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Investigation of osteoblast-like MC3T3-E1 cells on a novel recombinant collagen-like protein surface with triple helix structure

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Abstract Fol-8Col is a novel recombinant collagen-like protein incorporated with foldon sequences derived from the native T4 phage fibritin. In this paper, we examined the potential of using Fol-8Col as scaffold for bone tissue engineering. Circular dichroism (CD) spectra indicate that the triple helix structure of Fol-8Col exists at temperatures ranging from 4 to 40 °C. Lactate dehydrogenase assay results and live/death cell staining of osteoblast-like MC3T3-E1 cells, cultivated on Fol-8Col for 24 h, showed evidence of cell cytocompatibility comparable to that of native type I collagen. Attachment and spreading of osteoblast-like MC3T3-E1 cells seeded on Fol-8Col were studied by immunofluorescence staining of cell nuclei, vinculin, and F-actin. Intensive focal adhesion patches and an elongated cortical actin cytoskeleton were observed after 24 hours' cultivation. Proliferation assays of MC3T3-E1 cells cultivated on Fol-8Col for 2 weeks revealed no consistent differences in rate and pattern compared to growth on type I collagen. Alkaline phosphatase activity assay and osteogenic gene expression, detected by RT-PCR, evaluated the osteogenic differentiation of MC3T3-E1 cells on Fol-8Col. This study shows that Fol-8Col, with a triple helix structure, has good potential for application in bone regeneration as a replacement for native collagen, thereby reducing the risk of contamination.

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Introduction

Bone, dentin, and cementum formation involve the apatite nucleation of collagenous matrix, through a special interaction with other collagen-interactive acidic phosphoproteins, secreted by osteoblasts, odontoblasts, and cementoblasts, respectively [1, 2]. The majority of the extracellular matrix (ECM) is composed of collagen. The function of collagen fibers as mechanical reinforcements of connective tissues is determined by the architectural structure of collagen. Collagen fibrils (mostly Type II collagen) in cartilage matrix form a random, loose network while tendon is composed of highly ordered ECM, in which tens of millions of collagen fibrils are synthesized parallel to the tendon long axis [3, 4]. Resultant research suggested that the reticular dermis processes approximately uniaxial orientation of the collagen fibers [5]. Various collagen-based scaffolds have been developed and functionalized for tissue engineering, owing to their capacity to modulate tissue growth [6–8]. However, considering the risk of contracting viruses and other infectious agents from collagen isolated from an animal source, recombinant collagen-like proteins are safe, mechanically stable, economically viable, biocompatible alternatives, with the potential for functionalization with bioactive groups [9, 10].

In previous work, we synthesized a recombinant collagen-like protein from *Escherichia coli*, named Fol-8Col, which has numerous advantages for cell growth, including

rapid proliferation, high-level protein expression, simple media requirements, and cost-effectiveness [11]. Results showed that the C-terminal incorporated foldon sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL, derived from the phage T4 fibritin, is effective in forming the triple helix structure of Fol-8Col, thus increasing the thermal denaturation temperature by 7 °C over those without the sequence. Furthermore, with incorporation of the RGD (Arg-Gly-Asp) triplet, which has been shown to be effective for improving cell adhesion, initial response of fibroblasts to Fol-8Col was found to be higher than that of native collagen [11]. Based on previous designs, including the preparation and characterization of Fol-8Col, the focus of this work was mainly to investigate its potential for use as a structural matrix in bone regeneration. We believe that the acidic amino acids (Glu, Asp, Asn) in Fol-8Col may contribute to promoting nucleation activity in de novo biomineralization of bone [11].

Recent studies have shown the role of apatite–collagen composites in the formation of biominerals in the human and animal body. The ordering in favor of an apatite crystal structure was shown to be predominantly induced by the triple helix of tropocollagen [12]. In this work, we confirm the stability of the triple helix structure in Fol-8Col at different temperatures, ranging from 4 to 40 °C.

The mouse osteoblast-like MC3T3-E1 cell line was selected for cellular investigations as morphologically, it is a well-characterized model. Osteoblasts play an important role in the regulation of bone metabolism during osteogenesis, differentiation, and normal physiology [13]. Furthermore, MC3T3-E1 has a high level of alkaline phosphatase (ALP) activity, which is one of the most well-known markers of osteoblast differentiation [14]. In this study, we conducted preliminary investigations on the viability, attachment, proliferation, and differentiation of MC3T3-E1 cells on Fol-8Col matrix, to evaluate the potential of Fol-8Col for application in bone tissue engineering.

Materials and methods

Materials

In our previous work, Fol-8Col was designed based on amino acid sequence (GPRGDNGPAGSVGPTGDTGPEG FRGDSGPHGEAGAS)$_8$(GYIPEAPRDGQAYVRKDGEW VLLSTFL), where (GYIPEAPRDGQAYVRKDGEWVLL STFL) in the C-terminus is a T4 phage fibritin-derived foldon sequence [11]. Batch cultures were performed in a BioFlo 110 fermentor (New Brunswick Scientific Ltd, USA) at 37 °C, using enriched medium (TB, Novagen, Germany) containing 0.8 mmol isopropyl-thio-2-D-galactopyranoside (IPTG), for the induction of protein expression. The recombinant protein was purified under native conditions by immobilized nickel chelate affinity chromatography, selective for the $[His]_6$ sequences located in the N- and C-terminal sequences [11]. The yield of purified Fol-8Col was 90 ± 10 mg/L in TB medium. Purified Fol-8Col was loaded onto a 12% polyacrylamide gel for SDS-PAGE, following the method of Laemmli [15].

Native type I collagen (Bovine placenta) was purchased from Funakoshi Co. (Tokyo, Japan). MC3T3-E1 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). All other chemicals were analytical grade reagents purchased from Wako (Japan), unless otherwise mentioned.

Circular dichroism (CD) measurements

Circular dichroism (CD) spectrum data of Fol-8Col was recorded on a J-600 (JASCO Corporation, Japan) spectropolarimeter. The instrument settings were as follows: scan range 200–250 nm; scan speed 50 nm/min; quartz cell length 10 mm; sensitivity 5 mdeg; response 0.5 s; resolution 0.1 nm; accumulation times 4; temperature 4, 10, 20, and 40 °C. Fol-8Col was prepared in 17 mM phosphate-buffered saline (PBS).

Preparation of Fol-8Col-coated dishes

The preparation of Fol-8Col- and type I collagen-coated culture dishes were based on a cross-linking procedure using water-soluble carbodiimide (WSC) [16]. 150 μL of 0.1% Fol-8Col was pipetted and spread evenly over the bottom of a 12-well tissue culture dish (TCD; Thermo Fisher Scientific, Denmark). Dishes were left half covered, without ventilation at room temperature overnight. After crosslinking with 150 μL WSC (1%) at 4 °C overnight, the coated surface was carefully rinsed twice with double distilled water. The plates were then examined to ensure that the protein layer was a thin flat surface, without any cracks or breaks, prior to cell cultivation.

Cell culture

MC3T3-E1 cells, being cultured on dishes prepared as shown in the above section, were used to test cell viability in vitro. Dishes were incubated at 37 °C, in a 5% CO_2 atmosphere incubator, using α-modified minimal essential medium (α-MEM; GIBCO). The medium comprised of 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. For all cell investigations, MC3T3-E1 cells cultured on type I collagen and TCDs were evaluated as controls. TCDs are composed of optically clear, high-grade polystyrene Nunc™ Dishes, prepared using a vacuum-gas plasma sterilization treatment. The incorporation of nitrogen-containing cations

on the surface of the dishes has been correlated with the attachment and spreading of primary endothelial cells in a clonal cell-growth assay [17].

Cytotoxicity assay and live/dead cell staining

Cells were seeded (2.5×10^4 cells/well) on coated dishes, as mentioned in "Preparation of Fol-8Col-coated dishes" section and cultivated for 24 h for the quantitative analysis of cell death, using lactate dehydrogenase (LDH) assay. LDH activity is measured by determining the concentration of LDH liberated from cells after chemically lysing the cell membrane, using a LDH-Cytotoxicity Kit (Wako Pure Chemical Industries Ltd).

Cell live/dead staining was performed to determine the number of viable and non-viable MC3TC-E1 cells, using a Cellstain-Double Staining Kit (Dojindo Laboratories, Japan). After 24 hours' cultivation on Fol-8Col- and native type I collagen-coated dishes, MC3TC-E1 cells were washed thoroughly with PBS and stained with PBS containing 2 μM Calcein AM and 4 μM propidium iodide (PI), for 15 min at 37 °C. Calcein AM reacts with intracellular esterase to produce green fluorescence at 490 nm, while PI enters only dead cells with damaged membranes to produce red fluorescence at 545 nm, upon binding to nucleic acids [18]. Digital images of viable (green) and dead (red) cells in selected areas were visualized using a Zeiss Axio Imager M1 fluorescence microscope, equipped with AxioCam MRm (Carl Zeiss MicroImaging GmbH, Germany).

Cell attachment

Cells were seeded and cultured as under the same conditions as in "Cytotoxicity assay and live/dead cell staining" section. Cells in culture medium were counted (Nm) after 1, 3, 6, and 24 h of incubation. The cell adhesion ratio for each condition was calculated using the following equation:

$$\text{Adhesion ratio } (\%) = \left(1 - \text{Nm}/2.5 \times 10^4\right) \times 100$$

All data reported were the mean of three examinations.

Immunocytofluorescence staining for nuclei, vinculin, and filamentous actin

After seeding and 24 hours' cultivation as described in "Cell attachment" section, samples being used for fluorescent staining were fixed with acetone for 40 min at −20 °C, followed by washing twice with 0.1 M PBS. Samples were blocked with 10% bovine serum albumin (BSA) for 30 min to reduce non-specific background staining. Immunostaining for vinculin was performed using a monoclonal mouse anti-vinculin IgG1 primary antibody (1:400 dilution; Sigma-Aldrich, USA) and a goat anti-mouse IgG (H + L) Alexa Fluor 568-conjugated secondary antibody (1:800 dilution; Molecular Probes Europe BV, The Netherlands). The cell cytoskeleton, F-actin was labeled with 5 U/mL fluorescein phalloidin (a high-affinity probe for F-actin that is made from a mushroom toxin conjugated to the green-fluorescent dye, Fluorescein isothiocyanate, Molecular Probes Europe BV), for 1 h at room temperature. Finally, cells were mounted for 1 h at room temperature, using a DAPI (4′,6-diamidino-2-phenylindole, Dojindo Molecular Technologies, Japan) nuclear stain. Staining was visualized using a Zeiss Axio Imager M1 fluorescence microscope equipped with AxioCam MRm. Alexa-568 produces red fluorescence at 568 nm; FITC produces green fluorescence at 490 nm; and DAPI produces blue fluorescence at 345 nm.

Proliferation assays

MC3T3-E1 cells were seeded (2×10^4 cells/well) on coated dishes prepared as described in "Preparation of Fol-8Col-coated dishes" section. After incubation for 1, 3, 5, 7, 10, and 14 days, 10 μL of TetraColor ONE reagent containing tetrazolium monosodium salt (Seikagaku Corporation, Japan) was added to each well and cells were incubated for an additional 2 h. Absorbance at 450 nm was measured using a Biotrack II plate reader.

Assessment of differentiation

The level of cell differentiation on Fol-8Col films was assessed by determining the level of ALP activity. Cells were seeded and cultured as described in "Proliferation assays" section. α-MEM, supplemented with 0.1% β-glycerophosphate, was used to induce osteoblastic differentiation. ALP activity was measured after 3, 5, 7, 10, 14, and 21 days cultivation. 500 μL of β-nitrophenyl phosphate solution containing 1 mM $MgCl_2$ (Sigma, USA) was added to the medium and incubated for 10 min at 37 °C. The enzymatic reaction was stopped by adding 500 μL of 0.2 N NaOH. Finally, the absorbance was read at 405 nm.

Reverse transcription PCR

Expressions of the osteoblastic markers, Runt-related transcription factor 2 (Runx2), type I collagen (ColI), alkaline phosphatase (ALP), osteopontin (OP), bone sialoprotein (BSP), and osteocalcin (OC) were evaluated using RT-PCR analysis. After 21 days culturing as described in "Assessment of differentiation" section, confluent MC3T3-E1 cell monolayers were washed with PBS and total RNA was isolated using a RNA-isolation kit (Isogen, Molecular Research Center, Inc). Reverse

Table 1 Oligonucleotide primer sequences and PCR conditions for regular RT-PCR

Gene, fragment size (bp)	Primer sequence (5′ to 3′)	PCR conditions			
		Denaturation	Annealing	Elongation	Cycles
Runx2 (487)	F: GCAGTGCCCCGATTGAGG	94 °C, 30 s	57 °C, 60 s	72 °C, 90 s	35
	R: CATACTGGGATGAGGAATGCG				
ColI (269)	F: TCTCCACTCTTCTAGTTCCT	94 °C, 30 s	55 °C, 60 s	72 °C, 60 s	20
	R: TTGGGTCATTTCCACATGC				
ALP (372)	F: GCCCTCTCCAAGACATATA	94 °C, 30 s	55 °C, 60 s	72 °C, 60 s	20
	R: CCATGATCACGTCGATATCC				
OP (240)	F: ACACTTTCACTCCAATCGTCC	94 °C, 30 s	55 °C, 60 s	72 °C, 60 s	20
	R: TGCCCTTTCCGTTGTTGTCC				
BSP (384)	F: CTCGGGTGTAACAGCTAGCTAC	94 °C, 30 s	57 °C, 60 s	72 °C, 90 s	35
	R: CGTTCAGAAGGACAGCTGTCTG				
OC (371)	F: CAAGTCCCACACAGCAGCTT	94 °C, 30 s	55 °C, 60 s	72 °C, 60 s	22
	R: AAAGCCGAGCTGCCAGAGTT				
GAPDH (267)	F: ACTTTGTCAAGCTCATTTCC	94 °C, 30 s	55 °C, 45 s	72 °C, 60 s	25
	R: TGCAGCGAACTTTATTGATG				

transcription of isolated mRNA was performed using Omniscript RT Kit (QIAGEN), under the following reaction conditions: reverse transcriptase reaction (one cycle) 37 °C for 60 min, followed by 93 °C for 5 min. PCR amplification of Runx2, ColI, ALP, OP, BSP, OC, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; internal control) cDNA was conducted using Taq PCR CoreKit (QIAGEN) in a PCR-Thermo cycler (Gene Amp, PCR System 9700, Applied Biosystems, USA), with gene-specific primers and reaction conditions as shown in Table 1. The reaction products were analyzed by electrophoresis of 10 μL samples in 2% agarose gels. The amplified DNA fragments were stained with ethidium bromide and photographed under UV illumination (AE-6933 FXCF, ATTO Corporation, Japan). The experiment was performed in triplicate for each sample.

Statistical analysis

The data collected were expressed as mean ± standard deviation (S.D). The two-tailed Student's *T*-test (*T*-test) was employed to obtain *p* values, enabling determination of the level of significance of the data. *p*-values of less than 0.05 ($p < 0.05$) were considered to be of significant difference.

Results

Expression and purification of Fol-8Col in *E. coli*

Figure 1 shows the SDS-PAGE analysis of the recombinant protein dissolved in PBS. A single band located around 90 kDa (noted as Fol-8Col) was observed. The molecular weight of single chain Fol-8Col, calculated from the amino acid sequences $(GPRGDNGPAGSVGPTGDTGPEGFRGDSGPHGEAGAS)_8$(GYIPEAPRDGQAYVRKDGEWVLLSTFL), is approximately 33 kDa, with the triple helix structure of Fol-8Col giving a molecular weight of approximately 100 kDa. However, heat denaturation

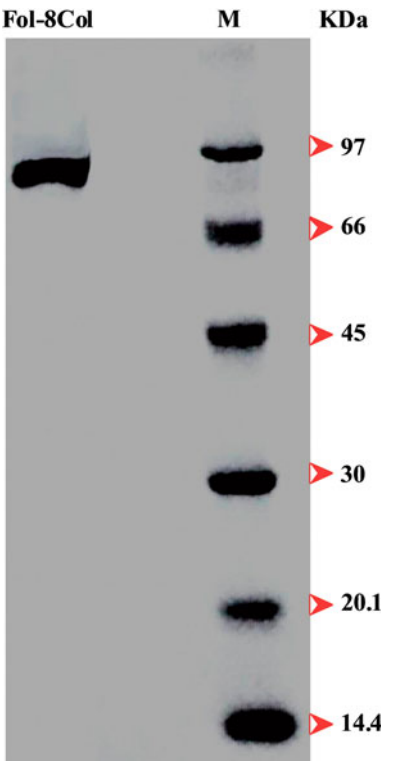

Fig. 1 SDS-PAGE results of the recombinant collagen-like protein, Fol-8Col, after purification by Nickel chelate chromatography

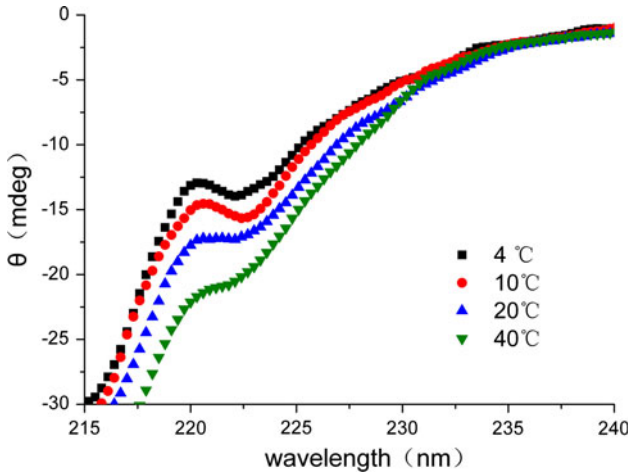

Fig. 2 CD spectra of Fol-8Col in PBS (pH 7.8) at different temperatures. Spectra were recorded at a protein concentration of 17 μM

during SDS-PAGE possibly leads to a certain degree of thermal degradation resulting in a band near 90 kDa.

CD spectra of Fol-8Col

Figure 2 shows the far UV spectra of Fol-8Col at a concentration of 17 mM in PBS at 4, 10, 20, and 40 °C. The results show characteristic peaks of collagen-like peptides at 220 nm in a triple helix state [9]. Although the molecular weight of Fol-8Col is significantly lower than that of native type I collagen, an obvious peak could still be observed with temperatures as high as 40 °C. The triple helix structure is more stable at low temperatures, as can be seen from the peak intensity at 220 nm, indicating that Fol-8Col may undergo a more significant thermal unfolding with temperature increases above 40 °C.

Cytotoxicity assay

Lactate dehydrogenase (LDH) leakage assay results in Fig. 3-I suggest that cell culturing on Fol-8Col causes a 7% LDH release while the LDH release is nearly 5% on type I collagen. These data are consistent with those from previous studies indicating that the concentration of type I collagen does not affect cell viability, where a maximum of 7% cell death was observed [19].

From the live/dead fluorescence micrographs in Fig. 3-II, the majority of cells incubated for 24 h on Fol-8Col (a), type I collagen (b), and TCD (c) were alive and revealed polygonal-shaped morphology. This figure also semi-qualitatively shows more than 90% viable MC3T3-E1 cells attached to Fol-8Col after 24 hours' cultivation, with 7% LDH release. Cytotoxicity assays indicate that MC3T3-E1 cells have comparable viability on Fol-8Col matrix as on native type I collagen.

Adhesion ratio and cell morphology

The adhesion ratio of MC3T3-E1 cells on Fol-8Col is shown in Fig. 4-I. Although after 6 h, the cell adhesion

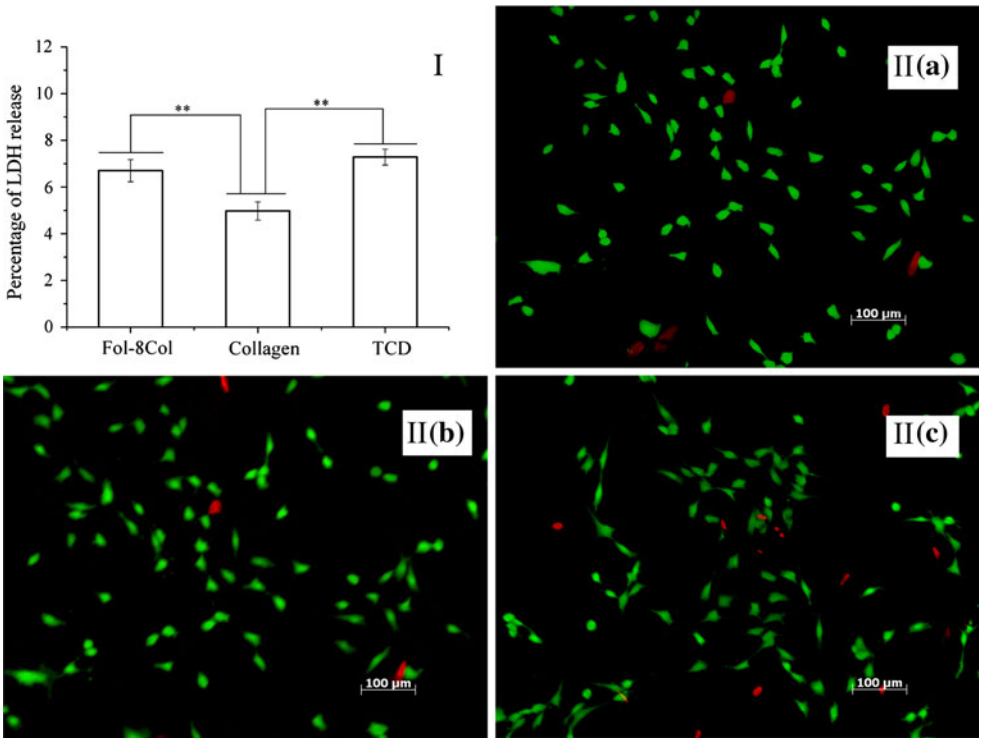

Fig. 3 **I** % LDH release and **II** fluorescence micrographs of Calcein AM/PI-stained MC3TC-E1 cells with live cells fluorescing *green* and dead cells fluorescing *red* after 24 h culture on the Fol-8Col- (**a**), native type I collagen (**b**)-coated dishes, and TCDs (**c**) as a control. Significant difference between different material groups were denoted as * ($p < 0.05$), ** ($p < 0.02$) (Color figure online)

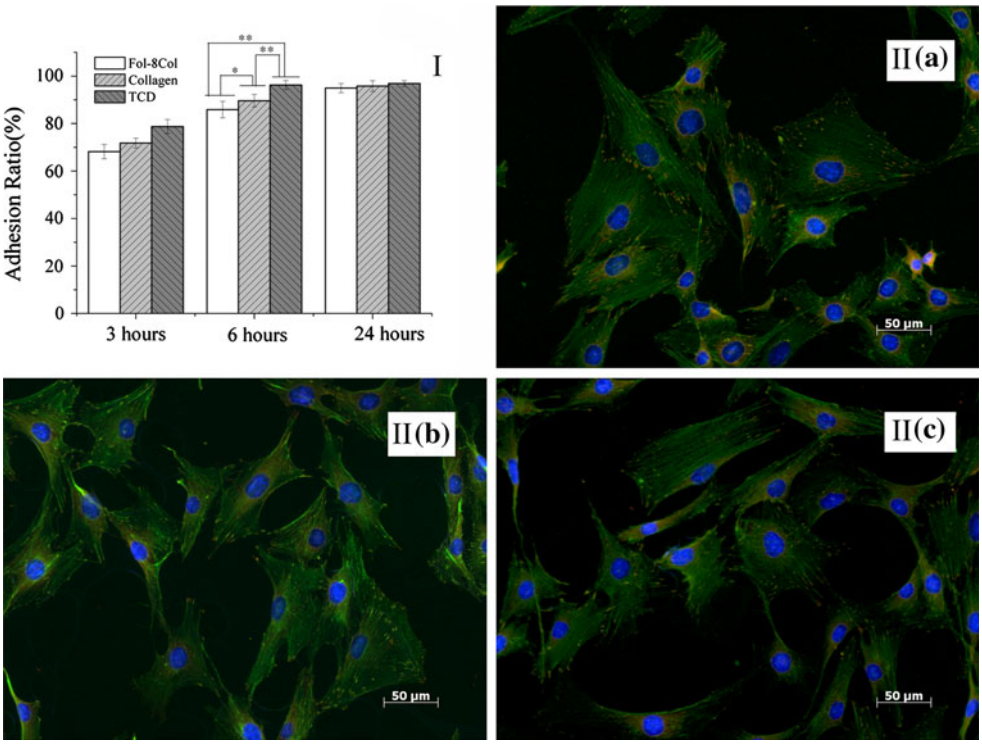

Fig. 4 **I** The adhesion ratio and **II** fluorescent staining of F-actin (*green*), vinculin (*red*), and cell nuclei (*blue*) for MC3T3-E1 cells after 1 day culture on Fol-8Col (**a**), native type I collagen (**b**), and TCD (**c**). Significant difference between different material groups were denoted as * ($p < 0.05$), ** ($p < 0.02$) (Color figure online)

ratio on Fol-8Col-coated dishes (85.9 ± 3.4%) was slightly lower than that on collagen (89.6 ± 2.6%), they reached as high as 100% after 24 hours' cultivation without difference. Immunofluorescence microscopy of polygonal elongated MC3T3-E1 cells grown on Fol-8Col (a), native type I collagen-coated (b) dishes, and TCDs (c) are shown in Fig. 4-II. Blue fluorescence of cell nuclei revealed round, well-spaced, and regularly distributed nuclei across the surface of the culture dishes. Immunofluorescence of F-actin, displaying cytoskeletal organization (green), demonstrates the presence of stress fibers composed of actin filaments. These cortical filament bundles are seen at the cell periphery, oriented in a parallel direction following the main cellular axis. MC3T3-E1 cells on Fol-8Col show intensive vinculin signals along extended actin microfilaments, with the highest concentration seen at the extremities of cellular extensions, resulting in focal adhesion patches.

Proliferation assay

Figure 5 shows the results of the proliferation assay. After 5 days, cells on Fol-8Col are significantly higher than on type I collagen. Cells on all samples were fully confluent after 7 days of culturing, suggesting that Fol-8Col-coated film is conducive for cell proliferation, which is comparable to type I collagen.

ALP activity

Alkaline phosphatase (ALP)-hydrolyzed phosphate esters play an essential role in the initiation of the cell differentiation process [20]. Thus, ALP activity is a marker of osteoblastic activity and a standard to evaluate the differentiation of MC3TC-E1 cells. Figure 6-I suggests that after 7 days, when the proliferation of cells on Fol-8Col reached its highest level as shown in Fig. 5, ALP activity begins to

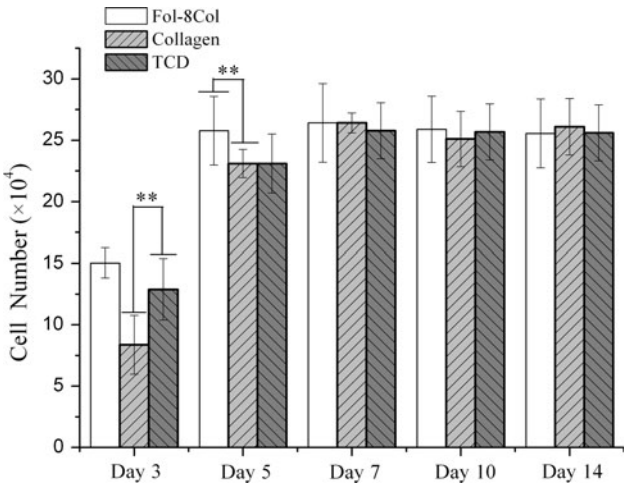

Fig. 5 Proliferation analysis of MC3T3-E1 cells after 14 days of culture on Fol-8Col, native type I collagen and TCD. Significant difference between different material groups were denoted as * ($p < 0.05$), ** ($p < 0.02$)

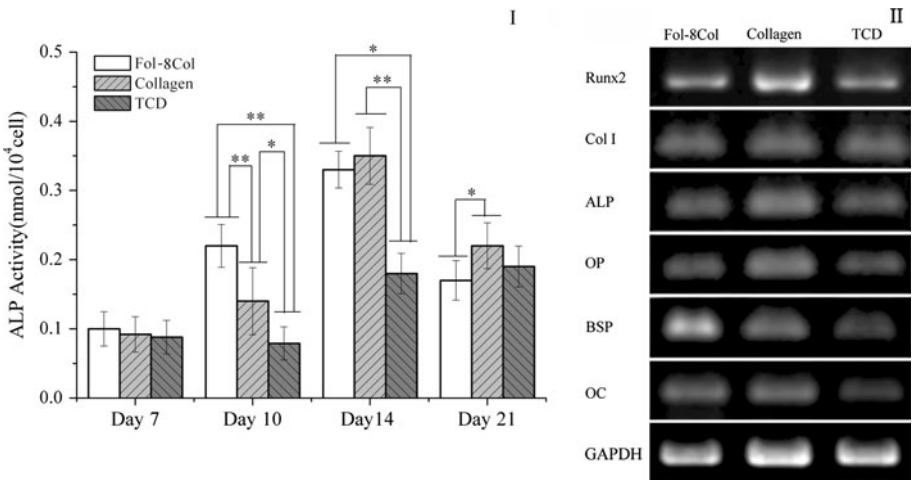

Fig. 6 **I** ALP activity and **II** expression of mouse Runx2, ColI, ALP, OP, BSP, and OC mRNA of MC3T3-E1 cells after 21 days culture on Fol-8Col, native type I collagen and TCD. Significant difference between different material groups were denoted as * ($p < 0.05$), ** ($p < 0.02$)

increase until reaching its highest level after 14 days of cultivation. The differentiation rate of MC3T3-E1 cells on Fol-8Col is significantly higher than that on collagen after 10 days of cultivation although it turned to be lower than the latter after 14 days of cultivation.

RT-PCR of osteoblastic genes

Figure 6-II shows 2% agarose electrophoresis results of MC3T3-E1 cells expressing the osteoblastic genes Runx2, ColI, ALP, OP, BSP, and OC after 21 days incubation on Fol-8Col. Since these genes have been reported to be necessary for osteoblast differentiation [21], the results are consistent with the ALP activity results in Fig. 6-I. Runx2 is a downstream osteogenic master transcription factor, which in turn works sequentially with/without bone marker genes to induce their expression, thereby representing transdifferentiation [22]. ALP and ColI represent the beginning of osteoblastic differentiation, as once the matrix synthesis begins in MC3T3-E1 cells, the cells differentiate in accordance with the activation of osteoblast marker genes, such as ALP and type I collagen [23]. The expression of osteocalcin and bone sialoprotein genes, as the typical osteoblastic phenotype, was initiated only after cells reached the mineralized tissue-formation stage, thus marking the different stages of osteoblast maturation [24, 25]. By participating in the nucleation of hydroxyapatite, osteopontin, a phosphorylated glycoprotein, is secreted by osteoblasts into the mineralizing ECM during bone development [26]. Osteocalcin is a late stage marker of bone cell differentiation, closely related to osteoblast maturation. Furthermore, its association with bone mineralization correlates with its ability to bind calcium and hydroxyapatite [27]. GAPDH was used as an internal control [28].

Type I collagen and ALP mRNA expression levels of MC3T3-E1 cells on Fol-8Col are as effective as those on type I collagen. Together with the results of ALP activity in Fig. 6-I, this shows that Fol-8Col has similar osteoblastic properties to type I collagen as a cell culture coating. Strong mRNA expression of BSP observed after 3 weeks' cultivation on Fol-8Col suggests that fully differentiated osteoblasts are associated with the initial stages of bone formation. This shows that Fol-8Col has a comparable capability to support the cell differentiation as native type I collagen.

Discussion

The incorporation of foldon sequence to stabilize the triple helix of Fol-8Col expressed in *E. coli*

Compared with the majority of self-assembling peptides in use which have been produced through chemical synthesis, recombinant proteins produced in various expression systems have the advantage of providing sustainable sources of biomaterials [10]. Nokelainen et al. [29] have developed a yeast *Pichia pastoris* expression system which can probably be optimized for high-level production of recombinant human type I collagen for numerous medical applications. Nevertheless, limitation of biological peptide production is using the 20 naturally occurring amino acids. Moreover, recombinant expression of collagens is often difficult, as specific post-translational enzymes, in particular prolyl 4-hydroxylase, are required for the biosynthesis of Hyp (hydroxyproline)—a major contributor to triple helix stability in animal collagens [30]. Thus, the prokaryotic expression systems, e.g., the valuable *E. coli* organism, lack prolyl 4-hydroxylase that makes more arduous their recombinant collagen production with triple helix structure. In recent years, frontier research has focused on clarifying the mechanism of stabilization of collagen triple helix in the absence of Hyp [31, 32]. A novel approach to the production of hydroxylated collagen

not involving P4H co-expression was achieved by supplementing the cultures with hydroxyproline while growing the cells in hyperosmotic media [33].

In order to make the most of bacterium *E. coli* expression system which has advantages of rapid cell growth, high-level protein expression, simple media, and low cost, our previous research successfully produced Fol-8Col with yield of 90 mg/L culture in *E. coli* [11]. Fol-8Col was incorporated with a C-terminal foldon sequence derived from the phage T4 fibritin to stabilize the triple helix. CD spectra of Fol-8Col in this study indicate that the triple helix structure of Fol-8Col exists at temperatures near 40 °C. Previous studies suggest that the excellent biocompatibility of type I collagen is due to its triple helix structure which specifically binds to the integrin receptors of cells [34]. This approach to synthesize collagen-like protein in *E. coli* by incorporating foldon sequence reported here might open a new avenue to design functional recombinant collagen-like protein with stable triple helix and other helical fibrous protein in prokaryotic expression system in the absence of prolyl 4-hydroxylase.

RGD binding sequence: biologically active motif for osteoblastic application

During the last decades, artificial self-assembling peptide-based biomaterials are being developed for therapeutic drug-release application and tissue engineering scaffolds. However, most of these have exploited are chemically synthesized peptide [10]. For example, Horii et al. [35] designed a class of peptide nanofiber scaffolds based on RADA16 (the first commercially available self-assembling peptide) for osteoblasts application. As for biologically recombinant collagen-like protein, Peng et al. [36] examined the cytotoxicity and immunogenicity of the collagen-like domain from *Streptococcus pyogenes* Scl12 protein which shows potential for tissue engineering as a scaffold. A major advantage of recombinant collagen-like protein for biomaterial application is the ability to easily modify the expressed construct, to introduce desirable biological activities within or adjacent to the triple helix protein. Yanagisawa et al. produced genetically modified silk that contained partial sequences of collagen or fibronectin (RGD) by *E. coli*. Compared with the original silk, the resultant modified silk produced by the *Nd-s*^{D} mutant inserted RGD sequence showed a 6 times higher affinity to mouse fibroblast cell BALB/3T3 [37].

The novel Fol-8Col in the present study is designed to be functionalized with bioactive group RGD for bone tissue engineering too. The results of the adhesion ratio and immunolabeling analysis show that Fol-8Col can support the attachment and stretching of MC3T3-E1 cells, which may facilitate cell proliferation and matrix production. RGD sequences in Fol-8Col, which been proven effective at improving cell adhesion [38], possibly contribute to a more intensive vinculin signal from MC3T3-E1 cells on Fol-8Col compared to type I collagen.

Future application of Fol-8Col in promoting bone tissue engineering

The structure of fibrillar collagens makes these proteins unique in their ability to interact with other macromolecules and cells since most biological processes in which fibrillar collagens take part depend on site-specific interactions [39]. Our previous research results show that the morphology of Fol-8Col after the lyophilization preferred to aggregate and form the fibrils—the structure which is similar to that of native procollagen molecular. Moreover, the incorporation of foldon sequence in Fol-8Col at the C-terminal significantly improved its thermostability that offering new possibilities for future application as a biomaterial [11].

The present paper aimed to study the potential of using Fol-8Col as scaffold for osteoblast tissue engineering. Figure 5 of proliferation assay results shows that Fol-8Col is conducive for the proliferation of MC3T3-E1 cells. Furthermore, differentiation rate and osteoblastic genes (Runx2, ColI, ALP, OP, BSP, and OC) RT-PCR analysis of MC3T3-E1 cells show that Fol-8Col has a comparable capability to support the osteoblast-like cell differentiation as native type I collagen. Zhai and Cui [40] report a recombinant human-like type I collagen, an acidic protein, can direct growth of hydroxyapatite (HA) nanocrystals in vitro in the form of self-assembly of nano-fibrils of mineralized collagen resembling ECM. Thus, the acidic amino acids (Glu, Asp, Asn) in Fol-8Col and its triple helix structure may be responsible for the nucleation activity and enhance de novo biomineralization when used as a scaffold for bone tissue engineering.

Very recently, the electrospinning nanoribbons of a bioengineered silk-elastin-like protein (SELP) from aqueous solution has been reported by Ner et al. [41]. Considering the future application, the water-soluble (data not shown) Fol-8Col is available to be fabricate into three-dimensional scaffold that would considerably expand the use of such biomaterials in bone tissue engineering.

Conclusions

Recombinant ECM proteins expressed in prokaryotic systems are in general insoluble and require denaturation, followed by in vitro refolding, which might present problems for full-length and multidomain proteins [30]. However, in this study, we confirmed the existence and stability

of the triple helix structure in soluble Fol-8Col, at temperatures approximate to body temperature, using CD spectra. Investigation of osteoblast-like MC3T3-E1 cells on Fol-8Col-coated dishes not only showed good viability but also more intensive focal adhesion patches than those on native type I collagen. Proliferation and differentiation analysis revealed that Fol-8Col is conducive to osteoblast-like MC3T3-E1 cells, which is comparable to type I collagen. Considering that Fol-8Col has been expressed economically in *E. coli*, it is an excellent alternative to native collagen as a scaffold for bone regeneration applications. However, full-length recombinant collagen-like proteins from *E. coli* still encounter problems with complex post-translational modifications and poor productivity, as well as convenience [30, 33]. The combination of Fol-8Col with other biopolymers and the processing of these material systems into three-dimensional scaffolds will greatly expand the potential for tissue regeneration applications of recombinant collagen-like proteins with a triple helix structure.

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