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Generation and characterization of a fusion protein of single-chain fragment variable antibody against hemagglutinin antigen of avian influenza virus and truncated protamine

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ABSTRACT

The hemagglutinin antigen (HA) of avian influenza virus (AIV) is an immunogen abundant on the surfaces of infected cells, and can be used as a target for specific antibodies to clear viral infection. Protamine has been demonstrated to deliver DNA into cells effectively. Accordingly, a fusion protein of anti-HA single-chain fragment variable (scFv) and truncated protamine (tP) may be used as a vehicle for delivering the anti-AIV siRNA into the AIV-infected cells for gene therapy. To test this hypothesis, we constructed a novel recombinant plasmid, pET28-scFv-tP, by connecting the genes for anti-H5N1 AIV HA-specific scFv with synthesized oligonucleotides encoding the 22 amino acids of human tP and a linker. Furthermore, the recombinant scFV-tP was expressed and purified, with a yield of 7–8 mg of scFv-tP and a purity of >92% from 1 L of bacterial culture. Characterization of its bioactivity revealed that scFv-tP recognized HA, similar to its scFv control, in a dose-dependent manner and that the scFv-tP, but not its scFv control, bound to DNA and delivered plasmid and oligonucleotide DNA into the AIV-infected MDCK cells effectively. More importantly, transfection with the mixture of the scFv-tP and plasmid for the NP-specific siRNA significantly inhibited the replication of AIV in MDCK cells, as compared with that transfection with the scFv–plasmid mixture, even with the plasmid in liposome. Our data demonstrated that the recombinant scFv-tP retained the functions of both scFv and tP, and might be potentially used for delivering genetic materials for targeting therapy of AIV infection *in vivo*.

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1. Introduction

In recent years, frequent outbreaks of H5N1-subtype avian influenza virus (AIV) in poultry and humans have become serious health and economic concerns in China. AIV is composed of three transmembrane proteins: hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2) [1]. The HA protein is the principal immunogen on the envelope of AIV and is crucial for the infectivity of AIV. The HA protein is abundant on the surface of the infected cells, and is currently the main target of specific antibodies [2]. Recently, the siRNA-based gene therapy has shown the potential to inhibit the replication of high-virulent AIV [3,4]. However, little is known about selective targeting of inhibitory siRNA and other genetic materials to AIV-infected cells.

Single-chain fragment variable (scFv) format antibody is a small antibody engineered by connecting the gene fragments for the variable regions of the heavy and light chains of an immunoglobin with a linker. The resulting scFv antibody usually retains the affinity and specificity of its parent antibody. Human scFvs have been shown to have potent neutralizing activity against AIV [5,6]. The scFv antibody has many advantages in diagnostic and therapeutic applications because the scFv is small molecule and has targeting drug-delivery agent potential [7]. Protamine, a major component of salmon sperm nucleus, contains many basic arginine residues. Protamine can bind to and deliver DNA into cells efficiently and be used for gene therapy [8,9]. The 22 amino-acid form of truncated protamine (tP) containing the arginine-enriched domain usually retains protamine's ability to bind to and deliver DNA. Accordingly, a fusion protein of scFv specifically against AIV and tP may facilitate delivery of genetic materials, such as the AIV-specific siRNA, into the AIV-infected cells, inhibiting the AIV replication.

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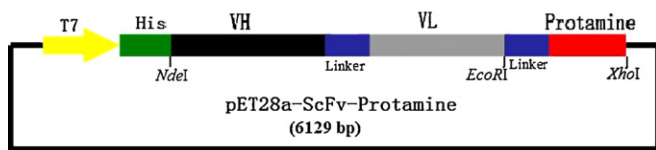

Fig. 1. Schematic structure of pET28a-scFv-tP recombinant plasmid.

In our previous study, we have successfully generated monoclonal antibody (mAb) against the HA of H5N1 tiger-born influenza A virus [10]. We then cloned and expressed the HA-specific scFv [10]. In this study, we constructed a novel plasmid that could express the scFv-tP fusion protein in *Escherichia coli*. Furthermore, we characterized whether the recombinant scFv-tP could retain its capacity to recognize its specific antigen, and bind to and deliver DNA into the AIV-infected cells. Finally, we evaluated whether scFv-tP could facilitate the NP-specific siRNA into the AIV-infected cells and enhance the antiviral activity of siRNA in inhibiting the replication of AIV *in vitro*. We found that the generated scFv-tP recognized the antigen and delivered DNA into the AIV-infected cells effectively, enhancing the inhibition of NP-specific siRNA on the AIV replication in Madin–Darby canine kidney (MDCK) cells.

2. Materials and methods

2.1. Construction of the pET28a-scFv-tP plasmid

The plasmid of pMD18-VHL-HA contained the DNA fragments for the HA-specific VH and VL. The scFv gene was amplified by PCR using the specific primers of forward 5′-CAGCCATATGGATAT TGTGTTTACACAGTC-3′ (underlined sequence for NdeI) and reverse 5′-GAATTCGGAGA CGGTGACTGAGGTTC-3′ (underlined sequence for EcoRI). A DNA fragment for the 22 amino acids of truncated human protamine (position 8–29, tP) and a specific linker was synthesized and its sense sequence was: 5′-GAATTCGGTGGTGGTGGTTCTGGCGGCGGCGGCTCCGGTGG TGGTGGATCCCGCAGCCAGAGCCGGAGCAGATATTACCGCCAGAGA-CAAAGAAGTCGCAGACGAAGGAGGCGGAGCTAACTCGAG-3′. After being cloned into pMD18 T (Takara, Japan), the DNA fragments for scFv and tP were obtained by digesting them with NdeI/EcoRI and EcoRI/XhoI, respectively. The fragments were then cloned into the pET28a (Invitrogen, USA) to generate recombinant plasmid pET28a-scFv-tP, and sequenced. In addition, the scFv gene fragment was cloned into pET28a to generate the recombinant plasmid pET28a-scFv. The schematic diagram of the pET28a-scFv-tP is shown in Fig. 1.

2.2. Expression, purification, and refolding of fusion protein

To induce the recombinant scFv-tP expression, the plasmid pET28a-scFv-tP was transformed into *E. coli* BL21 (DE3) competent cells, and the expression of scFv-tP was induced using 1 mM IPTG for 6 h at 35 °C. The bacteria were harvested and lyzed, and the inclusion bodies were enriched by centrifuging, followed by washing with buffer (2 M urea, 50 mM Tris, 1% Triton X-100, and 100 mM NaCl, pH 7.5) [11]. Subsequently, the inclusion bodies were solubilized in 50 mM Tris (pH 8.0) containing 8 M urea overnight at 4 °C, centrifuged at 15,000 × g for 30 min at 4 °C and filtered through a 0.45 μm membrane. The scFv-tP protein was further purified by affinity chromatography using a HiTrap Ni–agarose column (GE Healthcare, USA) and 250 mM imidazole for elution. The eluted fusion protein was dialyzed against 2 L of refolding buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM reduced glutathione, and 0.4 mM oxidized glutathione, pH 8.0) containing decreasing urea gradient (8.0 M, 4.0 M, 2.0 M, and 0.0 M) at 4 °C for 24 h. After centrifuging, the fusion protein solution was concentrated with a Spin Concentrator (6 mL/10 kDa, Novagen, UK). The expression, purity, and immunoreactivity of the scFv-tP fusion protein were characterized by SDS-PAGE and Western blot assays using mouse anti-His IgG (1:1000, Merck, USA). The scFV protein was generated by the same procedure.

2.3. Influenza A virus-infected cell-based ELISA

MDCK cells (4.5 × 10^4/well) were cultured in 10% FCS DMEM medium in 96-well culture plates (Corning, USA) at 37 °C with 5% CO_2 overnight. The cells were uninfected (negative controls) or infected with 50 PFU of H5N1 virus (A/Tiger/HarBin/01/2002) for 20 h. The cells were fixed with 10% formalin for 15 min at room temperature and the remaining spaces in wells were blocked with 3% BSA in PBS at 37 °C for 1 h. Subsequently, the cells were in triplicate probed with different concentrations of scFv-tP or scFv at 37 °C for 2 h. After washing, the bound scFv or scFv-tP was detected using mouse anti-His IgG antibodies (1:1000) at 30 °C for 1 h and then developed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Merck, USA), and substrate TMB (TIANGEN, China), followed by adding 1 M H_2SO_4. The absorbance was measured at 450 nm using an ELISA reader (Thermo Fisher, USA).

2.4. Indirect immunofluorescence assay (IFA)

MDCK cells were uninfected (negative controls) or infected with AIV, and probed with 2.64 μg/mL of scFv-tP or scFv, followed by detection with anti-His IgG, as described above. The immunoreactivity of scFv-tP to H5N1 virus was characterized by FITC-conjugated goat anti-mouse IgG (Sigma, USA) and observed under a fluorescent microscope (Olympus, Japan).

2.5. Gel-shift assay and electrophoretic mobility shift assay (EMSA)

The plasmid pET28a (1 μg) was mixed with or without different amounts of scFv or scFv-tP in 0.2 M NaCl solution at room temperature for 30 min. The mobility of individual samples was characterized by electrophoresis on 1% agarose gels and imaged. In addition, the HA gene (about 1800 bp) was amplified from the recombinant plasmid pMD18T-HA in the presence of ^{32}P-dCTP for radiolabeling (Furui, China). The radiolabeled PCR products were interacted with or without different amounts of the scFv or scFv-tP in 0.2 M NaCl solution at room temperature for 30 min. The mobility of individual samples was then characterized by electrophoresis on 5% polyacrylamide gels, followed by auto-radiography.

2.6. MDCK cells transfection assay

The scFv-tP-mediated DNA transfection was determined using the EGFP-expressing plasmid pGenesil-1 (GeneSil, China). MDCK cells (4.5 × 10^4/well) were cultured in 10% FCS DMEM in 96-well culture plates overnight and then uninfected (negative controls) or infected with 50 PFU of AIV for 10 h. Subsequently, the cells were transfected with 30 pM plasmid pGenesil-1 in liposome (Invitrogen, USA) or with a mixture of 30 pM plasmid pGenesil-1 with scFv or scFv-tP at a molar ratio of 1:6 in DMEM, and then cultured in complex medium for 12–24 h at 37 °C. The expression of EGFP was observed under a fluorescent microscope.

2.7. Flow cytometry assay

To determine whether the scFv-tP could deliver DNA preferably into the AIV-infected cells, MDCK cells were cultured in 6-well culture plates and infected with AIV for 12 h. The uninfected MDCK

cells were used as negative controls. Subsequently, the cells were further transfected with 30 pM FITC-ODN in liposome or a mixture of 30 pM FITC-ODN with scFv or scFv-tP at a molar ratio of 1:6 for 6 h. The cells were harvested, and the transfection efficacy was analyzed by flow cytometry assay on a BD FACSCalibur (BD, USA). The sequence of FITC-ODN was 5′-GTGTAGTCCGCTTTGGTG-3′.

2.8. Assay of virus titers in MDCK cells

To examine whether the scFv-tP could facilitate the AIV-specific siRNA transfection, increasing the antiviral activity of siRNA, we constructed the plasmids that could express the siRNA NP1496 (GGAUCUUAUUUCUUCGGAGTTTTCCUAGAAUAAAGAAGCCUC) or control siRNA (ACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGT), respectively, as previously described [12]. MDCK cells were cultured in 6-well culture plates and infected with AIV for 12 h. The uninfected MDCK cells were used as negative controls. After washing, the cells were transfected with 30 pM of the siRNA plasmid mixed with scFv or scFv-tP at a molar ratio of 1:6 for 24 h. The supernatants were harvested and the titers of AIV were measured for 50% tissue culture infective dose (TCID50). MDCK cells were infected in triplicate with or without serial diluted virus samples in 96-well culture plates for 2–3 days. The cytopathic effect (CPE) was examined under a light microscope and the titers of individual viral samples were calculated by Reed–Muench method [13].

The cells that had been transfected with the plasmid for control siRNA or NP-specific siRNA in liposome and a mixture of plasmid with scFv or scFv-tP, together with the control cells infected with AIV alone or infected with AIV and trasnfected with the mixture of plasmid for control siRNA and scFv-tP, were harvested. Total RNA was extracted from individual cell samples using Trizol reagent (GIBCO, USA) and reversely transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA) and poly-T oligonucleotide primer (5′-TTTTTTTTTTTTTT-3′, Takara, Japan). The levels of NP mRNA transcripts were determined by quantitative real-time PCR using SYBR Premix Ex Taq Kit (Takara, Japan). The PCR reactions (20 μL) was in duplicate performed at 94 °C for 2 min and subjected to 35 cycles of 94 °C for 15 s and 61 °C for 31 s on an ABI PRISM 7000 (Applied Biosystems, USA). The sequences of special primers were forward 5′-CTAATTCTGTACGACAAAGAGG-3′ and reverse 5′-ATATCATCAGGTGGGTAAGAC-3′ for NP (94 bp), and forward 5′-ATGTATCAGTTGTGGATCTGACCTG-3′ and reverse 5′-ATGCCTGCTTCACTACCTTCTTG-3′ for GAPDH (86 bp). The relative levels of NP mRNA transcripts to control GAPDH were analyzed with ABI PRISM 7000 software and calculated by double standard curves method.

2.9. Statistical analysis

The experimental data were expressed as mean ± SD. The difference among groups was analyzed by one-way ANOVA analysis and Student's *t*-tests using SPSS 13.0 software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and purification of scFv-tP

To generate recombinant scFv-tP protein, the DNA fragment, approximately 720 bp, for scFv was first amplified by PCR and cloned into the pET28a to generate the plasmid of pET28a-scFv. In addition, the synthesized oligonucleotides for the linker and the tP were inserted into pET28a-scFv to form the pET28a-scFv-tP. After sequencing, these plasmids were transformed into *E. coli* BL21, respectively, and the expression of recombinant scFv or scFv-tP proteins was induced by IPTG (Fig. 2A). The recombinant scFv and scFv-tP, as expected, had molecular weights of 32 kDa and 28 kDa, and occupied about 35% of total proteins primarily located on the insoluble inclusion body. Following sonication and washing with 2 M urea, the enriched inclusion bodies were solubilized in 8 M urea and subjected to purification by Ni^{2+}-IDA affinity chromatography. The purified scFv and scFV-tP were refolded by dialysis against gradually deceasing urea gradient. The generated scFv and scFv-tP had a purity of 92% and were recognized by anti-His IgG (Fig. 2B and C). Approximately, 7–8 mg of refolded scFv-tP and scFv were yielded from 1 L of bacterial culture in the experimental conditions. The successful generation of recombinant scFv and scFv-tP proteins provides useful reagents for testing their bioactivity.

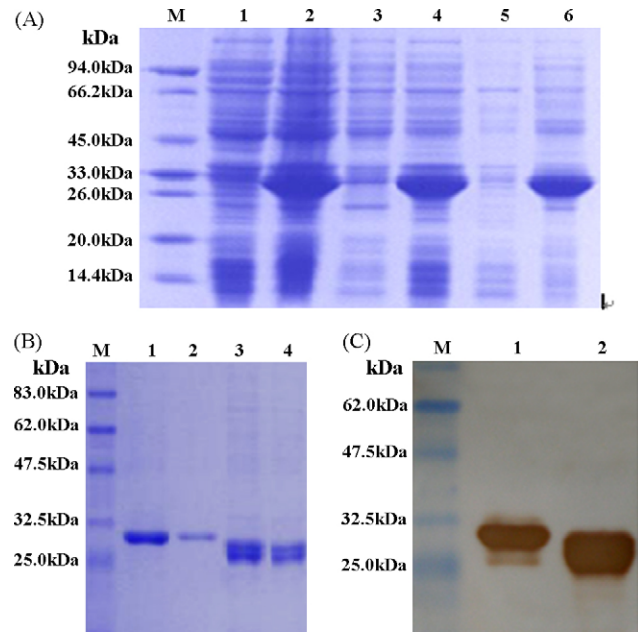

Fig. 2. Expression and purification of recombinant scFv-tP and scFv. The pET28a-scFv-tP and pET28a-scFv plasmids were transformed into *E. coli* BL21, respectively, and the expression of recombinant scFv-tP and scFv was induced by IPTG. The expressed recombinant proteins were resolved on 12% SDS–PAGE, stained with Coomassie blue R-250, and further analyzed by Western blot analysis. (A) SDS-PAGE analysis of recombinant proteins. M: protein markers; lane 1: the uninduced total bacteria proteins; lane 2: the induced total bacteria proteins; lane 3: the supernatant from IPTG induced bacterial culture; lane 4: lysates of inclusion bodies in 2 M urea; lane 5: the supernatant of inclusion bodies in 2 M urea after centrifugation; lane 6: lysates of inclusion bodies in 8 M urea. (B) Characterization of the purified recombinant proteins. Lane 1: the purified scFv-tP; lane 2: the refolded scFv-tP; lane 3: the purified scFv; lane 4: the refolded scFv. (C) Western blot analysis of recombinant scFv-tP and scFv. The recombinant scFv-tP and scFv were subjected to SDS-PAGE and transferred on NC membrane. After blocked, these proteins were probed with and anti-His antibodies and visualized using HRP-anti-mouse IgG and DBA. M: protein markers; lane 1: immunoblotting of scFv-tP; lane 2: immunoblotting of scFv. Data shown are representative images from three independent experiments.

3.2. Recombinant scFv and scFv-tP recognize AIV

To determine whether the recombinant scFv-tP and scFv retained their capacity to recognize the specific antigen of H5N1 virus, MDCK cells were uninfected (negative control) or infected with H5N1 AIV overnight. The immunoreactivity of the recombinant scFv-tP and scFv was determined by virus-infected cell-based ELISA using scFv or scFv-tP as the primary antibody, respectively. Both scFv and scFv-tP failed to bind to AIV-uninfected MDCK cells because the OD values in the scFv-tP-probed AIV-uninfected cells were similar to that of un-probed cells (Fig. 3A). Similar negative results were obtained by probing with scFV (data not shown).

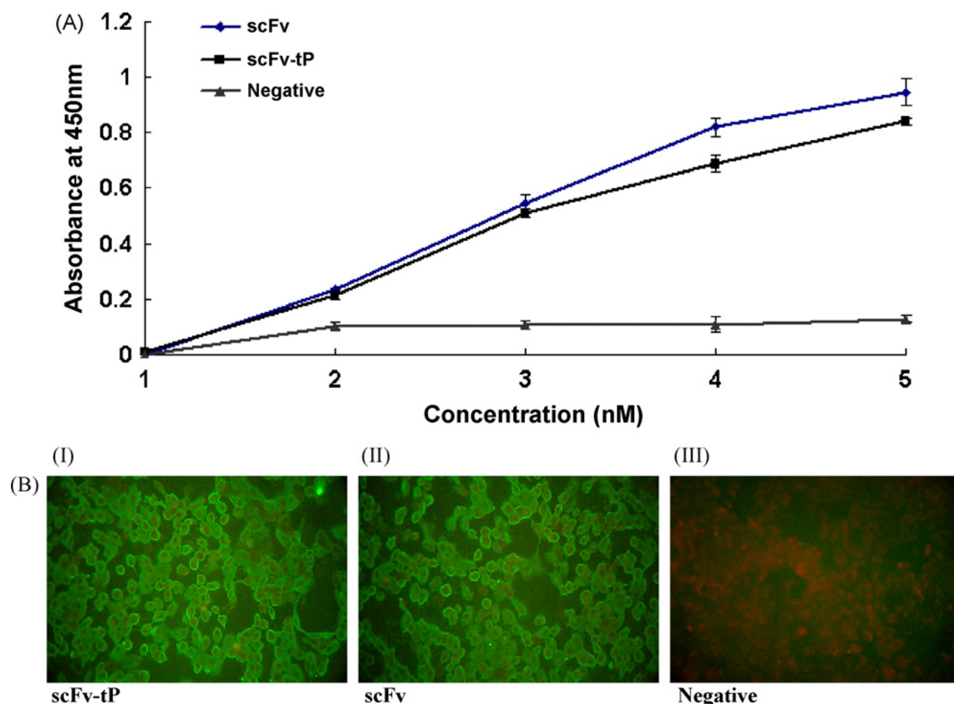

Fig. 3. Recombinant scFv-tP and scFv recognize the HA antigen. The immunoreactivity of recombinant scFv-tP and scFv was characterized by a cell-based ELISA and indirect immunofluorescent assays. (A) ELISA analysis of recombinant scFv-tP and scFv. MDCK cells were uninfected or infected with AIV and probed with different concentrations of scFv-tP or scFv. Subsequently, the bound scFv-tP and scFv were detected sequentially by anti-His, HRP-anti-mouse IgG, and TMB, followed by measurements at 450 nm. The AIV-uninfected cells probed with scFv-tP were used as negative controls. Similar background levels of OD values were observed in the cells uninfected with AIV and probed with scFV (data not shown). Data are expressed as mean ± SD of the OD values from three independent experiments. (B) Indirect immunofluorescent assay. MDCK cells were infected with AIV and probed with 2.64 μg/mL of scFv-tP or scFv, followed by detecting with FITC-anti-mouse IgG. The uninfected MDCK cells probed with scFV-tP were used as negative controls. The immunoreactivity of recombinant scFv-tP or scFv was visualized under a fluorescent microscope. I: scFv-tP; II: scFv; III: negative. Data shown are representative images of three separate experiments.

Furthermore, both scFv and scFv-tP had similar immunoreactivity against the HA on the AIV-infected MDCK cells and their antibody activities appeared to be dose-dependent (Fig. 3A). The OD values in the scFv and scFv-tP-probed cells were not significant difference at any of the tested concentrations ($p > 0.05$). In parallel, indirect immunofluorescent assay revealed that scFv-tP and scFv had comparable activity to recognize its specific antigen on the AIV-infected MDCK cells since similar fluorescent intensity was observed in both scFv and scFv-tP-probed AIV-infected cells (Fig. 3B). These data clearly indicate that scFv-tP can recognize its H5N1 viral antigen *in vitro* and has immunreactivity similar to that of scFv.

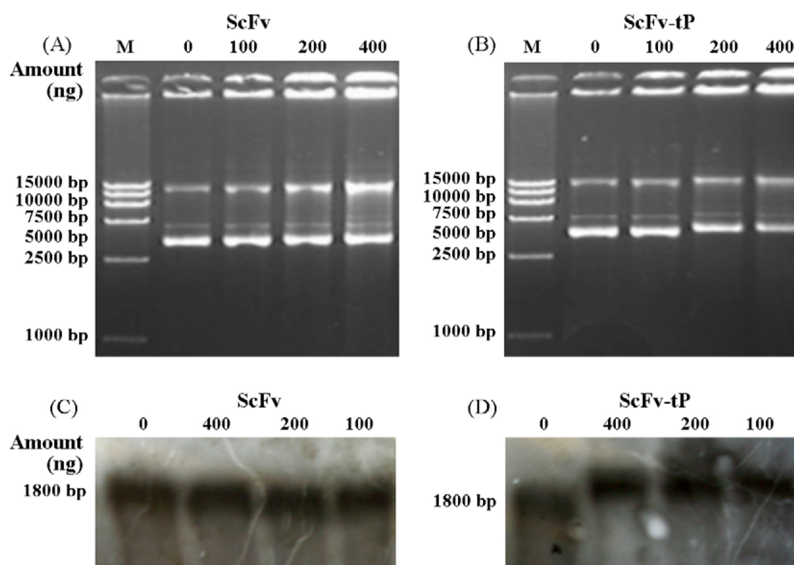

Fig. 4. Recombinant scFv-tP, but not scFv, binds to DNA. The binding ability of recombinant scFV-tP and scFv to DNA was characterized by EMSA. (A and B) The scFv-tP binds to plasmid DNA. Plasmid pET28 DNA 1 μg was mixed with different amounts of scFv-tP or scFv for 30 min, respectively and the mixtures were resolved on agarose gel electrophoresis. (C and D) The scFv-tP binds to DNA fragment. The DNA fragment of the partial HA gene (1.8 kb) was amplified in the presence of ^{32}P-dCTP for radiolabeling. After the purification, the radiolabeled DNA fragment was reacted with different amounts of scFv-tP or scFv for 30 min, respectively, and analyzed by electrophoresis, followed by radioautography. Data shown are representative images from three separate experiments.

3.3. Recombinant scFv-tP, but not scFv, binds to DNA

Protamine can bind to DNA and carries DNA through cell membrane [14]. To determine whether the generated recombinant scFv-tP and scFv could bind to DNA, the plasmid DNA was mixed with different doses of scFv-tP or control scFv, and their mobilizing behaviors were characterized by agarose gel electrophoresis (Fig. 4). The plasmid DNA migrated at the same rate regardless of whether it was mixed with scFv at any of the tested doses, indicating that scFv did not bind to the plasmid DNA (Fig. 4A). In contrast, when the plasmid DNA was mixed with scFv-tP, it moved slowly (Fig. 4B), suggesting that the scFv-tP bound to the plasmid DNA, forming the DNA/scFv-tP complex. Furthermore, the binding of scFv-tP to DNA was demonstrated by EMSA. While the radiolabeled HA DNA fragment that had been mixed with scFv migrated at the same rate as radiolabeled DNA alone, the movement of the radiolabeled HA DNA that had been mixed with scFv-tP was clearly slower than that of the DNA alone (Fig. 4C and D). Collectively, these data indicated that the scFv-tP, but not scFv, bound to DNA, forming the DNA/scFv-tP complex in our experimental system.

3.4. Recombinant scFv-tP, but not scFv, facilitates the DNA transfection preferably into H5N1 virus-infected MDCK cells in vitro

Given that the scFv-tP had anti-HA activity and could bind to DNA, it might facilitate the DNA transfection preferably into H5N1 virus-infected cells. MDCK cells were uninfected or infected with H5N1 virus and subsequently transfected with a mixture of the plasmid pGenesil with the scFv-tP, positive control liposome or negative control scFv. The transfection efficacy of EGFP-expressing cells was examined (Fig. 5A). The cells that had been transfected with the mixture of the plasmid and scFv had no EGFP-expressing cells regardless of whether these cells were infected with AIV, suggesting that the scFv was unable to mediate the transfection of plasmid pGenesil into MDCK cells. Furthermore, the cells that had

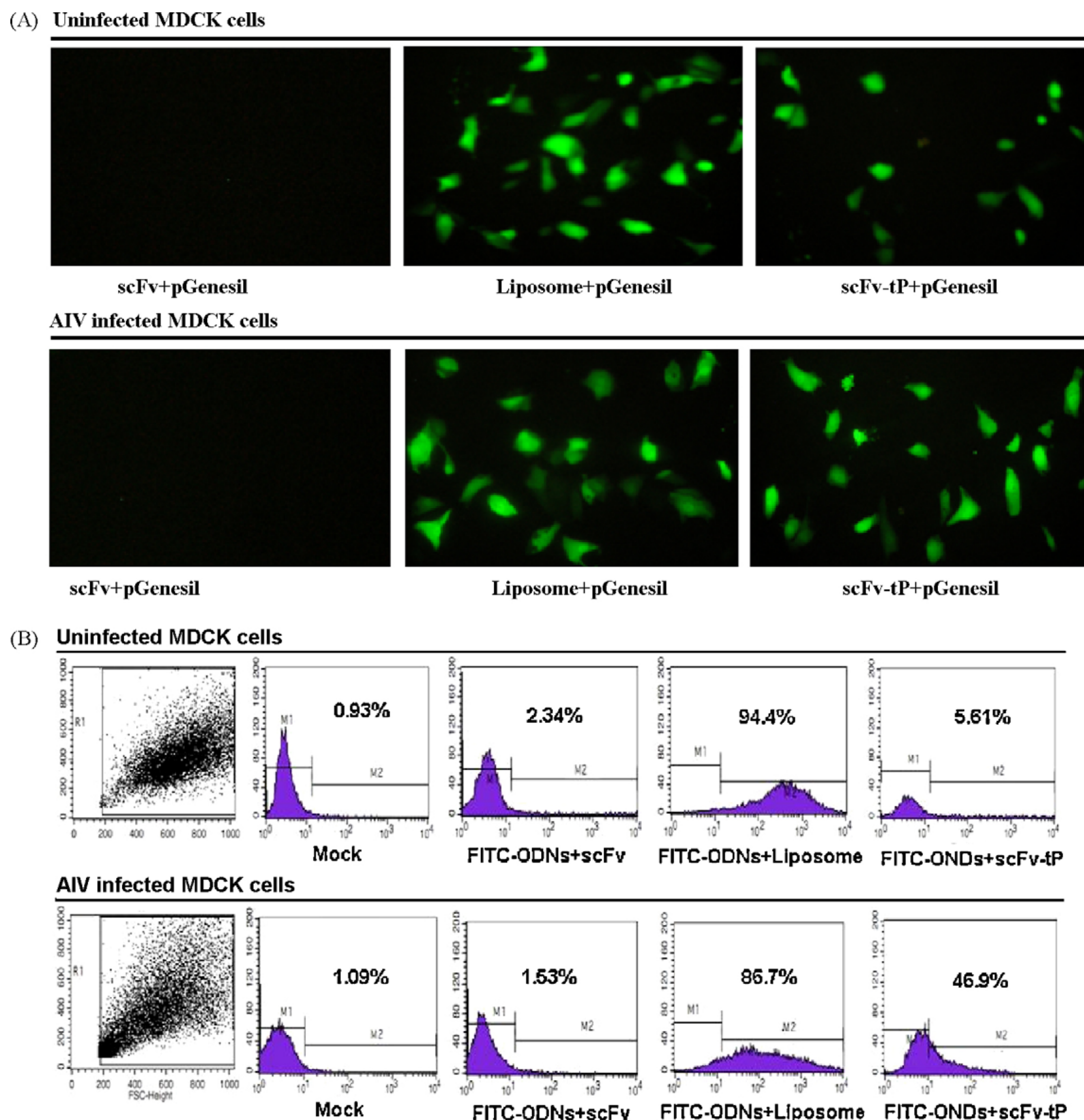

Fig. 5. Recombinant scFv-tP, but not scFv, delivers plasmid DNA and ODNs preferably to the AIV-infected MDCK cells. (A) The scFv-tP mediates the transfection of plasmid preferably into the AIV-infected cells. MDCK cells were uninfected or infected with AIV, and transfected with the plasmid of pGenesil mixed with liposome, scFv-tP or scFv, respectively, and the expression of EGFP in the transfected cells was observed under a fluorescent microscope. Similar density of cells was observed among these groups (data not shown). (B) The scFv-tP mediates the transfection of ODNs preferably into the AIV-infected cells. MDCK cells were uninfected or infected with AIV, and transfected with FITC-ODNs mixed with liposome, scFv-tP, or scFv, respectively. The transfection efficacy was determined by FACS analysis. Data shown are representative images of different groups of cells from three independent experiments. The AIV-infected and uninfected MDCK cells were simultaneously tested.

been transfected with the plasmid in liposome displayed comparable frequency of EGFP+ cells in both the H5N1 virus-infected and uninfected cells with similar fluorescent intensity, indicating that the cells infected with AIV were capable of accepting foreign DNA. Interestingly, while the AIV-uninfected cells that had been transfected with the mixture of plasmid and scFv-tP displayed lower frequency of EGFP+ cells, the AIV-infected cells transfected with the same amount of plasmid and scFv-tP presented high frequency of EGFP+ cells. The frequency of EGFP+ cells and their fluorescent intensity were similar to that of the positive controls.

To further determine whether scFv-tP was capable of mediating DNA transfection, the AIV-infected and uninfected MDCK cells were transfected with FITC-ODNs in liposome, mixed with scFv-tP or scFv and the transfection efficacy was determined by FACS analysis. While transfection with FITC-ODN/scFv did not effectively deliver FITC-ODNs into the cells, transfection of the cells with FITC-ODNs/liposome delivered the ODNs into 94.4% and 86.7% of uninfected and infected cells, respectively. Notably, while transfection with FITC-ODN/scFv-tP delivered ODNs into 5.61% of uninfected cells, the same transfection resulted in 46.9% of AIV-infected cells carrying the FITC-ODNs. The data clearly demonstrated that scFv-tP facilitated the plasmid and ODN transfection, preferably into the AIV-infected MDCK cells.

3.5. *Recombinant scFv-tP, but not scFv, enhances the anti-AIV activity of the NP-specific siRNA in MDCK cells*

We examined whether the scFv-tP could deliver the plasmid expressing the NP-specific siRNA into the AIV-infected MDCK cells and enhance the anti-AIV activity of a NP-specific siRNA against the viral replication. MDCK cells were infected with AIV and transfected with the plasmid in liposome, a mixture of plasmid with scFv or scFv-tP, respectively. MDCK cells that had been infected with AIV alone or infected with AIV and transfected with the plasmid for the control siRNA were used as negative controls. The AIV titers in the supernatants measured in Fig. 6A. The AIV titers in the cells transfected with the mixture of the plasmid for the NP-siRNA and scFv-tP were significantly lower than that of negative controls, even slightly lower than that of liposome controls. Further analysis of the relative levels of NP mRNA transcripts to control GAPDH revealed a similar pattern of transfection effects on viral replication (Fig. 6B). Therefore, the data indicated that scFv-tP enhanced the transfection efficacy and increased the antiviral activity of the NP-specific siRNA against AIV replication in MDCK cells.

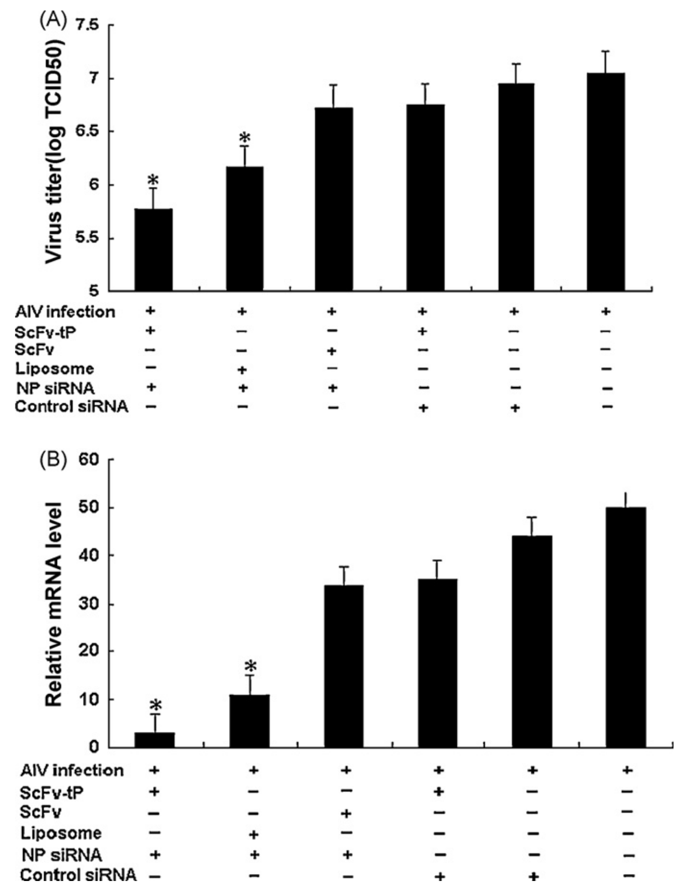

Fig. 6. Recombinant scFv-tP significantly enhances the anti-AIV activity of the NP-specific siRNA against the viral replication. (A) The AIV titers. MDCK cells were infected with AIV and transfected with the plasmid for control siRNA alone (negative control), the mixture of control siRNA plasmid and scFv-tP, the mixture of NP-specific siRNA plasmid with liposome, scFv-tP or scFv, respectively. The viral titers in the supernatants of cultured cells were determined by the CPE-based TCID50. Data are expressed as mean log TCID50/mL ± SEM of individual groups of cells from three separate experiments. (B) Analysis of the NP mRNA transcripts. The cells were harvested and the relative levels of NP mRNA transcripts to control GAPDH were determined quantitative RT-PCR. Data shown are mean ± SD of the relative levels of mRNA transcripts to control GAPDH of individual groups of cells from three separate experiments. The experimental and control groups of cells were tested simultaneously. $^{*}p < 0.05$ vs. control cells infected with AIV alone, determined by Student's *t*-test.

4. Discussion

Several strategies have been designed for targeting drug delivery [15–17], among which antibody-mediated delivery holds great promise. To successfully apply antibody-mediated delivery of the targeted drugs for therapy of influenza viral infection, it is important to identify the specific targets on the AIV-infected cells. During the AIV infection and replication, large amounts of AIV-specific HA molecules are presented on the surface of infected cells, providing an excellent target for drug delivery. Protamine is a relatively small polycationic peptide, and contains many arginine residues [14]. The protamine can condense DNA into compact particles, which promotes DNA uptake, increasing the transfection efficiency, and delivering the DNA from the cytoplasm into the nucleus of the cells [18]. Moreover, the protamine can also bind to siRNA to form protein/siRNA complex, delivering RNA into many types of cells, and the protamine-based systemic delivery has shown to be a reliable and safe approach to maximize effectiveness of siRNA delivery for treatment of cancer and viral disease [19,20]. Recently, treatment with the scFv-tP fusion proteins in combination with the specific siRNAs has been reported to specifically inhibit the replication of HIV and HBV and the proliferation of breast cancer cells [21,22]. These data indicate that these scFv-tP proteins have specific immunoreactivity against the antigen and can deliver the specific siRNA into the target cells effectively. Their immunoreactivities depend on the parent antibodies [15,16,18], and they have lower immunogenicity because of their simple monovalent structure and smaller molecule weight. However, currently there is no recombinant scFv-tP fusion protein for AIV.

In this study, we chose the *E. coli* system for the generation of these recombinant proteins because genetic engineering of foreign proteins in *E. coli* usually results in high levels of protein expression with low costs. Furthermore, high levels of foreign protein expression commonly accumulate as inclusion bodies in the cytoplasm of bacteria [23], even in SF-9 cells [24], which can be easily purified. Accordingly, we constructed new plasmids of pET28-scFv and pET28-scFv-tP by cloning the VH and LH genes for anti-HA monoclonal antibody alone, or linked with oligonucleotides for the 22-amino acid of the truncated protamine in pET28a, respectively, for induction of recombinant proteins in *E. coli*. After transformation and induction with IPTG, the expressed scFv-tP fusion proteins

consisted of about 35% of the total bacterial proteins and were predominantly located in the inclusion body of *E. coli*. Following extraction of inclusion body and washing with 2 M urea, the fusion protein-contained inclusion bodies were two-fold enriched. The scFv-tP and scFv proteins were purified by affinity chromatography using His-Ni+ column and refolded, yielding 7–8 mg from 1 L of bacterial culture with a purity of near 92%. The successful generation of recombinant scFv-tP and scFv against AIV provided useful reagents for biological study.

We characterized the HA-specific scFv-tP and found that the scFv-tP recognized the HA in the AIV-infected cells and its immunoreactivity was dose-dependent, similar to that of scFv although its immunoreactivity was lower than that of its native antibody. The low immunoreactivity of scFv-tP is likely stemmed from its monovalent nature [25,26]. Furthermore, scFv-tP, but not scFv, effectively bound to DNA and delivered the plasmid and ODN into the AIV-infected cells preferably, consistent with previous reports [21,22]. The transfection efficacy of plasmid and ODN mediated by the scFv-tP to the AIV-infected cells was similar to that of previous reports [21,22]. More importantly, scFv-tP, but not scFv, significantly enhanced the anti-AIV activity of the plasmid encoding NP-specific siRNA in inhibiting AIV replication in MDCK cells. It was shown that the viral titers and levels of NP mRNA transcripts in the cells that had been transfected with a mixture of the plasmid for the NP-specific siRNA and scFv-tP were significantly lower than that of the control cells infected with AIV alone or infected with AIV and transfected with the plasmid for control siRNA, even lower than that of the siRNA/liposome-transfected cells. This suggests that the ability of the scFv-tP to deliver the plasmid for NP-specific siRNA into the AIV-infected cells is higher than that of the liposome control. The enhanced effects of the scFv-tP were mediated by the facts that the scFv-tP not only effectively delivered the plasmid for NP-specific siRNA into the AIV-infected cells, but also might inhibit the spreading of AIV by neutralizing the virus slightly [5,6]. Furthermore, liposome has moderate levels of cytotoxicity against many types of cells [27,28]. Therefore, the HA-specific scFv-tP may be a better reagent for the delivery of the plasmid for AIV-specific siRNA for the gene therapy of AIV infection.

In summary, we successfully generated the recombinant HA-specific scFv-tP and demonstrated that the scFv-tP effectively recognized the HA of AIV, bound and delivered DNA into the AIV-infected cells *in vitro*. Furthermore, we found that scFv-tP significantly enhanced the anti-AIV activity of the plasmid for the NP-specific siRNA in inhibiting the replication of AIV in MDCK cells *in vitro*. We are interested in further evaluating its biological activity and safety *in vivo*.

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