF-fused fibroin proteins from degummed NK34 silkworm cocoons had little effect on cell proliferation (Supporting Information Fig. S1), the bFGF-fused fibroin was directly extracted from PSGs of fifth-instar NK34 silkworms. The PSGs were diced and immersed in 50 mM sodium phosphate buffer (PB; pH 7.4) on ice for 6–8 h to extract bFGF-fused fibroin proteins. The solution was filtered through an article membrane with 9.5-\(\mu\)m pores (#5B; Kiriyama glass, Japan) to remove impurities. Then, the supernatant was dialyzed against 50 mM PB (pH 7.4) using cellulose dialysis membranes (molecular weight cut off, 6–8000; Spectrum Laboratories, USA) for 3 days at 4°C, changing the buffer every 10–14 h. Insoluble portions were removed by centrifugation at 42,000g for 30 min at 4°C, and the fibroin concentration in the resulting solution was determined by bicinchoninic acid assay with fibroin protein standards.

NIH3T3 mouse embryonic fibroblast line, provided by RIKEN BRC through the National Bio-Resource Project of MEXT in Japan, was seeded onto wells of 96-well tissue culture plates (Asahi Glass, Japan) at a concentration of 2.0 \(\times\) 10\(^3\) cells/well and cultured with Eagle’s MEM (Nissui Pharmaceutical, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco Invitrogen, USA), 10 ng mL\(^{-1}\) kanamycin (Gibco Invitrogen), 0.2% sodium bicarbonate (Gibco Invitrogen), and 2 mM L-glutamine (Gibco Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\). After incubation for 24 h, the medium was replaced with Eagle's MEM containing 1% FBS, 10 ng mL\(^{-1}\) kanamycin, 0.2% sodium bicarbonate, 2 mM L-glutamine, 5 \(\mu\)g mL\(^{-1}\) sodium heparin (Wako Pure Chemical Industries), and different amounts of bFGF-fused fibroin or commercial recombinant human bFGF (rhbFGF; Wako Pure Chemical Industries). The fibroin or rhbFGF solutions were sterilized by filtering through a polyethersulfone membrane with 0.22-\(\mu\)m pores (Millipore, USA). After culturing for 2 days, the cells were incubated with WST-1 reagent (Roche Diagnostics, Germany) for 2 h, followed by absorbance measurement at 450 nm using a plate reader (VersaMax™; Molecular Devices LLC, USA). The absorbance at 650 nm was used as a reference. Three different wells were measured for each culture condition, respectively (n = 3).

Preparation of fibroin sponges

Wild-type fibroin sponges were prepared as previously described,\(^{21}\) with minor modifications. Briefly, a 25 mM PB solution (pH 7.4) containing 4.0% (w/v) degummed wild-type fibroin protein and 1% (v/v) dimethyl sulfoxide (DMSO) was placed in a mold (5 cm\(^2\), thickness, 1.5 mm) and frozen at −20°C for 17 h to form a spongy structure by phase separation. Fibroin sponges containing bFGF-fused fibroin proteins were formed from a 25 mM PB solution (pH 7.4) containing 3.7% (w/v) degummed wild-type fibroin protein, 0.3% (w/v) bFGF-fused fibroin protein extracted from the PSGs of NK34 silkworms, and 1% (v/v) DMSO using the same protocols for preparing the wild-type fibroin sponges. The concentration of the bFGF-fused fibroin protein (0.3% (w/v), which is equivalent to 7.5% (w/w) of the total (wild-type and bFGF-fused) fibroin protein) was sufficient to stimulate cell proliferation significantly (Supporting Information Fig. 2). The DMSO was removed by immersing the sponges in 25 mM PB (pH 7.4) for 3 days at 4°C, changing the buffer every 10–14 h. Then, the sponges were freeze-dried (FDU-1100; Tokyo Rikakikai, Japan), placed in sterilization pouches, and irradiated with an electron beam (EB) for sterilization. The EB sterilization was conducted by the Japan Electron Beam Irradiation Service, Japan. Fibroin sponges were placed on dry ice and irradiated using a Dynamitron® EB accelerator (IBA Industrial Inc., Belgium) with an acceleration voltage of 4.8 MV, an electron current of 20.0 mA, and a conveyor speed of 10.5 m min\(^{-1}\). The resultant electron dose was 26.1 kGy.

Fourier transform infrared (FTIR) spectroscopy analysis of fibroin sponges

FTIR spectroscopy analysis of EB sterilized wild-type and bFGF-fused fibroin sponges was performed using a spectrometer (FT/IR-620; Jasco, Japan), equipped with a five-times-reflection ZnSe attenuated total reflection attachment (ATR500/M; Jasco Corp.). The instrument was continuously purged with nitrogen gas to remove atmospheric water...
The spectra were recorded with 32 scans accumulated at a resolution of 4 cm\(^{-1}\).

**Solid-state \(^{13}\)C nuclear magnetic resonance (NMR) analysis of fibroin sponges**

High-resolution solid-state \(^{13}\)C cross polarization (CP)/magic angle spinning (MAS) NMR analysis of EB sterilized wild-type and bFGF-fused fibroin sponges was performed using an Avance 600 WB (Bruker Karlsruhe, Germany), with a magnetic field of 14.1 T. The spectrometers were operated at a \(^{13}\)C NMR frequency of 150.94 MHz. The sample were placed in a solid-state probe and spun at a MAS frequency of 10.0 kHz in a 4.0-mm-/zirconia rotor. In the CP experiments, a \(^1\)H 90° pulse length of 3.5 \mu s and \(^1\)H--\(^{13}\)C CP contact of 70 kHz were employed. The high-power \(^1\)H decoupling using the SPINAL-64 method was employed for CP method. The repetition time for the CP experiments was set at 3.0 seconds. All the spectra were calibrated using adamantane as the standard, and the chemical shift of the adamantane CH\(_2\) peak appearing at 29.5 ppm was referenced to the tetramethylsilane peak appearing at 0 ppm.

**Scanning electron microscopy (SEM) analysis of fibroin sponges**

A low-vacuum scanning electron microscope (TM-1000; Hitachi, Japan) was used to observe the topography of EB sterilized wild-type and bFGF-fused fibroin sponges. Top-view (cell-seeding side) and cross-sectional images of the sponges were photographed. The pore size was measured using ImageJ software (National Institute of Health, USA), with at least ten pores from the two top-view images analyzed.

**Mechanical testing of fibroin sponges**

The compressive properties of EB sterilized wild-type and bFGF-fused fibroin sponges were measured using a tensile tester (EZ test; Shimadzu, Japan), equipped with a 5 N load cell and an 8-mm diameter load plate, at 5 mm min\(^{-1}\) of head speed at room temperature; thickness measurements followed using a micrometer gauge. The samples were completely wetted in D-PBS, and the excess solution was wiped away using a tissue before measuring the thickness. The compressive modulus of the wet sponges was determined from the initial slope of the stress/strain curves at a strain of 0.5–5%. Five and four different samples were tested for the wild-type and bFGF-fused fibroin sponges, respectively (n = 5, wild type; n = 4, bFGF-fused).

**Culture of fibroblasts on fibroin sponges**

Wild-type and bFGF-fused fibroin sponges were cut into a cylindrical shape (diameter, 6 mm; thickness, 1.5 mm) and completely wetted by immersing in Eagle’s MEM containing 10 ng mL\(^{-1}\) kanamycin, 0.2% sodium bicarbonate, and 2 mM L-glutamine for 2 h. Then, NIH3T3 cells were seeded onto the fibroin sponges at a concentration of 1.0 \times 10^4 cells/sponge and cultured with Eagle’s MEM supplemented with 5% (v/v) FBS, 10 ng mL\(^{-1}\) kanamycin, 0.2% sodium bicarbonate, 2 mM L-glutamine, and 5 \mu g mL\(^{-1}\) sodium heparin. As the positive control, NIH3T3 cells were cultured on wild-type fibroin sponges with medium containing 1 ng mL\(^{-1}\) rhbFGF. The number of cells on the fibroin sponges was determined using the previously described lactate dehydrogenase (LDH) assay\(^{28}\) with standards with various concentrations of fibroblasts. Briefly, after incubation for 4 h, 1, 2, or 3 days, the cells were washed three times with D-PBS. The washed cells were then dissolved by incubating in 0.5% (w/v) Triton X-100 (Research Organics, USA) in D-PBS at 4°C. The LDH activity of the dissolved cells was measured from changes in the absorbance at 340 nm that are attributed to the kinetics of nicotinamide adenine dinucleotide-consuming reactions. Four different sponges were used for each time point (n = 4).
described. Briefly, articular cartilage tissues were aseptically harvested from the proximal humerus, distal femur, and proximal tibia of 4-week-old Japanese white rabbits (Oriental Bio Service, Japan). Chondrocytes were isolated via enzymatic digestion. After obtaining a cell suspension, the cells were cultured to 80% confluence in a tissue culture flask (Thermo Fisher Scientific, USA) with Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque) containing 10% (v/v) FBS and 1% (v/v) antibiotic mixture (10,000 U mL\(^{-1}\) penicillin, 10,000 µg mL\(^{-1}\) streptomycin, and 25 µg mL\(^{-1}\) amphotericin B; Nacalai Tesque) at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\) for 7 days. The medium was changed every 3 days.

After expansion in culture, chondrocytes were removed from the flask by mixing with 0.25% trypsin-EDTA (Nacalai Tesque) and washed twice with D-PBS. The cells (passage 1) were seeded onto wild-type and bFGF-fused fibroin sponges (diameter, 6 mm; thickness, 1.5 mm), which were pre-incubated in DMEM containing 1% (v/v) antibiotic mixture for 2 h. The cell seeding density was 1.0 × 10^5 cells/sponge. The chondrocytes on the fibroin sponges were cultured with DMEM containing 5% (v/v) FBS, 1% (v/v) antibiotic mixture, and 5 µg mL\(^{-1}\) sodium heparin at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\), for up to 7 days. The medium was changed after 4 days in culture. After culturing for 4 h, 1, 3, 5, or 7 days, the number of chondrocytes grown on the wild-type and bFGF-fused fibroin sponges was determined by the LDH assay with standards with various concentrations of chondrocytes as the control.

Population doubling time (PDT) of the fibroblasts was calculated using the following equation:

\[ \text{PDT} = \frac{\mu}{\ln 2} \]

where \( \mu \) is the specific growth rate determined from the exponential fitting of the growth curve.

**Culture of chondrocytes on fibroin sponges**

Rabbit articular chondrocytes were prepared as previously described. Briefly, articular cartilage tissues were aseptically harvested from the proximal humerus, distal femur, and proximal tibia of 4-week-old Japanese white rabbits (Oriental Bio Service, Japan). Chondrocytes were isolated via enzymatic digestion. After obtaining a cell suspension, the cells were cultured to 80% confluence in a tissue culture flask (Thermo Fisher Scientific, USA) with Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque) containing 10% (v/v) FBS and 1% (v/v) antibiotic mixture (10,000 U mL\(^{-1}\) penicillin, 10,000 µg mL\(^{-1}\) streptomycin, and 25 µg mL\(^{-1}\) amphotericin B; Nacalai Tesque) at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\) for 7 days. The medium was changed every 3 days.

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Population doubling time (PDT) of the fibroblasts was calculated using the following equation:

\[ \text{PDT} = \frac{\mu}{\ln 2} \]

where \( \mu \) is the specific growth rate determined from the exponential fitting of the growth curve.

**Statistical analysis**

Quantitative data were analyzed between wild-type and bFGF-fused fibroin experimental groups. Analysis of covariance was used to compare the growth rates of fibroblasts grown on wild-type and bFGF-fused fibroin sponges. Two-way analysis of variance was used to analyze the effects of the type of fibroin sponge and the culture period on chondrocyte density and gene expression level. This analysis was followed by Tukey’s post hoc comparison. For the analysis of mechanical properties of the fibroin sponges, the two-sided Aspin-Welch t test was used. A value of \( p < 0.05 \) was considered significant. Asterisks indicate statistical significances between wild-type and bFGF-fused fibroin groups.

**RESULTS**

**Transgenesis outcomes: Secretion and bioactivity of bFGF-fused fibroin**

Transformation vector pBac[3×P3-DsRed2afm]E1_pLC-col2bFGF (Fig. 1) was injected into 482 wild-type silkworm eggs together with helper plasmid pHA3PIG. Two hundred and sixty-seven eggs hatched (hatching rate, 55.4%) and larvae (G0) were allowed to develop into G0 moths. The moths were intercrossed, and there were 45 resulting G1 broods, where 11 broods had DsRed2-positive larvae. Thus, the success rate of transgenesis was 24.4%; the transgenic silkworm strain was designated as NK34.

We then analyzed the silk proteins produced by NK34 silkworms by SDS-PAGE and western blotting with an anti-human bFGF antibody (Fig. 2). On CBB-stained gels [Fig. 2(A)], no band was detected at the position corresponding to the predicted molecular weight of bFGF-fused fibroin L-chain (45 kDa; L-chain, 27 kDa + bFGF, 18 kDa). However, such a band was clearly observed on western blotting for
the silk proteins of NK34 silkworms but not for those of wild-type silkworms [Fig. 2(B)]. Based on analysis of band intensities, the amount of bFGF contained in the total fibroin protein produced by NK34 silkworms was estimated to be 0.1–0.2 wt %. In collagenase-treated NK34 samples, a band of around 18 kDa was detected by immunoblotting. This band could correspond to monomeric bFGF by comparison with the commercial rhbFGF. Thus, NK34 transgenic silkworms spun silk fibers containing the fibroin L-chain fused to human bFGF via the collagenase-cleavage site, PLGIAG.

The bioactivity of the bFGF-fused fibroin protein extracted from PSGs of fifth-instar NK34 silkworms was assessed by the addition of the protein to the culture medium of fibroblasts; the dose-dependent changes in absorbance, indicative of cell number, are shown in Figure 3. The bFGF-fused fibroin protein increased the growth of fibroblasts in a dose-dependent manner up to ~50 μg mL⁻¹ [Fig. 3(A)]. At higher concentrations, the bioactivity of the bFGF-fused fibroin from PSGs was suppressed, which is consistent with reports from other research groups that an overdose of the growth factor inhibits cell proliferation. Based on a comparison of the effects of bFGF-fused fibroin and rhbFGF [Fig. 3(B)], the amount of bioactive bFGF contained in the total fibroin protein produced by NK34 silkworms was estimated to be 0.2 wt %: the absorbance of culture medium with ~50 μg mL⁻¹ bFGF-fused fibroin was equivalent to that of culture medium with 100 ng/mL rhbFGF.

Effects of bFGF-fused fibroin on the physical properties of fibroin sponges
The bFGF-fused fibroin proteins extracted from PSGs were mixed with degummed wild-type fibroin protein at the weight ratio of 7.5:92.5; the mixture was fabricated into a fibroin spongy structure, which was then sterilized with a 26.1 kGy EB. The influence of the modified fibroin protein on the physical properties of fibroin sponges was evaluated using FTIR spectroscopy, solid-state ¹³C NMR, SEM, and compression testing.

Figure 4(A) presents FTIR spectra of the wild-type and bFGF-fused fibroin sponges at the amide I (1600–1700 cm⁻¹) and amide II (1500–1560 cm⁻¹) regions, which provide information related to the protein’s secondary structure (α-helix, 1650 cm⁻¹; β-sheet, 1624 cm⁻¹). According to the two spectra, proteins in both fibroin sponges had a similar secondary structure. This was confirmed by the amino acid Cα/Cβ peak region (0–80 ppm) of the solid-state ¹³C NMR spectra of the fibroin sponges, which showed similar peaks for Cα and Cβ in the Ala and Ser residues in the random coil, Silk I crystal, and β-sheet-rich Silk II crystal structures [Fig. 4(B)]. The results of these structural analyses of the fibroin sponges suggest that the growth factor contained in the bFGF-fused fibroin sponges was undetectable, likely because of the small amount present (<0.015% (w/w); 0.075 × 0.002 × 100). SEM of their structure showed that both the wild-type and bFGF-fused fibroin sponges appeared to be similar in morphology, with similar pore size, pore structure, and pore size distribution [Fig. 4(C)]. The average diameter of pores observed in the wild-type and bFGF-fused fibroin sponges, from the top view (cell-seeding side), was 25 ± 7 μm and 27 ± 11 μm, respectively. No statistically significant difference in the compressive modulus of the two kinds of fibroin sponges was revealed by mechanical testing, as shown in Figure 4(D). Therefore, the introduction of bFGF-fused fibroin proteins into a fibroin sponge had no influence on the structure and physical properties of the sponge.

Effects of bFGF-fused fibroin on fibroblast growth on fibroin sponges
Time-dependent changes in the number of NIH3T3 fibroblasts growing on the fibroin sponges are shown in Figure 5(A). Initial cell density (at 4 h post-seeding) varied among experimental groups possibly because of differences in fibroblast attachment to fibroin sponges. However, supplementation of the culture medium with rhbFGF stimulated fibroblast proliferation on the wild-type fibroin sponges, but cells on the bFGF-fused fibroin sponges exhibited significantly faster growth, with a shorter PDT (4.0 days) than

![Figure 3](image-url)

**Figure 3.** (A) Dose-dependent fibroblast growth-promoting activity of bFGF-fused fibroin protein extracted from posterior silk glands. NIH3T3 fibroblasts were cultured in medium containing 0.3, 0.6, 3, 6, 30, 60, 300, or 600 μg mL⁻¹ of either bFGF-fused or wild-type fibroin protein. Cell proliferation was assessed by WST-1 assay. The absorbance of the culture medium supplemented with wild-type fibroin was subtracted from that of the culture medium supplemented with bFGF-fused fibroin at each concentration. (B) Effects of commercial recombinant human bFGF (rhbFGF) were assayed at the same time; the absorbance of the cell culture medium without rhbFGF was made zero. Data are shown as the mean ± SD (n = 3).
those on the wild-type fibroin sponges (8.7 days). The PDT of cells on the bFGF-fused fibroin sponges was similar to that of cells grown on the wild-type fibroin sponges in medium supplemented with rhbFGF (4.1 days). The fold increase in cell number from the previous time point is shown in Figure 5(B). The rhbFGF supplement strongly promoted the growth of fibroblasts on wild-type fibroin sponges from 4 h to 1 day post-seeding but, thereafter, the fold increase was comparable to that without the supplement. In contrast, cells grown on bFGF-fused fibroin sponges showed a gradual increase in cell number, with the highest fold increases from 1–2 days to 2–3 days of the three sponge/treatment groups.

The morphology of fibroblasts cultured on the wild-type or bFGF-fused fibroin sponges was different on each sponge [Fig. 5(C)]; the morphology of fibroblasts grown for 3 days on the wild-type fibroin sponges without the rhbFGF supplement was round with an immature cytoskeleton [arrowheads in Fig. 5(C)]. On the other hand, the cells grown on the bFGF-fused fibroin sponges exhibited a spread-out shape with a developed cytoskeleton [arrows in Fig. 5(C)], as was

![Graph](image1)

![Graph](image2)

![Graph](image3)

FIGURE 4. Physicochemical properties of fibroin sponges generated from wild-type protein and fibroin sponges generated from wild-type protein and bFGF-fused protein. (A) FTIR spectra of the amide I and II regions of the fibroin sponges. The signals at 1650 cm\(^{-1}\) and 1624 cm\(^{-1}\) are attributed to the \(\alpha\)-helix and \(\beta\)-sheet conformations, respectively. (B) Amino acid C\(_{a}/C\(_{b}\) peak region of the solid-state \(^{13}\)C CP/MAS NMR spectra of the fibroin sponges. The signals are assigned for C\(_{a}\) and C\(_{b}\) in the Ala and Ser residues in the random coil, Silk I crystal, and \(\beta\)-sheet-rich Silk II crystal conformations. (C) Scanning electron micrographs of the fibroin sponges. Photographs were taken of cross-sectional and top (cell-seeding side) views. Scale bars are 1 mm, 300 \(\mu\)m, or 200 \(\mu\)m, as indicated. (D) Compression modulus of the fibroin sponges. Data are shown as the mean \(\pm\) SD (\(n = 5\), wild-type; \(n = 4\), bFGF-fused). No statistical differences were detected by the two-sided Aspin–Welch \(t\) test.
Effects of bFGF-fused fibroin on chondrocyte growth on fibroin sponges

Primary rabbit articular chondrocytes were cultured on wild-type and bFGF-fused fibroin sponges. Figure 6(A) shows the time-dependent changes in cell density. Initial chondrocyte attachment was analyzed at 4 h post-seeding and this analysis showed that almost the same number of seeded chondrocytes was trapped in the two sponge types, but the growth of chondrocytes was clearly different between the wild-type and bFGF-fused fibroin experimental groups. The cells on the bFGF-fused fibroin sponges rapidly proliferated for 3 days after seeding but, thereafter, their growth reached a plateau. In contrast, the cell density on the wild-type fibroin sponges was nearly unchanged throughout the culture period.

Expression of the gene encoding collagen type II, which is one of the constituents of the cartilage-specific extracellular matrix (ECM), was quantified by real-time PCR [Fig. 6(B)]. The expression levels at 3 and 7 days post seeding were normalized to the level before seeding. Chondrocytes grown on the bFGF-fused fibroin sponges showed a significantly lower expression level than those grown on wild-type fibroin sponges after 3 days of culture. However, after 7 days of culture, the level in the bFGF-fused fibroin experimental group was comparable to that in chondrocytes before seeding, and there was no significant difference between the wild-type and bFGF-fused fibroin groups.

The morphology of the cells was observed using SEM [Fig. 6(C)]. Most of the cells on the wild-type fibroin sponges were spherical in shape, whereas those on the bFGF-fused fibroin sponges showed spindle morphologies with a developed cytoskeleton after 7 days in culture. Thus, chondrocytes grown on the bFGF-fused fibroin sponges had higher growth activity than those on wild-type fibroin sponges.

DISCUSSION

In this study, we generated a transgenic silkworm strain (NK34) that produces fibroin fused to human bFGF via a collagenease-cleavage site (Fig. 2). Western blot analysis [Fig. 2(B)] showed that the total fibroin protein produced by NK34 silkworms contained bFGF at a concentration of 0.1–0.2 wt %. Thus, 50 µg of total fibroin protein contained 50–100 ng of bFGF. This estimate corresponded to that from the WST-1 assay (Fig. 3), suggesting that most of the growth factor fused to the fibroin L-chain maintained its bioactivity when the fusion protein was extracted from PSGs. Additionally, it is
inferred that no change in the specific activity of bFGF was induced by fusion to the L-chain. This is supported by the dose-dependent cell growth-promoting activity of the bFGF-fused fibroin, which was similar to that reported for bFGF alone. The bioactive bFGF-fused fibroin proteins were mixed with degummed wild-type fibroin proteins and processed into a spongy structure to be used for scaffolds for fibroblast and chondrocyte culture. Fibroblasts cultured on bFGF-fused fibroin sponges showed enhanced growth compared to those cultured on wild-type fibroin sponges, showing rapid proliferation and a spread-out morphology (Fig. 5). In addition, the secondary structure, topography, and mechanical properties of the fibroin sponges were almost identical between the wild-type and bFGF-fused fibroin sponges (Fig. 4). These results strongly suggest that differences in the fibroblast growth on the wild-type and bFGF-fused fibroin sponges were induced by the bioactivity of the bFGF fused to the fibroin L-chain protein. It is inferred that fusion of bFGF to the L-chain resulted in exposure of the growth factor on the surface of bFGF-fused fibroin sponges in wet conditions (e.g., in cell culture medium) because the L-chain is more hydrophilic than the fibroin heavy chain (H-chain); the grand average of hydropathicity values for the L- and H-chains, calculated using the ProtParam tool (http://web.expasy.org/protparam/), are -0.080 and 0.213, respectively. Therefore, the growth factor fused to the L-chain likely maintained its bioactivity, being able to affect cell behavior, even after the bFGF-fused fibroin proteins were fabricated into a spongy structure. Interestingly, the effects of the rhbFGF supplement were observed within 1 day post-seeding, while those of bFGF fused to the fibroin continued for 3 days. It is considered that bFGF added to the medium was consumed and/or inactivated within a short period. However, when fused to fibroin, the growth factor showed long-lasting effects; this might be due to the gradual release of the bFGF from the fibroin sponge triggered by the cleavage of the PLGIA sequence. This difference between the activity of bFGF fused to the fibroin L-chain and that of bFGF alone was not observed when the growth factor was added to the culture medium (Fig. 3). When used as a scaffold, the bFGF-fused fibroin likely enabled local and sustained diffusion of the growth factor, resulting in long-term enhancement of cell growth. This sustained activity might be related to the stabilizing effect of fibroin, especially the $\beta$-sheet-rich H-chain, on small molecules and proteins.

Primary articular chondrocytes show poor proliferative potential in 3-D culture conditions in vitro, which has been one of the major obstacles to tissue-engineered articular cartilage therapy with autologous chondrocytes. Fibroin sponges have been studied as scaffolds for cartilage regeneration.
and we have previously shown that chondrocytes grown on fibroin sponges can synthesize cartilage-specific ECM to form hyaline cartilage-like tissues. Although chondrocytes on a fibroin sponge proliferated more rapidly than those in a collagen gel, it took around 10 days for the initial cell density in the fibroin sponge to increase five-fold. Additionally, in vivo functions of a fibroin sponge in cartilage repair in a rabbit model were reported, but a 2-week in vitro culture period was required for implantation. The effect of bFGF fused to fibroin was more clearly shown with chondrocytes than with fibroblasts; the cell density of chondrocytes was five times higher on bFGF-fused fibroin sponges than on wild-type fibroin sponges at 3 days post-seeding [Fig. 6(A)]. It has been reported that bFGF promotes the proliferation of chondrocytes but also stimulates their dedifferentiation into fibroblast-like cells in vitro. In our study, chondrocytes grown on bFGF-fused fibroin sponges showed significantly reduced collagen type II gene expression at 3 days [Fig. 6(B)] and a fibroblast-like spindle shape at 7 days [Fig. 6(C)]. However, recovery of the expression level of the collagen type II gene in chondrocytes grown on bFGF-fused fibroin sponges was observed from day 3 to day 7; the level at 7 days was comparable to that in cells before seeding and was not significantly different from that in the wild-type fibroin experimental group [Fig. 6(B)].

The significance of this study is the establishment of processes to prepare fibroin sponges without losing the bioactivity of recombinant proteins fused to the fibroin, which is likely owing to the simple process used to form a fibroin sponge structure. The process requires a single cycle of freeze/thawing of a fibroin aqueous solution in the presence of 1 vol % DMSO. This organic solvent promotes β-sheet formation in the final fibroin sponge structure, which makes the fibroin sponge water-insoluble without methanol treatment. The β-sheet-rich conformation of bFGF could be stabilized by DMSO. It has been reported that such a low concentration of DMSO should not cause the unfolding of protein tertiary structure. Therefore, the low concentration of DMSO was unlikely to inactivate the bFGF fused to the fibroin L-chain. The EB sterilization seems to be the second key factor in maintaining the recombinant protein’s activity. In preliminary experiments, we had sought a sterilization method that had minimal influence on protein activity, resulting in recombinant enhanced green fluorescent protein fused to the fibroin L-chain that remained fluorescent after EB irradiation (Supporting Information Fig. S3). The properties of fibroin films, such as the protein’s secondary structure, are reported to be little affected by gamma irradiation, which generates secondary electrons and therefore sterilizes by the same mechanism as EB irradiation. In addition, because the EB irradiation was conducted on dry ice in this study, this method was considered to induce less thermal elevation than autoclaving and dry heating, resulting in the maintenance of the bioactivity of the recombinant protein fused to the fibroin L-chain. No contamination was observed in cell culture experiments with the fibroin sponges, therefore the EB irradiation is suggested to be a practical way to sterilize fibroin sponges containing bioactive factors.

We have not just successfully prepared a coating material, but a bulky fibroin sponge, with genetically fused bioactive bFGF. As the growth factor has been reported to promote the regeneration of various tissues, such as bone, peripheral nerve, and cardiac muscle, the bFGF-fused fibroin sponge has the potential for widespread application in the field of tissue engineering. Of especial note, the fibroin sponge would be applicable to tissues exposed to mechanical stress. This is because, in contrast to hydorgels containing bFGF, the bFGF-fused fibroin sponge shows high mechanical properties, without the use of a cross-linker like glutaraldehyde. We are now investigating the application of the bFGF-fused fibroin sponge as a useful scaffold for tissue regeneration.

**CONCLUSIONS**

The results of the present study demonstrated that bioactive recombinant bFGF was produced in a transgenic silkworm strain, fused to the fibroin L-chain, and its activity (e.g., cell-growth promotion) was maintained, even after the bFGF-fused fibroin was fabricated into a spongy structure. The physicochemical properties of fibroin sponges were unaffected by the introduction of the bFGF-fused fibroin. Because bFGF is known to promote the regeneration of various tissues, the fibroin sponge is expected to be applicable to tissue engineering. This study also found a method for preparing fibroin sponges that allows for the maintenance of fusion-protein functions.

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