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Advanced silk material spun by a transgenic silkworm promotes cell proliferation for biomedical application

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ABSTRACT

Natural silk fiber spun by the silkworm *Bombyx mori* is widely used not only for textile materials, but also for biofunctional materials. In the present study, we genetically engineered an advanced silk material, named hSFSV, using a transgenic silkworm, in which the recombinant human acidic fibroblast growth factor (hFGF1) protein was specifically synthesized in the middle silk gland and secreted into the sericin layer to surround the silk fiber using our previously optimized sericin1 expression system. The content of the recombinant hFGF1 in the hSFSV silk was estimated to be approximate 0.07% of the cocoon shell weight. The mechanical properties of hSFSV raw silk fiber were enhanced slightly compared to those of the wild-type raw silk fiber, probably due to the presence of the recombinant of hFGF1 in the sericin layer. Remarkably, the hSFSV raw silk significantly stimulated the cell growth and proliferation of NIH/3T3 mouse embryonic fibroblast cells, suggesting that the mitogenic activity of recombinant hFGF1 was well maintained and functioned in the sericin layer of hSFSV raw silk. These results show that the genetically engineered raw silk hSFSV could be used directly as a fine biomedical material for mass application. In addition, the strategy whereby functional recombinant proteins are expressed in the sericin layer of silk might be used to create more genetically engineered silks with various biofunctions and applications.

1. Introduction

The spun silk of the silkworm *Bombyx mori* is a natural fiber which is composed mainly of the proteins fibroin and sericin [1]. Fibroin constitutes the core of the silk fiber, while the glue-like sericin surrounds the fibroin core to form the outer layer of silk. Fibroin and sericin account for 75 and 25% of the weight of the silk, respectively [2]. Fibroin is a hydrophobic fibrous protein consisting of fibroin heavy chain (Fib-H), fibroin light chain (Fib-L) and fibrohexamerin (P25), with molecular masses of 370, 25 and 25 kDa, respectively [3,4]. Fib-H and Fib-L are covalently linked by S–S bonds and are associated with P25 by noncovalent interactions, forming an elementary unit that contributes to the secretion of fibroin into the lumen of the larval posterior silk gland [5]. Sericin, which is mainly encoded by *sericin* 1, *sericin* 2 and *sericin* 3 genes [6–8], is a hydrophilic protein that glues the fibroin threads together to form the cocoon [9].

Silk fibers have traditionally been used to make textiles. In addition, silk fiber has shown such advantageous properties as biocompatibility [10], material versatility [11], mechanical robustness [12] and controllable degradability [13], and has thus been widely utilized as a biomedical material to make surgical sutures [14], vascular grafts [15], drug delivery carriers and tissue engineering scaffolds [16]. Sericin, which was once removed as waste during silk degumming, has been found to be a suitable material for biological applications due to its various biological properties, such as biocompatibility, UV protective property, antibacterial activity, antioxidant, anti-tyrosinase activity and coagulant and moisturizing capabilities [17]. Furthermore, sericin had also been reported to stimulate cell migration through up-regulation and phosphorylation of c-Jun [18], and has been proven to cause no inflammation [19,20]. Sericin itself can form hydrogels, gel films and sponges, which make it suitable for use in biomedical applications, e.g. as a wound dressing [21,22]. When blended with other polymers, sericin can form a membrane or scaffold with improved

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characteristics that could be used for cosmetic and pharmaceutical applications [17,23]. In addition, sericin can be used as an improving reagent or coating material to improve the functional properties of a number of natural and synthetic fibers, fabrics and articles [17,24]. The applications of these silk materials mainly rely on the exploitation of the inherent properties of silk proteins. Further expanding the applications of silk will depend on the de novo engineering of silk with specific biological functions.

Genetic technology to generate transgenic silkworms by the *piggyBac* transposon derived from the lepidopteran *Trichoplusia ni* has been successfully established and is widely used for fundamental and applied research into the silkworm [25,26]. Various transgenic expression systems based on the usage of *fib*-H, *fib*-L, P25 and *sericin* 1 promoters have been established for the production of exogenous proteins in the silk of transgenic silkworms [27,28]. These transgenic resources make it possible to create genetically modified silk materials with improved mechanical properties and new biofunctionalities. For example, a modified chimeric silkworm/spider recombinant silk spun by transgenic silkworms using a *fib*-H promoter-derived transgenic construct was reported to be significantly tougher than ordinary silkworm fiber [29]. A colored fluorescent silk was made for textile application by fusion expression of three different fluorescent proteins with fibroin using *fib*-H promoter-originated transgenic vectors [30]. A fibroin solution-based material in which is contained a fusion protein of fibroin L chain and human basic fibroblast growth factor (bFGF) was reported to be biologically active as a new biomaterial for tissue engineering after refolding by the glutathione redox system [31]. Silk film containing collagen or fibronectin-derived peptides was made by transgenic silkworms and has been shown to have improved cell-adhesive properties [32]. These genetically engineered silk materials have greatly expanded the application of silk in biotechnological fields.

Since the fusion proteins of fibroin and functional foreign proteins are distributed in the core of silk fibers, the activity of which is barely exerted in the natural form of silks, application of these silks usually requires them to be remodeled into different forms, such as films, gels and sponges, to function properly [33]. To achieve this, a series of treatments, such as the removal of sericin, the dissolution of fibroin and a refolding process to recover the bioactivity of the functional protein, are involved [31]. These procedures are complicated and time-consuming, which greatly limits the large-scale application of these functional silk materials. Thus, it is important to create a new functional silk that can be directly and conveniently applied for large-scale production of textiles or biomedical materials without remodeling and processing the silk.

This study aims to genetically engineer a silk which could be directly used as a biomedical material for tissue engineering. Human acidic fibroblast growth factor (hFGF1), which belongs to the FGF protein family, is a heparin-binding protein [34]. It participates in a wide range of biological processes [35], and has potential for clinical applications in wound healing [36] and the treatment of ischemia via its mitogenic activity [37,38]. We generated a transgenic silkworm hSFSV which could specifically synthesize the recombinant hFGF1 proteins in the middle silk gland (MSG) of silkworms and spin them into the sericin layer of silk using our previously established sericin1 expression system [27]. The hSFSV raw silk was directly used to culture NIH/3T3 cells and was found to be biologically active in that it significantly promoted the proliferation of the NIH/3T3 cells. This genetically engineered silk could be used as a fine functional material for biomedical applications and this work provides a new strategy to create such genetically engineered silk for tissue engineering and biomedical applications.

2. Materials and methods

2.1. Cell lines and silkworm strains

The mouse embryonic fibroblast cell line NIH/3T3 was purchased and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10 vol.% fetal bovine serum (FBS; Gibco) at 37 °C in a 5% CO_2 atmosphere. The Dazao strain silkworm embryos were maintained in our laboratory.

2.2. Vector construction

The optimized hFGF1 gene with a silkworm codon bias fusing a His_6 tag in the C terminus was synthesized commercially (by GenScript) and inserted into an intermediate vector pSL1180{hSer1spDsRedSv40} [27] by *Bam*HI and *Not*I to replace the *DsRed*, then the open reading frame (ORF) of the resulting vector was released by *Asc*I and inserted into the pBac{3xp3EGFPaf} [39] basic transgenic vector cut by the same *Asc*I to generate the final transgenic vector phShFGF1Sv40.

2.3. Generation of transgenic silkworms

Transgenic silkworms were constructed by a previously reported method [25]. Briefly, the plasmid of phShFGF1Sv40 was purified using the Plasmid Mini kit (Qiagen) and microinjected (Eppendorf, Germany) into preblastoderm embryos of the Dazao strain silkworm with a pHA3PIG helper [25]. Hatched larvae were reared to moths and mated to induce oviposition. The G1 eggs at the body pigmentation stage were screened for expression of 3xp3EGFP in eyes using an Olympus SZX12 fluorescence stereomicroscope (Olympus). The G1-positive eggs were reared and backcrossed with wild-type (WT) Dazao silkworms to generate stable transgenic silkworms.

2.4. Histological examination

Samples for histological examination were prepared as described previously [40]. The MSGs and cocoons of silkworms were fixed overnight with 10 vol.% formalin, frozen in Tissue-Tek® O.C.T.™ compound (Sakura Finetechnical Co., Ltd.) and cut into 10 μm thick sections. The sections were immunoblotted with rabbit anti-FGF1 polyclonal antibody (BioVision), detected with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Beyotime) and observed under a fluorescence microscope (Nikon).

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Fresh MSG (~1 g) of silkworm larvae on the 6th day of the 5th instar were cut into small sections and dissolved in 1 ml of phosphate-buffered saline (PBS, pH 7.0) at 4 °C overnight. Cocoons were shattered into powder and added to 50 mM Tris–HCl and 8 M urea, pH 7.0, at a final concentration of 30 mg ml^{-1} overnight at 4 °C. The supernatant samples were collected by centrifugation at 18,000 rpm for 5 min, then 20 μl each of MSG and cocoon extracted protein samples were analyzed by SDS-PAGE (15% (w/v) polyacrylamide gel) and stained with Coomassie brilliant blue R250. For immunoblots, 5 μl of each sample together with an hFGF1 standard (Biovision) was subjected to SDS-PAGE (15% (w/v) polyacrylamide gel) and transferred electrophoretically onto a polyvinylidene fluoride membrane, immunoreacted with anti-hFGF1 antibody (Abcam) and visualized by ECL plus (Amersham Biosciences). The images were recorded using a Chemiscope Series (Clinx Science Instruments). The content of recombinant hFGF1