Design, expression and characterization of single chain Fv, Mms13 and the single chain Fv-mms13 fusion protein

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Abstract. Single chain Fv (scFv) antibodies are attractive as tumor-targeting vehicles due to their smaller size compared with intact antibody molecules. Mms13 is a putative membrane anchor protein of magnetosome. The present study fused the scFV gene of type IV collagenase to *mms13* using the splicing by overlap extension polymerase chain reaction technique. The genes of scFv, *mms13* and the scFv-*mms13* fusion gene were cloned into a pET30a(+) vector to construct pET30a(+)-scFv, pET30a(+)-*mms13* and pET30a(+)-scFv-*mms13* expression vectors. The three protein compositions were confirmed by DNA sequencing and western blot analysis, and their cellular locations were determined using SDS-PAGE. The results of enzyme-linked immunosorbent assays and immunofluorescence demonstrated that the ScFv and ScFv-*mms13* fusion proteins bound to the type IV collagenase and the antigen-associated cancer cells SMMC-7721, MCF-7 and HepG2 cells, in a dose-dependent and saturable manner. Although the immunoreactivities of ScFv-*mms13* to the type IV collagenase and associated tumor cells were marginally lower than the corresponding scFv (3G11), considerable binding ability to the antigen by ScFv-*mms13* remained.

Introduction

It has been demonstrated that heterologous proteins can be displayed directly on the surface of magnetosomes through genetic fusion to magnetosome membrane proteins (MMPs) (1). A number of MMP associated with the synthesis of nanoparticles have been identified, and several of these are used as anchor proteins, including MpsA, MagA, Mms13 and Mms6 (MamC, Mam12) (2,3). A previous report indicated that *mms13* (*mamC*) is a putative membrane anchor gene (4,5).

Type IV collagenase, also termed gelatinase, including gelatinase A (MMP-2; 72 kDa) and gelatinase B (MMP-9; 92 kDa), is an important member of the MMP family. Type IV collagenase is abundantly expressed in proliferating endothelial cells and in several types of malignant tumor, where it is involved in cancer invasion, metastasis and angiogenesis (6). Therefore, type IV collagenase is a potential target in cancer therapy. As reported, the 3G11 type IV collagenase monoclonal antibody and its single chain Fv fragment (scFv) exhibit specific binding to target enzymes and can prevent tumor growth, invasion and metastasis (6,7). scFv antibodies have potential advantages over whole antibodies, including their small size, minimal antigenicity, high penetrability and their ability to be manipulated by genetic engineering (8). Therefore, these antibodies present as a relatively ideal tumor-targeting agent.

The present study involved the construction, expression, purification and characterization of ScFv, Mms13 and the ScFv-*mms13* fusion protein.

Materials and methods

Cell culture. The *Magnetospirillum magneticum* AMB-1 strain (American Type Culture Collection, Manassas, VA, USA) was grown microaerobically at 28˚C in modified enriched mangnetic spirillum growth medium (EMSGM) (9). For plate cultivation, agar was added (1.5% wt/vol) to the EMSGM. The *Escherichia coli* DH5α strain (DH5α-competent cells; Takara Bio, Inc., Dalian, Liaoning, China) was used for DNA cloning and the Rosetta (DE3) *E. coli* strain (Novagen, Heidelberg, Germany) was used for protein expression. The *E. coli* DH5α strain was grown on Luria-Bertani (LB) medium at 37˚C, supplemented with kanamycin (50 μg/ml) or ampicillin (50 μg/ml) and 1.5% (wt/vol) agar, if appropriate (10).

Human breast carcinoma MCF-7 cells, human hepatoma SMMC-7721 cells and HepG2 cells were obtained from China Medical Culture Collection Center (Beijing, China), and

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Abbreviations: scFv, single chain Fv; SOE-PCR, splicing by overlap extension polymerase chain reaction; MMPs, magnetosome membrane proteins; EMSGM, enriched mangnetic spirillum growth medium

Key words: single chain Fv, *mms*13, fusion protein, splicing by overlap extension polymerase chain reaction

grown in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Beyotime Biotechnology, Beijing, China), penicillin G (100 U/ml) and streptomycin (100 mg/ml) at 37˚C in an atmosphere of 5% CO_2. All cell lines were passaged every 3 days and were maintained in exponential growth to ~80% confluence for the subsequent experiments.

*Construction of the pET30a(+)-*scFv *and pET30a(+)-mms13 expression vectors*. The primers and the gene of the anti-type IV collagenase scFv were synthesized, according to the GenBank database (accession no. FJ037775 by Takara Bio, Inc. (11) and cloned into the pMD18-T vector (Thermo Fisher Scientific, San Jose, CA, USA) to create the pMD-scFv vector. DNA fragments were amplified by polymerase chain reaction (PCR) for subsequent cloning using a T-Gradient Thermoblock PCR cycler (Biometra, Gottingen, Germany) and PCR MasterMix (Hangzhou Biosci Biotech Co., Ltd., Hangzhou, China). The scFv gene was amplified from the pMD-scFv plasmid by PCR using the following primers: P1, forward 5'-GGAATTCCATATGCAGGTGAAGCTGCAG-3', introducing an *Nde*I restriction site, and P2, reverse 5'-CCGCTCGAGACGTTTGATTTCCAGCTT-3, introducing an *Xho*I site to the 3' end of the scFv gene. The cycling conditions were as follows: For *mms13*, 30 cycles of 94˚C for 30 sec, 48˚C for 45 sec and 72˚C for 1 min; for scFv, 30 cycles of 94˚C for 30 sec, 55˚C for 45 sec and 72˚C for 1 min; and for scFv-*mms13*, 30 cycles of 94˚C for 30 sec, 53˚C for 45 sec and 72˚C for 1 min. The 763 bp PCR product was purified and digested by *Nde*I/*Xho*I (Thermo Fisher Scientific) and then ligated into an *Nde*I/*Xho*I-cleaved pET-30a(+) (Novagen) to produce the pET30a(+)-scFv expression vector. Sequences were analyzed by Sangon Biotech Co., Ltd. (Shanghai, China).

The genomic DNA of the *M. magneticum* AMB-1 was extracted using a MiniBEST Bacterial Genomic DNA Extraction kit (Takara Bio, Inc.). The *mms13* gene was amplified from the genome of *M. magneticum* using the following primers: P3, forward 5'-GGAATTCCATATGCCCTTTCACCTTG-3', introducing an *Nde*I restriction site, and P4, reverse 5'-CCGCTCGAGGGCCAGTTCGTCCCG-3', introducing an *Xho*I site to the 3' end of the *mms13* gene. The 388 bp PCR product was cloned into pET-30a(+) to produce pET30a(+)-*mms13*, similar to the previously constructed pET30a(+)-scFv.

*Construction of the pET30a(+)-*scFv-mms*13 expression vector*. The scFv-*mms13* fusion gene was constructed using the splicing by overlap-extension PCR (SOE-PCR) technique. The scFv gene was amplified by PCR using the following primers: P5, forward 5'-ATGCAGGTGAAGCTGCAG-3', and P6, reverse 5'-GGAGCCGCCGCCGCCAGAACCACCACCACCACGTT TGATTTCCAGCTT-3', and the *mms13* gene was amplified using the following primers: P7, forward 5'-GGCGGCGGCG-GCTCCGGTGGTGGTGGTTCTATGCCCTTTCACCTTG-3' and P8 reverse 5'-GGCCAGTTCGTCCCG-3'. The PCR products of scFv and *mms13* were purified using the TIANquick Midi Purification kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) and mixed. The assembly reaction included one cycle of denaturation, annealing and extension, in the absence of primers, to facilitate the assembly of chains, followed by 30 cycles of amplification reactions, in the presence of the scFv forward primer and *mms13* reverse primers. The scFv reverse primer and *mms13* forward primer included an artificial region of overlap to enable the formation of a flexible segment, corresponding to the G4S3 linker. The product of the SOE-PCR was purified and cloned into the pMD18-T vector to create the pMD-scfv-*mms13* expression vector. The scFv-*mms13* fusion gene was then amplified from the pMD-scfv-*mms13* using the following primers: P1, forward 5'-GGAATTCCATATGCAG-GTGAAGCTGCAG-3', introducing an *Nde*I restriction site, and P4, reverse 5'-CCGCTCGAGGGCCAGTTCGTCCCG-3', introducing an *Xho*I restriction site. The PCR product was cloned into pET-30a(+) to produce pET30a(+)-scFv-*mms13*.

Expression and cellular location of the ScFv*, Mms13 and* ScFv-*mms13 proteins by western blot analysis*. The procedures for the growth of the DE3 *E. coli* strain, transformed with pET30a(+)-scFv, pET30a(+)-*mms13* or pET30a(+)-scFv-*mms*13 were performed, according to standard protocol. The strain was cultured in LB medium at 37˚C, supplemented with kanamycin (50 μg/ml). Following induction of the target proteins at 37˚C for 4 h with 0.2 mM isopropylthio-β-D-thiogalactopyranoside (IPTG; Takara Bio, Inc.), the four fractions, including the medium, periplasmic, soluble cytoplasmic and insoluble samples, were obtained, according to the pET system manual (10th ed; http://www.merckmillipore.com/). The samples were electrophoresed on 15% SDS-polyacrylamide gels, and the proteins were then transblotted onto a nitrocellulose membrane (Millipore, Bedford, MA, USA), blocked with 5% bovine serum albumin (BSA)/Tris-buffered saline with Tween 20 (TBST; 25 mM Tris-HCl at pH 8.0, 125 mM NaCl and 0.05% Tween-20; Beyotime Institute of Biotechnology) for 1 h and incubated with a 1:1,000 dilution of anti-His-Tag monoclonal mouse primary antibody (cat. no. AH367; Beyotime Institute of Biotechnology) overnight at 4˚C, followed by incubation with 1:1,000 horseradish peroxidase-labeled goat anti-mouse polyclonal immunoglobulin (Ig)G secondary antibody (cat. no. AO216; Beyotime Institute of Biotechnology) at 37˚C for 1 h. The membrane was washed with TBST 5 times for 5 min each time and the antibody reactions were visualized using a Super signal West Pico Trial kit (Thermo Fisher Scientific).

Purification and refolding of the ScFv*, Mms13 and* ScFv-*mms13 proteins*. The induced bacterial cells (~10^8 cells/ml) were centrifuged at 10,000 g for 10 min (HC-2518R; Anhui USTC Zonkia Scientific Instruments Co., Ltd., Hefei, China). The cells pellet was resuspended in binding buffer, containing 20 mmol/l imidazole, 0.5 mol/l NaCl and 20 mmol/l NaH_2PO_4 (pH 7.5), and sonicated (JY92-2D; Ningbo Scientz Bio, Inc., Ningbo, China), followed by centrifugation of the cell lysate at 12,000 g for 10 min at 4˚C. The pellet was then resuspended and incubated in binding buffer, containing 8 mol/l urea, on ice for 1 h. The insoluble material was then removed by centrifugation at 12,000 g for 20 min. The supernatant was filtered through a 0.45 nm membrane and purified using HisTrap affinity columns (GE Healthcare, Amersham, UK), under denaturing conditions, according to the manufacturer's instructions. The column was washed with distilled water with a flow rate of 1 ml/min and was equilibrated with binding

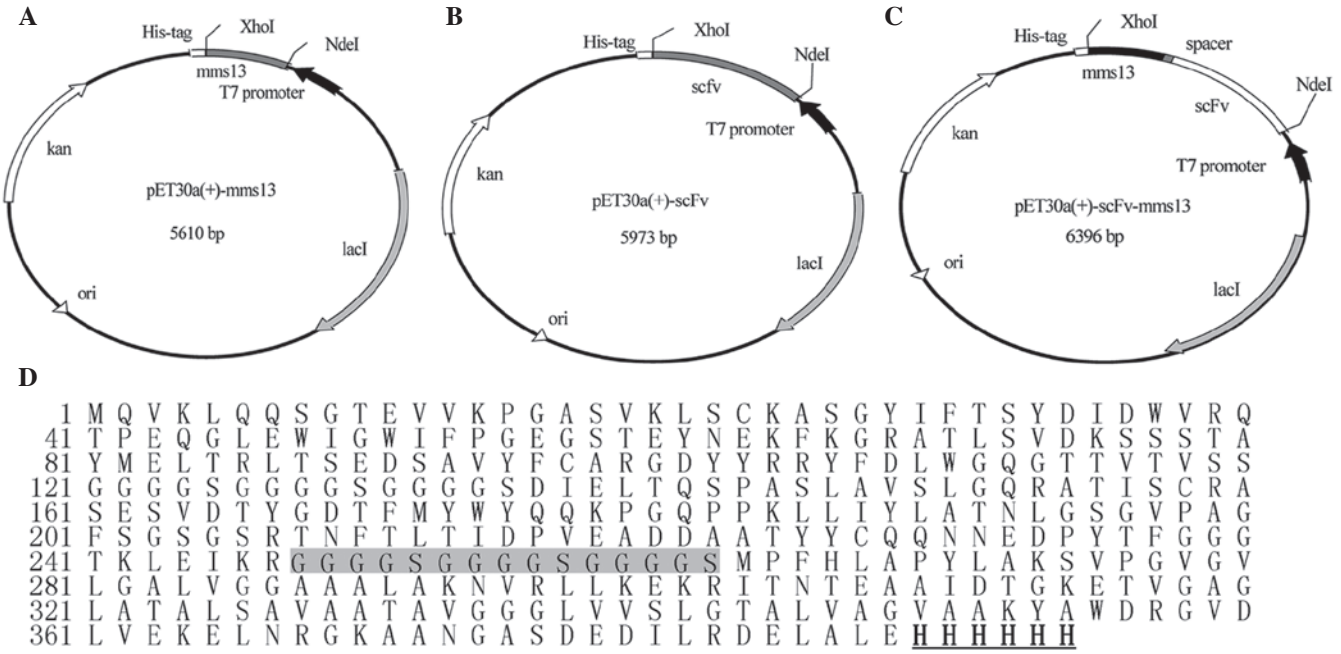

Figure 1. Construction of pET30a(+)-*mms*13, pET30a(+)-scFv and pET30a(+)-scFv-*mms*13 expression vectors. The genes of *mms*13, scFv and scFv-*mms*13 were cloned into the *Nde*I/*Xho*I restriction sites of pET-30a(+) to produce the expression vectors. The three genes were under the control of the T7 promoter, and a His_6-tag was introduced at the C-terminus of the constructs. (A) Construction of the pET30a(+)-*mms*13 expression vector. (B) Construction of the pET30a(+)-scFv expression vector. (C) Construction of the pET30a(+)-scFv-*mms*13 expression vector. (D) Amino acid sequence of the ScFv-*mms13* fusion protein. The shaded amino acids indicate the G_4S_3 spacer, the underlined amino acids indicate the, His_6-tag. ScFv, single chain Fv.

buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 8 M urea, pH 7.4). The column was then washed with binding buffer and eluted with elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, 8 M urea, pH 7.4). The purified ScFv and ScFv-*mms13* proteins were refolded using step-wise dialysis, as reported previously (12). In brief, β-mercaptoethanol (Tianjin Guangfu Fine Chemical Research Institute, Tianjin, China) was added to the protein solution at 1 M. The protein solution was dialysed with 50 fold refolding buffer (50 mM Tris-HCl, 1 mM EDAT, 200 mM NCl, pH 8.0) with 8 M urea (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China) at 4˚C for 12 h. The refolding buffer was replaced with step-wise concentrations of urea (4, 2, 1, 0.5 and 0 M). Oxidized glutathione (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was added at 50 μM and L-arginine (Sangon Biotech Co., Ltd.) at 400 mM was added with the 1 M urea step.

The protein concentrations of the fractions was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) using various concentrations of BSA (2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and 0 mg/m1; 25 μl/well; Beyotime Institute of Biotechnology) as a standard. The proteins were analyzed throughout using SDS-PAGE and the gels were stained with Coomassie brilliant blue (2.5 g with 500 ml methanol and 100 ml glacial acetic acid made up with 1 L ddH_2O; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

Enzyme-linked immunosorbent assays (ELISA). The cells ($2x10^4$ cells/well) were grown in 96-well plates to confluence, washed with phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology) and fixed for 30 min with 50 μl/well methanol at 4˚C. For gelatinase, 96-well microtiter plates were coated with 100 μl/well of 10 μg/ml type IV collagenase (Thermo Fisher Scientific) at 4˚C overnight. The plates containing type IV collagenase or the fixed cells were washed three times with PBS and blocked with 1% BSA/PBS at 4˚C overnight. The wells were emptied and 50 μl ScFv, Mms13 or ScFv-*mms13* were added in two-fold serial dilutions at concentrations ranging between 0.1 and 50 μmol/l for 2 h at 37˚C. Following washing with PBS, the wells were incubated with 50 μl/well 1:1,500 anti-His tag mAb (Beyotime Institute of Biotechnology), as a primary antibody, at 37˚C 1 h. The wells were then overlaid with 50 μl/well 1:2,000 horseradish peroxidase-labeled goat anti-mouse IgG (Beyotime Institute of Biotechnology), as a secondary antibody, at 37˚C 1 h following washing with PBS. Color development was achieved using 100 μl o-Phenylenediamine solution, which was terminated after 10 min with 100 μl 2 mol/l H_2SO_4. The absorbance was measured at 490 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific). All assays were performed in triplicate.

Immunofluorescent cytochemical staining of the SMMC-7721 and MCF-7 cells. Immunofluorescent staining was performed on the antigen-positive SMMC-7721 and MCF-7 cells. The cells were grown on slides and fixed in ice-cold methanol for 30 min. Nonspecific binding was inhibited using 200 μl/well 1% BSA/PBS at 4˚C overnight. Following washing with PBS, the cells were incubated with 50 μl/well ScFv, Mms13 or ScFv-*mms13*. The cells were then overlaid with 1:1,500 mouse anti-His tag monoclonal antibodies (Beyotime Institute of Biotechnology) following washing with PBS. A final washing step with PBS was performed, and the slides were mounted

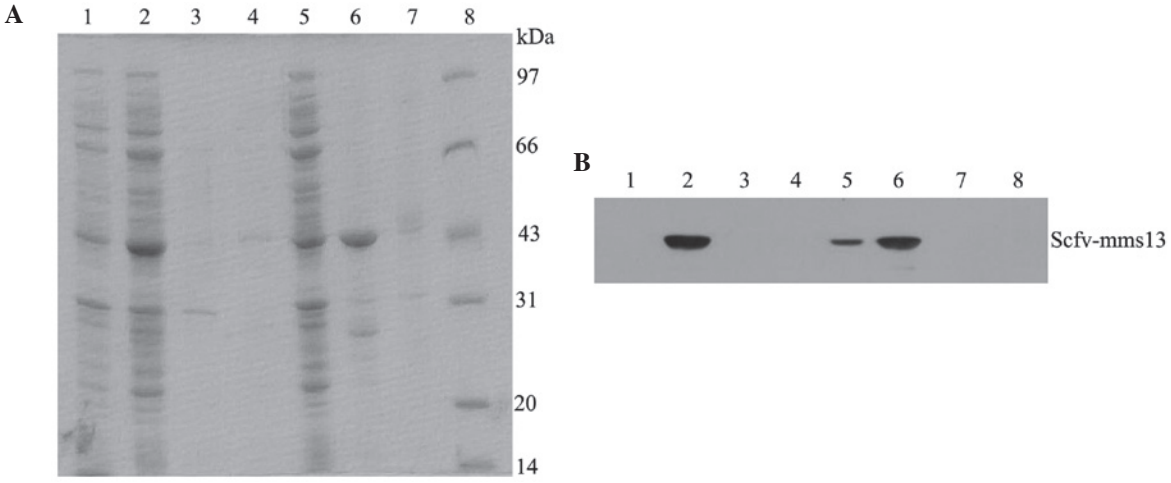

Figure 2. SDS-PAGE assay and western blot analysis of the ScFv-*mms13* fusion protein. The (A) SDS-PAGE and (B) western blot of each fraction of the *E. coli* Rosetta (DE3) cells expressing the ScFv-*mms13* fusion protein. Lane 1, total proteins of *E. coli* containing the pET-30a(+) plasmid following IPTG induction; Lane 2, total proteins of *E. coli* containing the pET-30a(+)-scFv-*mms*13 plasmid following IPTG induction; Lane 3, medium sample; Lane 4, periplasmic fraction; Lane 5, cytoplasmic soluble fraction; Lane 6, cytoplasmic insoluble fraction; Lane 7, cytoplasmic insoluble fraction of *E. coli* containing the pET30a(+)-scFv-*mms*13 plasmid without IPTG induction; Lane 8, molecular weight marker. ScFv, single chain Fv; IPTG, isopropylthio-β-D-thiogalacto pyranoside.

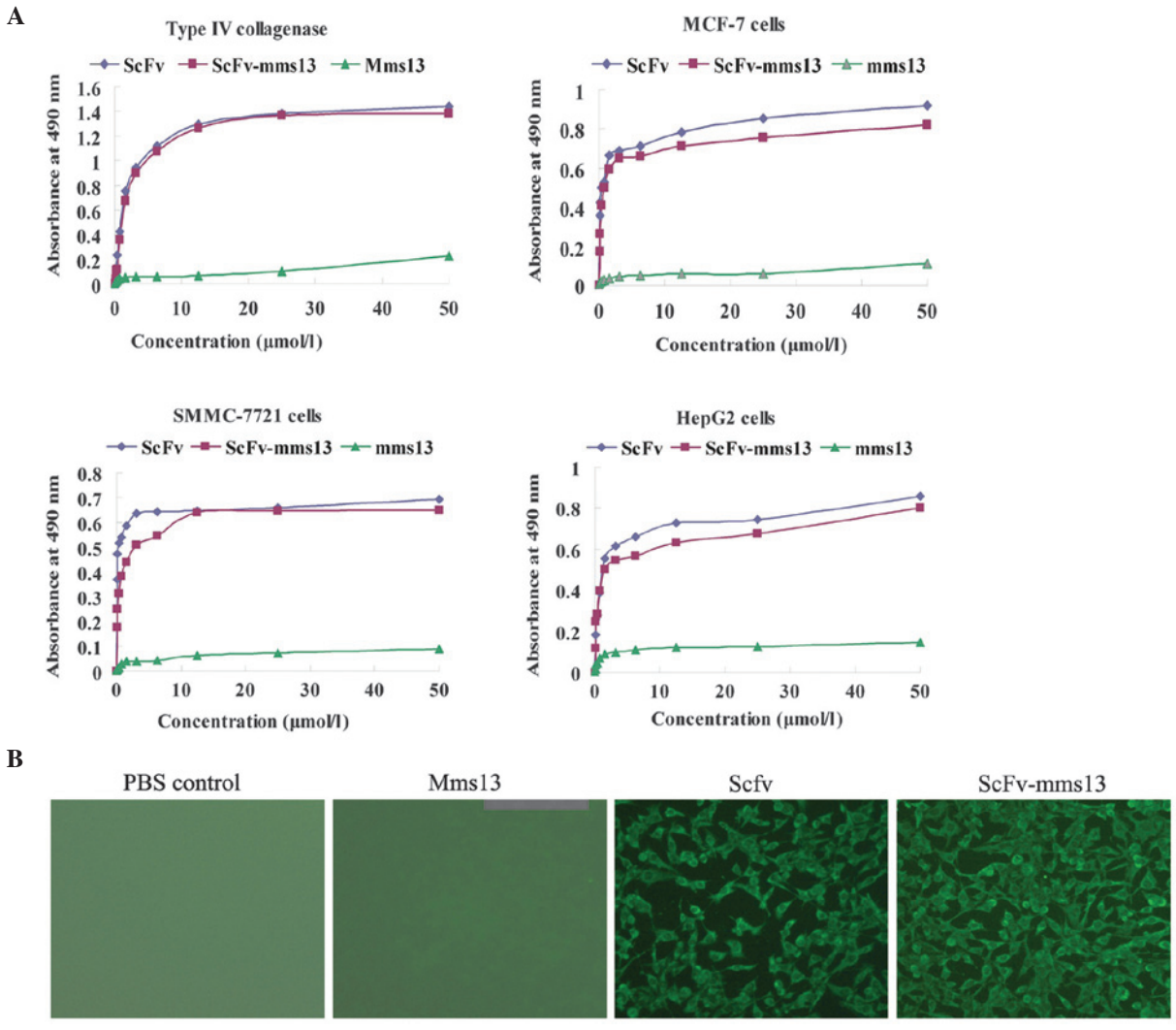

Figure 3. Immunoactivity analyses of ScFv, Mms13 and ScFv-*mms13*. (A) Immunoreactivity to type IV collagenase, MCF-7 cells, SMMC-7721 cells and HepG2 cells were assessed using enzyme-linked immunosorbent assays. The ScFv and ScFv-*mms13* proteins bound to type IV collagenase and the antigen-associated cancer cells in a dose-dependent and saturable manner. (B) Immunofluorescence detection of the SMMC-7721 cells using fluorescein isothiocyanate-conjugated goat anti-mouse antibody (magnification, x100). The ScFv and ScFv-*mms13* fusion protein exhibited fluorescence, however, no fluorescence was observed in the PBS control and Mms13 groups. ScFv, single chain Fv; PBS, phosphate-buffered saline.

with 1:2,000 fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Beyotime Institute of Biotechnology) and fluorescence images were captured using an Olympus BX60 microscope (magnification, x100), equipped with an Olympus DP71 camera and Olympus DP-Controller software, version 2.1 (Olympus Corporation, Tokyo, Japan).

Results

*Construction of the pET30a(+)-*mms*13, pET30a(+)-*scFv *and pET30a(+)-*scFv-mms*13 expression vectors*. The DNA sequences encoding the *mms13* gene and the scFv of the 3G11 mAb were cloned into the *Nde*I/*Xho*I restriction sites of pET-30a(+), producing the pET30a(+)-*mms13* (Fig. 1A) and pET30a(+)-scFv (Fig. 1B) expression vectors. The *mms13* and scFv genes were amplified by PCR, and were linked using SOE-PCR to yield the scFv-*mms13* fusion gene. A 15 amino acid spacer $(G_4S)_3$ was present between the C-terminus of the scFv and the N-terminus of the *mms13* genes. The scFv-*mms13* fusion gene was cloned into the *Nde*I/*Xho*I restriction sites of pET-30a(+) to create the pET30a(+)-scfv-*mms13* expression vector (Fig. 1C). Sequence analyses confirmed all the expected DNA sequences. All the three genes were under the control of the T7 promoter, and a $(His)_6$-tag was introduced at the C-terminus of the constructs to facilitate purification using immobilized metal affinity chromatography. The Mms13, ScFv and ScFv-*mms13* fusion protein were composed of 132, 253 and 394 amino acids (Fig. 1D), with theoretical molecular weights of 13.4, 27.4 and 40.9 kDa respectively.

Expression, purification and refolding of the ScFv, *Mms13 and* ScFv-*mms13 proteins*. The three expression vectors were transformed into the DE3 *E. coli* strain, and the target proteins were induced by the addition of IPTG. The results of the Coomassie Blue-stained gel indicated that Mms13 was present in the cytoplasmic soluble fraction, ScFv was present in the insoluble fractions (data not shown) and the ScFv-*mms13* fusion protein was present in the cytoplasmic soluble fraction and insoluble fractions (Fig. 2A). The three proteins were further confirmed using western blot analysis with an anti-His-Tag antibody (Fig. 2B). The three proteins were purified using immobilized metal-affinity chromatography resin under denaturing conditions, and the purified ScFv and ScFv-*mms13* proteins were refolded using step-wise dialysis, as reported previously (12).

ELISA and immunofluorescent cytochemical staining. To confirm the correct folding and functional binding of the fusion protein, the abilities of ScFv, Mms13 and ScFv-*mms13* to bind to the target antigen type IV collagenase or antigen-relevant tumor cells were examined using ELISA. The data indicated that ScFv and ScFv-*mms13* bound to the type IV collagenase or the antigen-associated cancer cells, including SMMC-7721, MCF-7 and HepG2 cells, in a dose-dependent and saturable manner (Fig. 3A). Although the immunoreactivities of ScFv-*mms13* to the type IV collagenase and associated tumor cells were marginally lower than the corresponding scFv (3G11), there remained considerable binding ability to the antigen by ScFv-*mms13* (Fig. 3A).

Immunofluorescence staining was performed on the ScFv-, *mms13*- and ScFv-*mms13*-treated SMMC-7721 and MCF-7 cells. As shown in Fig. 3B, the ScFv and ScFv-*mms13* fusion protein exhibited green fluorescence, whereas no fluorescence was observed in the PBS control or Mms13, which further confirmed the immunoreactivity of ScFv-*mms13*.

Discussion

ScFv antibodies retaining the binding characteristics of the parent immunoglobulin have been preferred in clinical and diagnostic applications due to their prominent advantages, including lower molecular weight, superior penetration of tumor tissue, improved pharmacokinetics and a reduction in immunogenicity (13). The single-chain antibody of type IV collagenase, which is associated with the invasion, metastasis and angiogenesis of malignant types of tumor, may not only serve as a tumor targeting vehicle, but also exert its own anti-tumor activity by inhibiting target enzymes (6,7). A number of heterologous proteins have been fused to certain MMPs and displayed on the surface of magnetosomes (4,5,14,15). As the most abundant magnetosome protein, Mms13 has been demonstrated as an efficient anchor protein (4,5).

It has been previously reported that ScFv antibodies have low solubility, which imposes a significant limitation in their diagnostic and clinical implication (16). The co-expression of ScFv with affinity tags or molecular chaperones, including glutathione S-transferase (17), green fluorescent protein (18) or maltose binding protein (19) can enhance the solubility of a number of the fusion proteins. Therefore, the preset study hypothesized that the difference in the solubility of ScFv-*mms13* from the general isolated ScFv may be associated with the occurrence of Mms13. Mms13 itself was highly soluble in aqueous environments (data not shown), and the present study demonstrated that the ScFv-*mms13* fusion protein was present in the cytoplasmic soluble fraction (~30%) and insoluble fractions (Fig. 2a). It is possible that the existence of Mms13 may affect the solubility behavior and partly prevent the aggregation of the ScFv-*mms13* fusion protein. A putative explanation is that the linking of ScFv to Mms13 partly covers the exposed hydrophobic surface of ScFv and, therefore, prevents its aggregation.

Rosenblum *et al* (20) previously described immunotoxins, in which the single-chain antibody of ZME-018 was fused to the ribosome-inactivating plant toxin, gelonin, and found that the recombinant immunotoxin preserved the cytotoxicity and antigen-binding activity. The results of the present study were consistent with these findings. The ELISA results revealed that the ScFv-*mms13* fusion protein retained the antigen binding activity of ScFv and interacted with type IV collagenase and several antigen-relevant tumor cells. The results of the immunofluorescence analysis also demonstrated the immunoreactivity of the ScFv-*mms13* fusion protein with various antigen-associated cancer cells. These data suggested that the ScFv-*mms13* fusion protein contained sufficient structural information for specific antigen recognition.

In conclusion, the engineered ScFv-*mms13* fusion protein demonstrated antigen-binding activity, and presents as a promising candidate for the display of ScFv on magnetosome surfaces.

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