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Expression, Purification, and Characterization of Recombinant Fibulin-5 in a Prokaryote Expression System

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Fibulin-5 is a widely expressed, integrin-binding extracellular matrix protein that mediates endothelial cell adhesion and scaffolds cells to elastic fibers. To investigate anti-angiogenesis activities and context-specific activities on responsive cells of recombinant fibulin-5 (rfibulin-5) expressed in *Escherichia coli*, the cDNA of fibulin-5 cloned from a human placenta cDNA library was inserted into the pET32a (+) vector to allow fibulin-5 expression as a Trx fusion protein. The fusion protein Trx-fibulin-5, expressed as insoluble inclusion bodies, was solubilized and its resulting expression level reached to 15% of the total cell protein. The Trx-fibulin-5 was purified effectively by N^{2+}-chelating chromatography and then identified by Western blotting analysis with an anti-His tag antibody. The purified Trx-fibulin-5 was refolded by dialysis against redox reagents, and the rfibulin-5 released from the fusion protein by enterokinase cleavage was purified using a RESOURCE RPC column. The final purified rfibulin-5 effectively inhibited angiogenesis in chicken embryos in a dose-dependent manner through a chorioallantoic membrane (CAM) assay. Additionally, rfibulin-5 potently suppressed *in vitro* proliferation of human umbilical vein endothelial cells, but stimulated that of human dermal fibroblasts. The expression and *in vitro* refolding of rfibulin-5 resulted in production of an active molecule with a yield of 2.1 mg/L.

***Keywords*:** fibulins, anti-angiogenesis, CAM assay, fusion protein, prokaryotic expression, protein purification

Fibulins are a family of extracellular glycoproteins characterized by tandem repeats of calcium-binding epidermal growth factor (cbEGF)-like modules and a C-terminal fibulin domain (Argraves *et al*., 2003; Timpl *et al*., 2003). Five distinct fibulin genes, encoding at least nine protein products generated by alternative splicing, have been identified. Fibulins modulate cell morphology, growth, adhesion, and motility (Gallagher *et al*., 2005). Fibulin-5 (also known as DANCE, developing arteries and neural crest EGF-like/EVEC, embryonic vascular EGF-like repeat-containing protein) is a secreted 66-kDa extracellular matrix (ECM) protein belonging to the fibulin family, whose members figure prominently in the formation and stabilization of basement membranes, elastic fibers, and loose connective tissues (Nakamura *et al*., 1999; Yanagisawa *et al*., 2002). In addition to their structural functions, evidence now implicates fibulins as mediators of cell-cell and cell-matrix communication (Kowal *et al*., 1999). Fibulin-5 deficient mice show human aging phenotypes, such as loose skin, emphysematous lungs, and stiff arteries because of disorganized elastic fibers. Further studies have shown that fibulin-5 binds to elastic fiber components, such as tropoelastin (Nakamura *et al*., 2002; Kobayashi *et al*., 2007; Nonaka *et al*., 2009), fibrillin-1 (Freeman *et al*., 2005), emilin (Zanetti *et al*., 2004), and lysyl oxidases (Liu *et al*., 2004). Moreover, fibulin-5 can induce elastogenesis in cell culture (Nakamura *et al*., 2002; Yanagisawa *et al*., 2002; Hirai *et al*., 2007), antagonize vascular endothelial growth factor signaling in endothelial cells, and induce thrombospondin 1 expression (Albig *et al*., 2006). Fibulin-5 also suppresses tumor formation by controlling cancer cell proliferation, motility, and angiogenic sprouting (Albig and Schiemann, 2004, 2005; Albig *et al*., 2006).

It is difficult to produce functionally active proteins from recombinant proteins expressed in a bacterial expression system due to the lack of post-translational processing such as chemical modification and correct refolding (Hou *et al*., 2006). Overproduction of proteins often leads to the formation of insoluble aggregates, referred to as inclusion bodies, in the cytoplasmic or periplasmic space. In many cases, the inclusion bodies are made of expressed proteins (Asano *et al*., 2002). If these proteins can be refolded, large quantities can be made available for industrial and therapeutic needs (Levine *et al*., 1995; Tsumoto *et al*., 1998). The successful construction of several proteins using bacterial expression systems has been reported, and some proteins are now used for therapeutic purposes (Arndt *et al*., 1999; Takemura *et al*., 2000; Hudson and Souriau, 2001). Therefore, a stable and convenient refolding system would benefit the preparation of recombinant proteins.

In this study, we report vector construction and expression of human fibulin-5 in bacterial cells and its refolding and purification *in vitro* to recover active fibulin-5. The purified recombinant fibulin-5 (rfibulin-5) showed inhibition of angiogenesis on chicken embryo chorioallantoic membranes (CAMs), proliferation of fibroblast cells, and suppression of endothelial cells.

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Materials and Methods

Reagents and materials

E. coli strain DH5α (Novagen, USA) was used as the host for cloning. PCR reagents, restriction enzymes, and T4 DNA ligase were purchased from Promega (USA). Plasmid extraction kits and PCR production purification kits came from Bioneer (USA). Human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs) were purchased from the American Type Culture Collection (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Invitrogen (USA), and other chemical reagents were of analytical grade.

Molecular cloning of human fibulin-5

The DNA fragment containing the human fibulin-5 gene (GenBank accession no. AF112152) was amplified by PCR using the human placental cDNA library (Clontech, USA) as the template. For the cDNA encoding, the mature fibulin-5, the forward primer (5′-GGGGTACC*GACGACGACGACAAG*GCACAGGCACAGTGCAC-3′) introduced a *Kpn*I site (underlined) and an enterokinase cleavage site (italicized). The designed reverse primer was 5′-CCGCTCGAG TCAGAATGGGTACTGCGA-3′ in which *Xho*I site (underlined) and a stop codon were included. PCR conditions consisted of 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The amplified cDNA fragment was digested with the appropriate enzymes and inserted into the *E. coli* expression vector pET32a (+) (Novagen) at the corresponding restriction sites to construct the pET-fibulin-5 prokaryotic expression vector.

Expression of fusion protein Trx-fibulin-5

The recombinant pET-fibulin-5 plasmid was transformed into competent *E. coli* strain BL21 (DE3) pLysS (Novagen). A single colony was inoculated into LB medium containing 100 μg/ml ampicillin and was cultured overnight at 37°C. The overnight cultures were diluted 1:100, grown until the OD_{600} reached 0.6, and then induced by adding IPTG (Sigma, USA) to a final concentration of 1 mM. The expression of fusion protein Trx-fibulin-5 was conducted for 5 h at 30°C under vigorous shaking. The cells were finally harvested by centrifugation at 8,000×g for 10 min at 4°C and stored them at -80°C.

Solubilization of inclusion bodies

Two grams of pellet (wet weight from 1 L culture) was resuspended in 40 ml of lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 5 mM EDTA, pH 7.4) and lysed by sonication. The whole cell lysate was then centrifuged at 1,2000×g for 10 min at 4°C to separate the inclusion body. After decanting the supernatant, the inclusion-body pellet was washed with 40 ml of washing buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 5 mM EDTA, and 1% Triton X-100, pH 7.4), and centrifuged at 8,000×g for 10 min at 4°C. This washing step was performed twice. Washed inclusion bodies were dissolved in 40 ml of solubilizing buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM DTT, and 8 M urea, pH 7.4) for 12 h at room temperature.

Purification and Refolding of Trx-fibulin-5

The dissolved inclusion body was loaded onto the HiTrap FF crude column (GE Healthcare, Sweden), which was previously charged with $NiSO_4$ and equilibrated with binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM DTT, and 5 mM imidazole, pH 7.4). After extensive washing with an identical buffer but containing 60 mM imidazole, the fusion protein was eluted with five column volumes of elution buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 mM DTT, and 300 mM imidazole, pH 7.4). After collecting the eluted fractions and analyzing them by SDS-PAGE, the denatured fusion protein, refolded by dialysis against 1× dialysis buffer (20 mM Tris-HCl, pH 8.5) containing 0.1 mM dithiothreitiol, 1× dialysis buffer, 1× dialysis buffer with 1 mM GSH (reduced form of glutathione; Sigma, USA), and 0.25 mM GSSG (oxidized form of glutathione; Sigma), respectively. The refolded fusion protein was dialyzed against a cleavage buffer (20 mM Tris-HCl, 50 mM NaCl, and 2 mM $CaCl_2$, pH 7.4) and then lyophilized.

Release and purification of rfibulin-5

To release rfibulin-5 from the fusion protein, the lyophilized Trx-fibulin-5 was resuspended in cleavage buffer to give a final fusion protein concentration of 1 mg/ml. Enterokinase (Novagen) (1 U/μl) was added to the protein pool producing 1 U enzyme per 50 μg fusion protein. After a 16-h incubation at 25°C, the reaction mixture was loaded onto the HiTrap FF crude column again to remove the Trx. The flow-through was pooled, dialyzed, and applied to reverse-phase fast performance liquid chromatography on a RESOURCE RPC column (GE Healthcare) that had been previously equilibrated with 0.1% TFA. Elution was performed with a linear gradient of 20-40% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The rfibulin-5 containing peaks, monitored at 280 nm, were identified by SDS-PAGE. The rfibulin-5 was desalted for the activity assay using a PD-10 column (GE Healthcare), and the yield was determined by a conventional Bradford protein assay (Bradford, 1976).

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed essentially by the methods of Laemmli (1970) and Towbin *et al*. (1979), respectively. Briefly, Trx-tag, as a positive control, and Trx-fibulin-5 were separated by 12% SDS-PAGE under reducing conditions and then transferred onto a PVDF membrane by electroblotting. After blocking with 2% skim milk, the fusion protein was incubated with anti-His tag antibody (GE Healthcare) as a primary antibody. The secondary antibody was an anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad, USA), diluted 1:15,000, and alkaline phosphatase activity was detected according to the manufacturer's instructions.

Chicken embryo chorioallantoic membrane (CAM) assay

Fertilized chicken eggs were kept in a humidified incubator at 37°C. After a 3-day incubation, about 2 ml of albumin was aspirated from the eggs, and the shell covering the air sac was punched out and removed with forceps. Rfibulin-5 was applied to sterile 6-mm diameter Thermanox discs and allowed to dry under laminar flow conditions. The loaded discs were inverted and applied to the CAM surface of 5-day-old embryos through the windows. PBS was used as a control. The air sac ends of the embryo with shell were sealed with tape. Two days later, an appropriate volume of a 10% fat emulsion was injected into the 7-day embryo chorioallantois. The vascularization degree was quantified by counting the number of branches in the blood vessels using a microscope (Nikon, USA), a charge-coupled device imaging system, and photographed at a magnification of ×40. The inhibition rate of angiogenesis = [1-(vessel branch points of test group)/(vessel branch point of negative control]×100%. The assay was performed twice to ensure reproducibility.

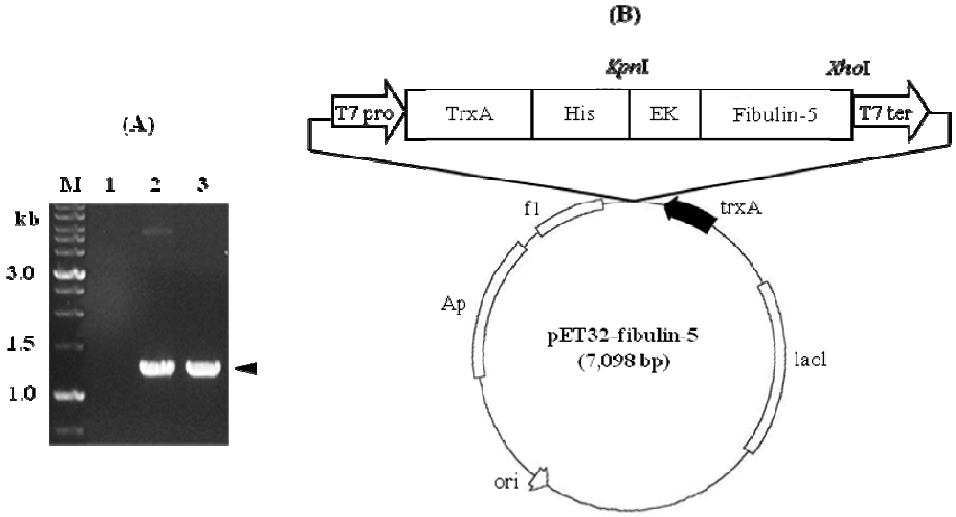

Fig. 1. Schematic representation of the pET32-fibulin-5 expression vector. (A) PCR screening of human fibulin-5. Lanes: 1, a negative control; 2, PCR products from human placenta cDNA library as the template; 3, PCR products from pET-fibulin-5; and M, DNA size markers. (B) The *Kpn*I-*Xho*I fragment (1,310 bp) containing fibulin-5 cDNA was ligated into the commercial vector pET-32a (+).

***In vitro* proliferation assay**

HUVECs and HDFs were grown to confluence in DMEM with 15% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were harvested by trypsinization at 37°C for 5 min. A suspension of a 4×10^3 cells/well in 100 μl DMEM with 5% FCS was added in triplicate to each well of 96-well tissue culture plates and incubated at 37°C for 24 h with 5% CO_2 and 95% humidity. The medium was removed and replaced with 150 μl fresh DMEM containing 5% FCS. Samples of recombinant fibulin-5 in 50 μl PBS at different concentrations were added to each well and an equal volume of PBS was added to the blank control wells. After a 72-h incubation, 20 μl MTT (Chemicon, USA) was added to each well and incubated for another 4 h at 37°C with 5% CO_2, and 95% humidity. The medium was pipetted out from each well, and 150 μl DMSO was added. The absorbance A_{595}, which correlated to the number of cells, was measured on a microplate reader at a wavelength of 595 nm.

Results and Discussion

Construction of the pET-fibulin-5expression vector

The gene for human fibulin-5 was amplified by PCR using a human placental cDNA library as the template. As shown in Fig. 1A, 1,310 bp of the PCR product coded for fibulin-5, combining the enterokinase cleavage site at its N-terminus, which facilitated the release of rfibulin-5 from the fusion protein by means of enterokinase. One pair of endonuclease restriction sites, *Kpn*I and *Xho*I, flanked the upstream and downstream of the PCR product, respectively. To construct an effective expression system for fibulin-5, Trx·$(His)_6$-tag under the control of the T7 promoter was selected as the fusion partner and, therefore, a recombinant pET-fibulin-5 vector was constructed for expressing the Trx-fibulin-5 fusion protein by modifying pET32a (+), as described in Fig. 1B. The existence of a $(His)_6$-tag between the Trx and fibulin-5 coding sequence was beneficial to facilitate purification of the Trx-fibulin-5 fusion protein by metal affinity chromatography. An enterokinase cleavage site immediately upstream to fibulin-5 was also created to facilitate enzymatic cleavage of the fusion protein for fibulin-5 release. The resulting plasmid was confirmed by restriction endonuclease digestion and DNA sequencing.

Expression and solubilization of Trx-fibulin-5

Heterologous expression of foreign genes in *E. coli* often leads to production of the expressed proteins in insoluble inclusion bodies. Inclusion bodies can potentially be a good starting point for protein purification, as they contain almost pure protein in different states of aggregation in an inactive form (Schoner *et al*., 1992; Werner *et al*., 1994; Rudolph and Lilie, 1996). However, the main problem lies in the correct refolding of fully active protein, as this very often produces insolubility or poor yield (Cao *et al*., 2005). The constructed pET-fibulin-5 expression vector was transformed into *E. coli* BL21 (DE3) pLysS. Several inducing factors affecting the expression of the fusion protein were analyzed, including bacterial cell density (OD_{600}=0.1-1.0), IPTG concentrations (0.1-1 mM), induction time (2-10 h), and temperature (20-37°C). Optimal expression of the fusion protein was obtained at a cell density of OD_{600}=0.6 with 1 mM IPTG after a 5 h induction at 30°C, and the resulting expression of the fusion protein accounted for approximately 15% of total bacterial protein. Both the cell lysate supernatant and pellet were examined after sonication to detect the fusion protein. Most of the fusion proteins were expressed in inclusion bodies of the pellet and were dissolved in solubilizing buffer containing 8 M urea (Fig. 2). Based on the amino acid sequence, the calculated molecular weight of the Trx-fibulin-5 was 65 kDa, which was consistent with the SDS-PAGE result.

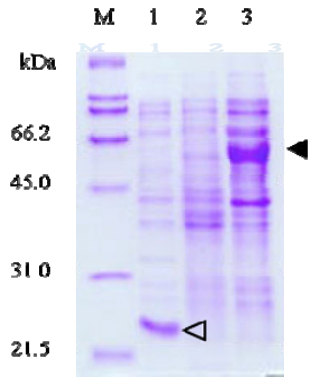

Fig. 2. Expression of the Trx-fibulin-5 fusion protein in *E. coli*. Lanes: 1, cell lysate supernatant containing the pET32a (+) expression vector as a positive control; 2 and 3, cell lysate supernatant and solubilized pellet containing Trx-fibulin-5, respectively; and M, protein size markers.

Purification and refolding of Trx-fibulin-5

Solubilized inclusion bodies were successfully purified using a HisTrap FF crude column, in which most of the fusion protein was eluted by 200 mM imidazole and easily purified to homogeneity. Western blotting analysis further confirmed this to be the Trx-fibulin-5 target fusion protein (Figs. 3A and B). One-step affinity chromatography purified about 16 mg of fusion protein from 1 L of culture medium to a purity of 73%. The purified Trx-fibulin-5 proteins were refolded by dialysis in a variety of dialysis buffers, according to the procedures described above. However, some of the Trx-fibulin-5 proteins precipitated out of solution during the refolding process, so the proteins present in solution were used for the next step.

Cleavage and purification of rfibulin-5

To generate rfibulin-5, the refolded Trx-fibulin-5 protein was treated with enterokinase at 22℃ and pH 7.4. Complete

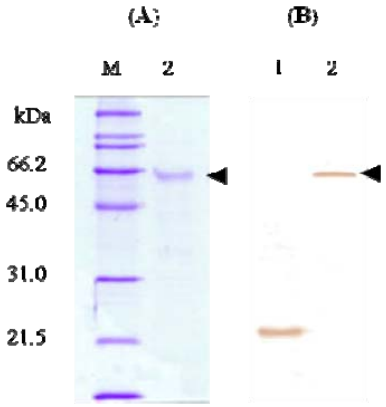

Fig. 3. SDS-PAGE (A) and Western blotting analysis of Trx-fibulin-5 fusion protein purified with an Ni^{2+}-chelating column (B). Western blotting analysis of fusion product using an anti-His tag antibody. Lanes: 1, Trx-tag as positive control; 2, Trx-fibilin-5 fusion protein purified with an Ni^{2+}-chelating column; and M, protein size markers.

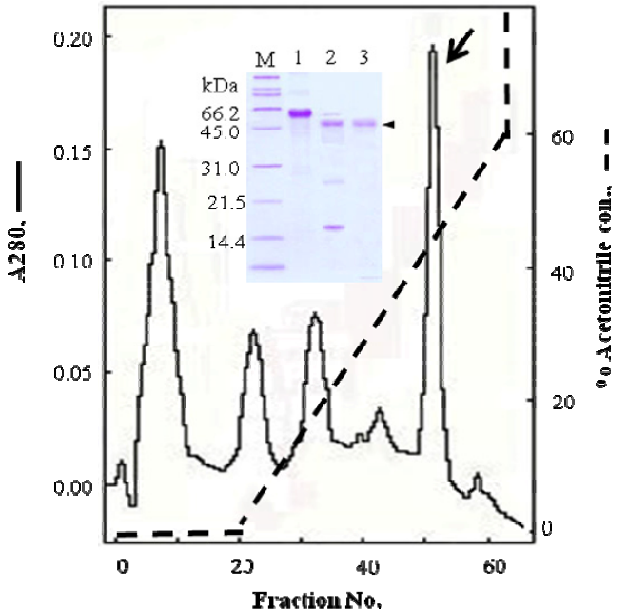

Fig. 4. Final purification of the rfibulin-5 by a RESOURCE RPC column. The rfibulin-5 peak is indicated by an arrow. Inset: SDS-PAGE for purified rfibulin-5. Lanes: 1, Trx-fibulin-5 fusion protein; 2, fusion protein cleaved by enterokinase; 3, purified rfibulin-5; and M, protein size markers.

digestion was achieved after a 12-h incubation of 0.5 mg fusion protein per unit enterokinase. After removing the Trx·$(His)_6$-tag and undigested fusion protein using a Ni^{2+}-chelating column, rfibulin-5 was purified to homogeneity using a RESOURCE RPC column. These procedures were highly efficient for producing large amounts of pure fibulin-5, and 2.10 mg of pure fibulin-5 was obtained from 1 L of culture medium. Fibulin-5 consists of a 448 amino acid glycoprotein, resulting in the secretion of a 66 kDa protein product considerably larger than that encoded by its cDNA (i.e., ~50 kDa) (Nakamura *et al*., 1999). Our fibulin-5 was produced in an *E. coli* system, which contained 426 amino acids of mature peptide. As shown in Fig. 4, the fraction eluted at around 44% acetonitrile on SDS-PAGE and weighed about 48 kDa, which was identical to the anticipated theoretical molecular mass of fibulin-5 (47.9 kDa), which likely reflects differences between *E. coli* and mammalian posttranslational machinery. Table 1

Table 1. Purification of the Trx-fibulin-5 overexpressed in *E. coli*

Purification step	Total protein (mg)[a]	Protein of interest (mg)[b]	Purity (%)	Yield (%)[c]
Crude extracts[d]	190	29	15	100
HiTrap FF crude column	22	16	73	55.2
RESOURCE RPC	2.2	2.1	96	7.2

[a] Total protein concentration was determined by Bradford protein assay, using bovine serum albumin as a standard.
[b] The amount of protein of interest was determined by quantifying the amount in each gel lane by densitometry.
[c] The purification yield is calculated based on the amount of protein of interest.
[d] The starting material was crude extracts from the lysis of 2 g (from 1 L culture) bacterial of *E. coli* BL21 (DE3) pLysS.

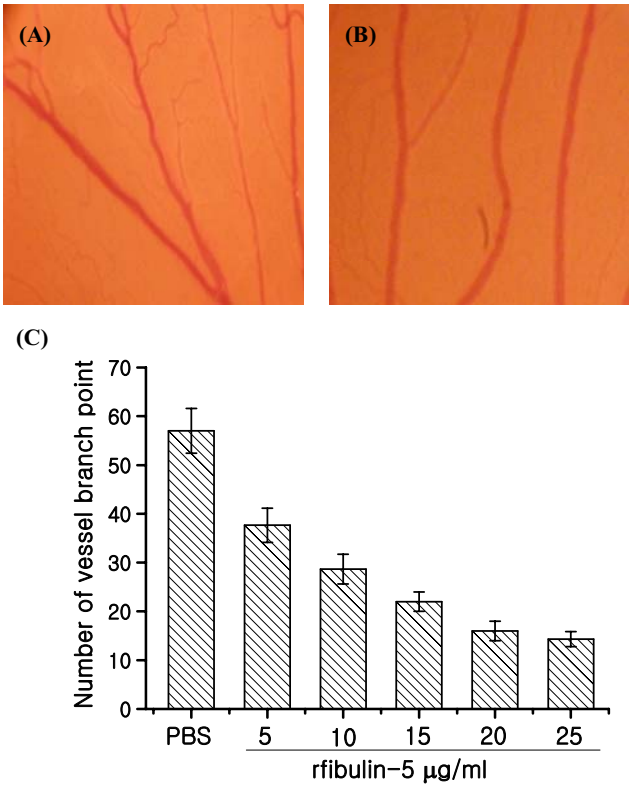

Fig. 5. Inhibition of angiogenesis by rfibulin-5 on the chorioallantoic membranes (CAMs). (A) Control CAM assay with disc soaked in PBS alone. (B) CAM assay with disc containing 20 μg rfibulin-5. (C) The number of vessel branch points markedly decreased with rfibulin-5 in a dose-dependent manner compared to PBS control. $n=6$, n represents the CAM number of each tested group. $p<0.001$ compared to PBS alone.

summarizes the yields and purity of the fusion protein and rfibulin-5 at some key steps of the purification. Finally, the purity of the purified recombinant fibulin-5 was nearly 96%.

CAM assay

CAM assays were used to determine the *in vivo* anti-angiogenic effects of rfibulin-5. In the PBS controls, blood vessels in CAM formed densely branching vascular networks (Fig. 5A). The rfibulin-5 was applied to the CAM of 5-day-old chicken embryos, and anti-angiogenic activity was evaluated by measuring the frequency of the avascular zone, which appeared white 2 days after sample implantation (Fig. 5B). A large number of newly formed blood vessels had significantly regressed within the avascular zones. The inhibition effect was dose-dependent within the range of 5-25 μg of rfibulin-5 per embryo, compared to the PBS control (Fig. 5C). These experimental results suggest that the refolded and purified rfibulin-5 was in a functionally active form, which permits further study on its inhibitory activity of tumor growth, molecular mechanism, and potential application for anti-angiogenesis therapy.

Proliferation assay

Fibulin-5 is a multifunctional signaling molecule that regulates

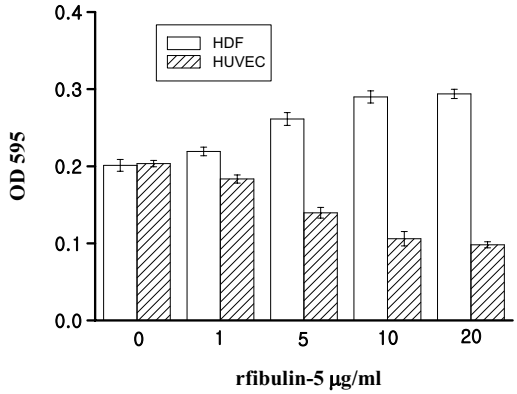

Fig. 6. Effects of rfibulin-5 on proliferation of human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs). 0, 1, 5, 10, 20 mg/ml of rfibulin-5 was applied to each cell individually. Cell density was measured at 595 nm. The columns show the result (mean values, with indicating S.D.) of triplicate determinations by MTT assay.

cell proliferation and motility in a context-specific manner (Schiemann *et al*., 2002). For example, it stimulates DNA synthesis and motility in fibroblasts and fibrosarcoma cells but inhibits these processes in epithelial (Schiemann *et al*., 2002) and endothelial (Albig and Schiemann, 2004) cells. HUVECs and HDFs cultured with 10% FCS were exposed to different amounts of rfibulin-5 *in vitro* to evaluate the context-specific effects of our rfibulin-5 on responsive cells. As shown in Fig. 6, rfibulin-5 inhibited endothelial cell proliferation at a concentration of 5 μg/ml, whereas 10 μg/ml of rfibulin-5 significantly inhibited endothelial cell proliferation. Cells treated with either 10 or 20 μg/ml fibulin-5 had a 52.1% or 48.3% proliferation rate, respectively, relative to the positive control. In contrast, fibulin-5 effectively stimulated the proliferation of fibroblast cells at doses from 5 to 10 μg/ml, suggesting a potential involvement in the control of cell proliferation in a context-dependent manner.

We reported a method for producing fibulin-5 protein based on its expression in a prokaryote system and subsequent refolding. A combination of a high yield expression system and an efficient refolding protocol made it possible to prepare sufficient quantities of rfibulin-5 protein. Our results may help in further functional studies investigating how fibulin-5 not only modulates angiogenesis and endothelial cell activities but also plays a role as a multifunctional ECM protein.

Acknowledgements

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