Construction of a disulfide-stabilized diabody against fibroblast growth factor-2 and the inhibition activity in targeting breast cancer

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Fibroblast growth factor-2 (FGF-2) is one of the most important angiogenic factors to promote tumor growth, progression and metastasis. Neutralizing antibodies against FGF-2 may suppress the growth of tumor cells by blocking the FGF-2 signaling pathway. In this study, a disulfide-stabilized diabody (ds-Diabody) that specifically targets FGF-2 was designed. Compared to its parent antibody, the introduction of disulfide bonds in the diabody could significantly increase the stability of ds-Diabody and maintain its antigen binding activity. The ds-Diabody against FGF-2 could effectively inhibit the tube formation and migration of vascular endothelial cells and block the proliferation and invasion of human breast cancer cells. In the mouse model of breast cancer xenograft tumors, the ds-Diabody against FGF-2 could significantly inhibit the growth of tumor cells. Moreover, the densities of microvessels stained with CD31 and lymphatic vessels stained with LYVE1 in tumors showed a significant decrease following treatment with the ds-Diabody against FGF-2. Our data indicated that the ds-Diabody against FGF-2 could inhibit tumor angiogenesis, lymphangiogenesis and tumor growth.

Angiogenesis plays a pivotal role in tumor growth, progression and metastasis.[1] Strategies targeting angiogenesis have been extensively studied, providing substantial data supporting the potential of angiogenesis targeting for cancer therapy and prevention.[2] Among many angiogenic factors overexpressed in tumors, the fibroblast growth factor 2 (FGF-2) and the vascular endothelial growth factor (VEGF) are the most important factors.[3] Agents that target VEGF and its receptors have shown promising activity in clinical trials and clinic therapy. The anti-VEGF monoclonal antibody (mAb) bevacizumab (Avastin; Genentech, South San Francisco, CA, USA) approved by the Food and Drug Administration of America (2004) has demonstrated good therapeutic effects in cancer therapy.[4] However, when used for a long time, patients may develop resistance to this antibody drug. One proposed mechanism of tumor escape from anti-VEGF therapy is the compensatory upregulation of fibroblast growth factor-2 (FGF-2) and platelet derived growth factor (PDGF).[5] Furthermore, as stated above,

FGF-2 blockade could impair tumor progression in the evasion phase of anti-VEGF therapy.[6]

Fibroblast growth factor-2 (also named basic fibroblast growth factor, bFGF) is a pleiotropic angiogenesis inducer belonging to the family of the heparin-binding FGF growth factors.[7] FGF-2 is highly expressed in numerous tumors and exerts its proangiogenic activity by interacting with tyrosine kinase receptors, heparin-sulfate proteoglycans and integrins expressed on the endothelial cells.[8] FGF-2/FGFR interaction leads to complex signal transduction pathways (including MAPK/ERK and PI3K/AKT) and activation of a "proangiogenic phenotype" in endothelium, which regulates proliferation, migration and survival of tumor cells.[9,10]

Blocking FGF-2/FGFR activity with antibodies should be a good therapeutic strategy, especially in tumor therapy.[11] The anti-FGF-2 murine mAbs can strongly inhibit tumor growth.[11–15] An FGF-2-neutralizing mAb was reported to inhibit angiogenesis induced by FGF-2 both *in vitro* and *in vivo*,[13] and block

vascularization and growth of chondrosarcoma in the rat.[14] Li (2010) reports that anti-bFGF-neutralizing mAbs could inhibit the growth of melanoma and tumor angiogenesis *in vivo*.[16] In our previous study, high affinity human antibodies of single chain Fv fragment (scFv) against FGF-2 were selected from a phage display library, and a full-length human antibody against FGF-2 was reconstructed.[17] The anti-FGF-2 human antibody could inhibit the proliferation and migration of glioma cells, and tube formation of vascular endothelia cells.[17]

Many attempts to reduce the size of the heterotetrameric IgG molecule (MW: 160 kDa) while retaining its antigen-binding properties have been made in relation to some critical factors for therapeutic antibodies (such as high-yield production, solubility, stability and small size).[18] This resulted in a series of antibody fragment constructs, such as diabody, which is a non-covalently associated bivalent molecule, created from scFv by shortening the polypeptide linker between the VH and VL domains.[19] Some diabodies were unstable and some showed lower affinity than full-length antibodies or Fabs because the diabody was non-covalently associated or the linker may have interfered with the antigen binding.[20] The best way to stabilize the diabody is to introduce the disulphide bond in the framework of VH and VL domains.[21–24]

Here, we report on the construction of the ds-Diabody against FGF-2 based on an scFv, the secretory expression of the target protein in *Picha pastoris*, and the effects on tumor angiogenesis and tumor growth of breast cancer.

Materials and Methods

Cells and animals. Human umbilical vein endothelial cells (HUVEC) were cultured in M199 medium (Gibco, Grand Island, NY, USA) supplemented with 2% Low Serum Growth Supplement (LSGS; Gibco), 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). Human breast cancer cells (MCF-7) were cultured in DMEM (Gibco) plus 10% FBS and 1% penicillin/streptomycin. All the cells were cultured in an incubator with 95% humidity and 5% CO_2 at 37°C.

BALB/c nude mice (female, 6–7 weeks) were purchased from the Laboratory Animal Center of Sun Yat-Sen University, Guangzhou, China. All the animals used in the experiments were treated humanely in accordance with the Institutional Animal Care and Use Committee Guidelines of Jinan University.

Construction of the ds-Diabody against fibroblast growth factor-2. The ds-Diabody against FGF-2 was constructed by site-directed mutation at the VH44 and VL100 positions by introducing cysteine residues. The mutation primers (Table S1) were synthesized in Invitrogen Life Technology (Guangzhou, China). The template of VH and VL domains for PCR was the scFv against FGF-2, which was selected from a phage display library in our lab.[17] The PCR was conducted according to the design procedures (Fig. 1). The mutation in the framework of VL100 (the Gly was replaced by Cys) was conducted by PCR with the complementary mutation primer pairs of P1/P2 and P3/P6 (Fig. 1). The full length of the VL-VH fragment (fragment III) with the mutation of VL100 was generated by splicing with overlap extension PCR (SOE PCR). Similarly, the mutation in the framework of VH44 was conducted by PCR with complementary mutation primer pairs of P1/P4 and P5/P6 (Fig. 1). The whole VL-VH fragments (fragment VI) mutated with Cys at VL100 and VH44 were assembled by SOE PCR to generate the anti-FGF-2 ds-Diabody fragment (Fig. 1). The anti-FGF-2 ds-Diabody fragment was inserted into a T vector
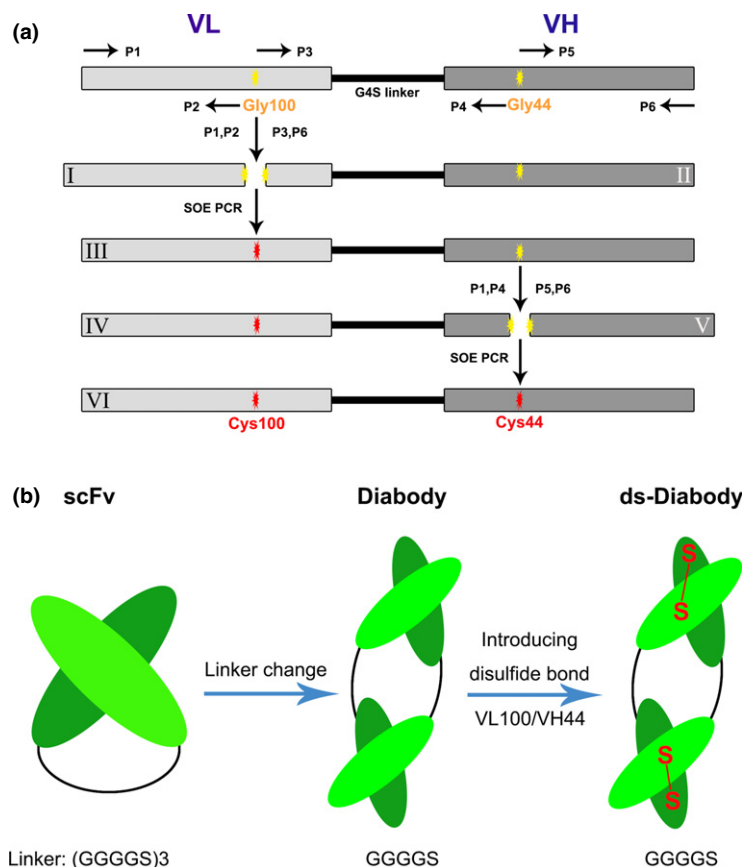
Fig. 1. Construction of the ds-Diabody against fibroblast growth factor-2 (FGF-2). (a) The ds-Diabody against FGF-2 was constructed by introducing disulfide bonds between VL and VH. (b) Schematic representation of the construction of ds-Diabody against FGF-2.

(Takara Bio, Shiga, Japan) and verified by sequencing (Sangon, Shanghai, China). The anti-FGF-2 ds-Diabody gene fragment was cut with restriction enzymes of *Not* I and *EcoR* I from the T vector and inserted into the vector of pPICZαA (Invitrogen) to construct expression recombinant plasmid pPICZαA-ds-Diabody against FGF-2.

Expression of ds-Diabody against fibroblast growth factor-2 in *Pichia pasporis*. The recombinant plasmid pPICZαA-ds-Diabody against FGF-2 was linearized with *Bgl* II and transformed into *Pichia pasporis* strain GS115 by electrotransformation. The transformants were selected in YPD (yeast extract peptone dextrose medium: 1% yeast extract, 2% peptone) plates with 50 μg/mL Zeocin (Invitrogen). The multi-copy transformants were selected by adding the concentration of Zeocin to 1 mg/mL.

The recombinant yeast transformants were cultured in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4 \times 10 - 5\%$ biotin, 1% glycerol) with 180 rpm shaking at 28°C until the OD_{600} reached approximately 20. The medium was changed to BMMY (1% methanol replaced the 1% glycerol in BMGY) for inducing culture with shaking (180 rpm) at 28°C. After 96 h of culture, the broth was collected and centrifuged with 10 000 *g* for 15 min. The supernatant was collected for purification or stored at −80°C.

Purification and identification of the ds-Diabody against fibroblast growth factor-2. The ds-Diabody against FGF-2 was purified from the expression supernatant by affinity chromatography and anion-exchange chromatography.[25–27] The target protein components were detected by reduced and non-reduced SDS-PAGE, using 12% acrylamide gels[28] followed by staining with Coomassie brilliant blue or immunoblotting. For the reduced SDS-PAGE, the samples were treated at 90°C for 10 min in loading sample buffer (250 mM pH 6.8 Tris-HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol) with 5% β-mercaptoethanol. For the non-reduced SDS-PAGE, the samples were treated at 90°C for 10 min in the loading sample buffer without β-mercaptoethanol. For western blot analysis the protein samples were separated by SDS-PAGE under reducing or non-reducing conditions and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk at 37°C for 1 h and incubated with a His-tag mouse mAb at 4°C overnight. The membrane was then incubated with the HRP-conjugated goat anti-mouse IgG for 1 h at 37°C. The blots were detected with an Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's protocol.

ELISA assay. The 96-well plates were coated with FGF-2 (50 ng/well; R&D, Minneapolis, MN, USA) at 4°C overnight and blocked with 5% non-fat milk. The purified ds-Diabody against FGF-2 and scFv against FGF-2 were added in different dilution and incubated for 1 h at 37°C. The secondary antibody of the anti-His tag monoclonal antibody was added to the plates and incubated for 1 h at 37°C. The HRP-conjugated goat anti-mouse IgG was added and incubated for 30 min at 37°C. The plates were stained with DAB and the absorbance values at 450 nm (A_{450}) were measured in an ELISA reader (BioTek, Highland Park, Winooski, VT, USA). The thermal stability of the ds-Diabody against FGF-2 and scFv against FGF-2 were evaluated by ELISA analysis of the antigen-binding activity after incubation at 37°C for several hours.

Cell proliferation assay. The breast cancer (MCF-7) cells (2000 cells/well) were transferred to 96-well plates and incubated overnight at 37°C. After serum-starved culture in DMEM with 0.5% FBS for 12 h, cells were treated with serially diluted ds-Diabodies against FGF-2 plus 15 ng/mL FGF-2 for 48 h. The controls were the full-length human IgG against FGF-2 and the irrelevant IgG. The proliferation of tumor cells was assayed with Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The A_{450} values were measured in an ELISA reader (BioTek).

Western blot assay of phosphorylation of Akt and MAPK. The MCF-7 cells (2×10^5 cells/well) were transferred in 6-well plates and serum-starved cultured in DMEM with 0.5% FBS overnight. The cells were exchanged with the medium of DMEM with 15 ng/mL FGF-2, 0.5% FBS and the ds-Diabody against FGF-2 and incubated for 30 min. The cells were washed with cold PBS and lysed in RIPA lysis buffer (Beyotime Biotechnology, Suzhou, China). The lysates were centrifuged at 12 000 *g* for 6 min at 4°C and the total proteins in the supernatant were quantified by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The proteins were separated by SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk at 37°C for 1 h and incubated with rabbit anti-t/p-MAPK (4695/4370; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-t/p-Akt (4691/4060; Cell Signaling Technology) at 4°C overnight. The membrane was then incubated with the HRP-conjugated goat anti-rabbit IgG for 1 h at 37°C. The blots were detected with an Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's protocol. The rabbit anti-GAPDH antibody (2118; Cell Signaling Technology) was used as the reference control.

Transwell assay. The effect of the ds-Diabody against FGF-2 on migration of HUVEC and monolayer invasion of tumor cells was assayed in transwell chambers (8-μm pore size; BD Biosciences, Bedford, MA, USA). HUVEC (5×10^4 cells/insert in serum-free medium containing 15 ng/mL FGF-2) were transferred to inserts of the transwell. The ds-Diabody against FGF-2 (100 μg/mL) was added to the inserts and incubated at 37°C for 16 h. The controls were the full-length human IgG against FGF-2 (100 μg/mL) and the irrelevant IgG (100 μg/mL) and the medium only. The lower chambers were DMEM with 10% FBS to act as a chemoattractant. At the end of the treatment, The cells on the upper side of the filters were mechanically removed, and those migrated into the lower side were fixed with 70% ethanol, stained by 0.1% crystal violet (Meryer, Shanghai, China) and imaged with a computerized imaging system. For the invasion assay, the upper side of the filters was coated with 45 μL Matrigel matrix (BD Biosciences) diluted (1:3) with serum-free DMEM. The MCF-7 cells were transferred to the inserts and treated with the ds-Diabody against FGF-2 as above. The invasion cancer cells were stained by crystal violet and imaged with a computerized imaging system.

Tube formation assay. Matrigel matrix (60 μL/well) was added into 96-well plates. The plates were incubated for 30 min at 37°C. HUVEC (2×10^4 cells/well) suspended in M199 complete medium plus LSGS (Gibco) and 15 ng/mL FGF-2 were transferred to each well. The purified ds-Diabody against FGF-2 (100 μg/mL) and the control antibodies were added in the wells and incubated for 6 h. The tube formation was observed under a microscope and the tube numbers were counted in five random high-power fields.

Anti-tumor activity of the ds-Diabody against fibroblast growth factor-2 *in vivo*. Human breast cancer MCF-7 cells (1×10^6 cells in 100 μL) were subcutaneously injected into

the right shoulder flank of BALB/c nude mice (15–20 g, $n = 6$). When palpable tumors (≥5 mm in diameter) developed, the mice were intravenously injected with the ds-Diabody against FGF-2 (10 mg/kg in 100 μL PBS) six times at 3 day intervals. The full-length human antibody (10 mg/kg) and the equal amount of PBS were controls. Tumor size was measured every 3 days in two dimensions using a vernier caliper. Mice were killed 24 h later after the last administration. The tumors were stripped for immunohistochemistry assays and weighed to calculate the tumor growth inhibition rate. Tumor volume (mm^3) was calculated as $V = 1/2(a \times b^2)$. a = tumor length, b = tumor width; tumor growth inhibition rate = (1 − the average tumor weight of treated groups/the average tumor weight of PBS group) × 100%.

Stability of the ds-Diabody against fibroblast growth factor-2 in vivo. The blood samples (20 μL) of mice were collected from the caudal vein at 1, 71, 143 and 215 h after the first injection of antibodies (namely, 1 h after the first injection, and 1 h before the second, the third and the fourth injection). The FGF-2 antigen-binding activity in blood samples were detected by ELISA. For the detection of the ds-Diabody against FGF-2, the secondary antibody was anti-His tag monoclonal antibody (1:5000 dilution). For the detection of full-length human IgG against FGF-2, the secondary antibody was HRP-conjugated goat anti-human IgG.

Immunohistochemical analysis. The expression of CD31 and LYVE1 in tumor tissues was analyzed by immunohistochemistry to determine the intratumoral microvessel density and lymphatic vessel density. Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut at 5 μm. After deparaffinization, the sections were heated for 10 min at 100°C in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval and treated with 3% H_2O_2 for 15 min at room temperature to block endogenous peroxidase activity. The sections were blocked in 3% BSA for 1 h and incubated with rabbit anti-CD31 polyclonal antibodies (1:50 dilution, ab28364; Abcam, Cambridge, UK) and rabbit anti-LYVE1 polyclonal antibody (1:500 dilution, ab14917; Abcam). The sections were incubated with secondary antibody of biotinylated goat anti-rabbit polyclonal antibody and avidin-HRP for 1 h. The sections were stained with DAB and hematoxylin. The vessels density was determined by counting the number of microvessels and lymphatic vessels in five random high-power fields within the sections, as described.[16]

Statistics analysis. Statistical comparisons were analyzed by one-way ANOVA followed by the least significant difference test. Data are presented as mean ± SD. P-values < 0.05 (*) and $P < 0.01$ (**) were considered statistically significant.

Results

Construct of the ds-Diabody against fibroblast growth factor-2. The ds-Diabody against FGF-2 was constructed based on the VL and VH domain of anti-FGF-2 scFv scanned from a phage display library.[17] The VL and VH domain were bound together with a 5 amino acid linker (GGGGS) to construct the diabody. Based on the diabody, site-directed mutation at the sites of VL100 and VH44 was conducted by introducing cysteine residues to construct the ds-Diabody against FGF-2 (Fig. 1a). The nucleotide sequence encoding the ds-Diabody against FGF-2 was verified by DNA sequencing. The Gly-100 of VL domain and Gly-44 of VH domain in the diabody were successfully replaced by cysteine (Fig. 1b).

Expression, purification and identification of the ds-Diabody against fibroblast growth factor-2. The gene fragment of the ds-Diabody against FGF-2 was inserted into the vector pPICZαA (Invitrogen) to construct recombinant plasmid pPICZαA-ds-Diabody against FGF-2. The expression vector was transformed to *Pichia pastoris* strain GS115 by electroporation. The ds-Diabody against FGF-2 was expressed by methanol induction and the products were assayed by SDS-PAGE and western blot. The results showed that the anti-FGF-2 ds-Diabody could be expressed in all the transformants and the expression of transformant No. 6 was the highest (Fig. 2a). The specific ds-Diabody against FGF-2 was purified from expression supernatant of yeast by affinity chromatography and anion-exchange chromatography. The purity of the specific ds-Diabody against FGF-2 could reach 95% after purifying by affinity chromatography and anion-exchange chromatography (Fig. 2b,c). The yield of purified ds-Diabody against FGF-2 was approximately 30–50 mg/L of culture volume with the purity of 95%. Under reducing conditions, the ds-Diabody against FGF-2 showed one band at approximately 35 kDa. Under the non-reducing conditions, the ds-Diabody against FGF-2 showed one band at approximately 60 kDa (Fig. 2d,e). However, the Diabody against FGF-2 showed one band at approximately 35 kDa under both reducing and non-reducing conditions (Fig. 2d,e). The results demonstrated that the disulphide bonds have been successfully intruduced in ds-Diabody against FGF-2. The molecular weight of the ds-Diabody against FGF-2 was lower than the calculated 70 kDa due to conformational effects of intact inter-chain and intra-chain disulfide bonds.

Antigen-binding activity of the ds-Diabody against fibroblast growth factor-2. The results of ELISA indicated that the introduction of the cysteine residues and the formation of disulphide bonds in the ds-Diabody against FGF-2 did not influence its antigen-binding activity (Fig. 3a).

Stability of the ds-Diabody aginst fibroblast growth factor-2 in vitro and in vivo. The thermal stability of the ds-Diabody against FGF-2 in vitro was evaluated by ELISA. The antigen-binding capacity of the ds-Diabody against FGF-2 was approximately 100% after incubation for 96 h and remained approximately 89% even after 240 h, while the residual antigen-binding activity of scFv against FGF-2 was reduced to 50% after incubation for approximately 9 h and only 5% for 24 h (Fig. 3b).

The stability of the ds-Diabody against FGF-2 in mice was evaluated according to the residual anti-FGF-2 antibody activity in the serum of BALB/c nude mice by ELISA. As shown in Figure 4, the anti-FGF-2 ds-Diabody remained relatively stable in the treatment period. Compared with the injections, the residual anti-FGF-2 antibody activity in mice of the ds-Diabody was approximately 30–40% in the treatment period, while the full-length human IgG against FGF-2 was about 45–60%. The results indicated that the ds-Diabody against FGF-2 could remain stable in mice.

Suppression of the ds-Diabody on the fibroblast growth factor-2-mediated cell proliferation and signaling. The FGF-2 mediated cell proliferation of MCF-7 cells was suppressed by anti-FGF-2 ds-Diabdoy in a dose-dependent manner. The inhibition rate was approximately 41.5% when the concentration of ds-Diabdoy against FGF-2 was 100 μg/mL (Fig. 5).

Fibroblast growth factor-2 could activate multiple signal pathways (including MAPK/ERK and PI3K/AKT). The anti-FGF-2 antibody may result in the phosphorylation inhibition of the molecules in the signal pathways. The western blot
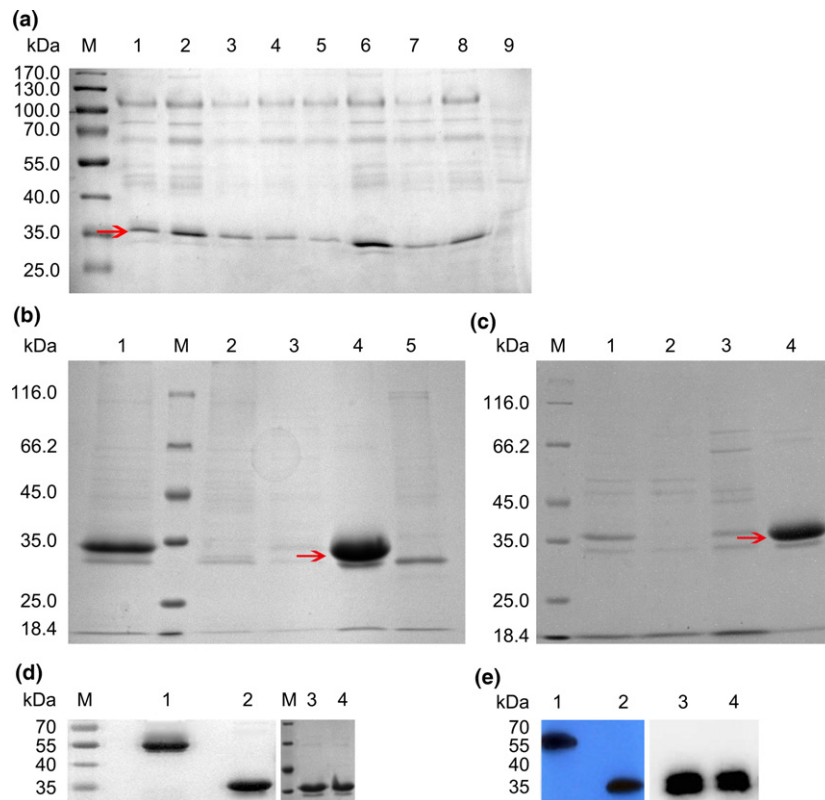
Fig. 2. Expression, purification and identification of the ds-Diabody against fibroblast growth factor-2 (FGF-2). (a) Expression analysis of the ds-Diabody against FGF-2 in different transformants by SDS-PAGE. Lane M, protein molecular weight marker; Lanes 1–8, the expression samples from different transformants; Lane 9, the expression sample of empty vector transformant. (b) SDS-PAGE analysis of ds-Diabody against FGF-2 purified by Ni Sepharose affinity chromatography. Lane 1, proteins from culture supernatant; Lanes 2–3, Fractions eluted with 50 mM imidazole; Lane 4, the target protein fractions eluted with 150 mM imidazole; Lane 5, fractions eluted with 400 mM imidazole. (c) SDS-PAGE analysis of ds-Diabody against FGF-2 purified by anion exchange chromatography. Lane 1, proteins from culture supernatant; Lanes 2–3, fractions eluted with 0.1–0.2 M NaCl; Lane 4, the target protein fractions eluted with 0.5 M NaCl. (d) The SDS-PAGE analysis of the ds-Diabody and Diabody against FGF-2 under reducing and non-reducing conditions. Lane 1, the purified ds-Diabody against FGF-2 under non-reducing condition; Lane 2, the purified ds-Diabody against FGF-2 under reducing condition; Lane 3, the purified Diabody against FGF-2 under non-reducing condition; Lane 4, the purified Diabody against FGF-2 under reducing condition. (e) Western blot analysis of the ds-Diabody and Diabody against FGF-2 under reducing and non-reducing conditions. Lane 1, the ds-Diabody against FGF-2 under non-reducing condition; Lane 2, the ds-Diabody against FGF-2 under reducing condition; Lane 3, the Diabody against FGF-2 under non-reducing condition; Lane 4, the Diabody against FGF-2 under reducing condition.

assay results showed that the ds-Diabody against FGF-2 could reduce the phosphorylation levels of Akt and MAPK in MCF-7 cells in a dose-dependent manner (Fig. 6). The results indicated that the ds-Diabody against FGF-2 could inhibit the proliferation of MCF-7 cells by blocking the signal pathway of Akt and MAPK triggered by FGF-2/FGFR.

Inhibition of the ds-Diabody against fibroblast growth factor-2 on the invasion of tumor cells. The invasive MCF-7 cells were assayed in porous membranes that had been coated with Matrigel matrix prior to loading cells in a transwell chamber. The cells in the upper chamber were chemoattracted by the serum in the lower chamber, and the invasion of MCF-7 cells was analyzed in the presence or absence of the ds-Diabody against FGF-2. The migration ratio of the MCF-7 cells in the groups of medium, the irrelevant IgG, the ds-Diaoby and the full-length human IgG against FGF-2 were 100%, 95.13 ± 2.82%, 25.13 ± 5.38% and 50.00 ± 6.92%, respectively. The results indicated that the ds-Diabody against FGF-2 could significantly inhibit the migration of MCF-7 cells to the lower chamber (Fig. 7).

Inhibition of the ds-Diabody on fibroblast growth factor-2-mediated capillary structure formation and migration of human umbilical vein endothelial cells. Angiogenesis is a complex procedure involving several kinds of cells. The maturation of endothelial cells to capillary tubes is a critical early step.[29,30] The tube formation assay was performed in Matrigel and the results showed that the anti-FGF-2 ds-Diabody could effectively inhibit the tube formation of HUVEC (Fig. 8). The rate of the tube formation in groups of medium, the irrelevant IgG, the ds-Diabody and the full-length human IgG against FGF-2 were 100%, 106.57 ± 3.14%, 50.00 ± 4.29% and 70.86 ± 2.86%, respectively.

Endothelial cell migration is a critical aspect of angiogenesis. The migration assays were also performed to estimate the ability of HUVEC to pass through the transwell membrane barrier in the presence or absence of the ds-Diabody against FGF-2. The HUVEC that had migrated to the lower chamber in the ds-Diabody against FGF-2 group were significantly reduced when compared to the irrelevant IgG group (Fig. 9). The migration rate of HUVEC in the ds-Diabody against FGF-2 group was 51.65%, while the full-length human IgG against
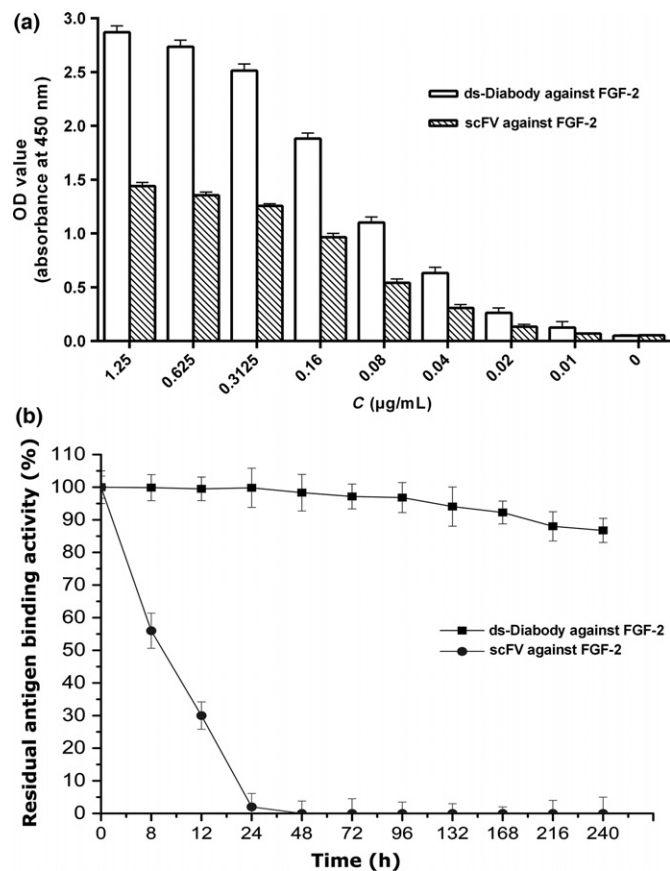
Fig. 3. Antigen-binding activity and thermal stability of the ds-Diabody and scFv against fibroblast growth factor-2 (FGF-2) were assayed by indirect ELISA. (a) Antigen-binding activity of the ds-Diabody and scFv against FGF-2. (b) The thermal stability of the ds-Diabody and scFv against FGF-2 under 37°C incubation. Data are presented as the mean ± SD of three independent experiments performed in triplicate.
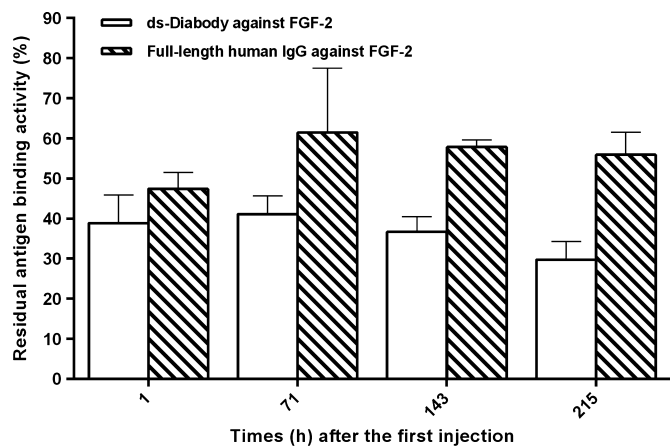

Fig. 4. The residual activity of antibodies in mouse blood. The residues antigen-binding activities of antibodies in the blood were measured by indirect ELISA at different time points. The results are presented as the means ± SD (error bars) from 6 animals.

FGF-2 group was 70.83%. This indicates that the ds-Diabody against FGF-2 could suppress the tube formation and migration of HUVEC more effectively than the full-length human IgG.

Inhibition of the ds-Diabody against fibroblast growth factor-2 on the growth of breast cancer *in vivo*. To investigate the
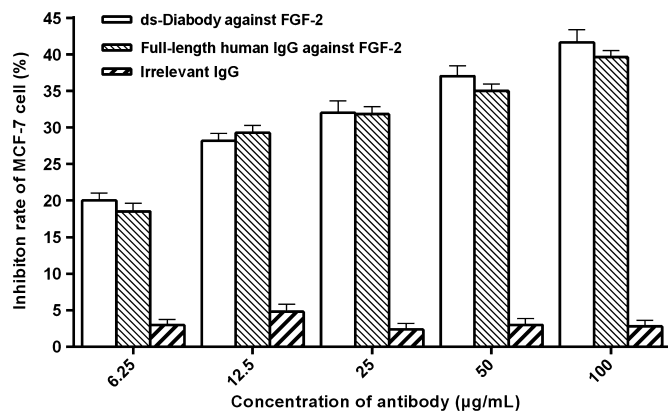
Fig. 5. Proliferation inhibition effects of the ds-Diabody against fibroblast growth factor-2 (FGF-2) on MCF-7 cells. The MCF-7 cells were transferred to 96-well plates. The cells were cultured with serum-starved in DMEM with 0.5% FBS overnight. The starved cells were treated with 15 ng/mL FGF-2 plus ds-Diabody against FGF-2 at the indicated concentrations and incubated for 48 h. The cell proliferation was assayed by CCK-8 kit. Data are presented as the mean ± SD of three independent experiments performed in triplicate.
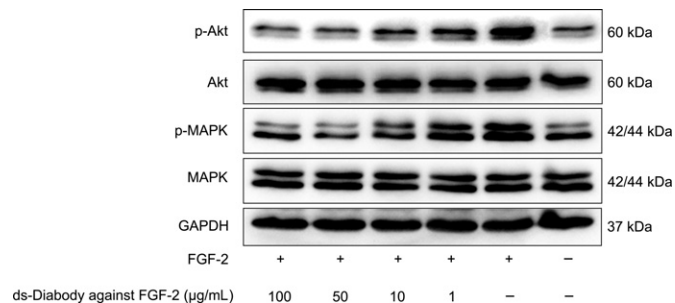

Fig. 6. Western blot assays of Akt and MAPK phosphorylation in MCF-7 cells treated with the ds-Diabody against fibroblast growth factor-2 (FGF-2). The primary antibodies were anti-MAPK, anti-p-MAPK, anti-Akt and anti-p-Akt, and GAPDH was served as the reference control.

in vivo effect of ds-Diabody against FGF-2 on MCF-7 cells growth, we established a model with MCF-7 cells implanted in BALB/c nude mice. The results showed that the ds-Diabody against FGF-2 could significantly reduce tumor burden and inhibit the growth of breast cancer in mice (Fig. 10a–c). The inhibition rate of tumor growth by the ds-Diabody against FGF-2 could reach 47.75%, while the inhibition rate of tumor growth by the full-length human IgG against FGF-2 was 17.99% (Fig. 10d).
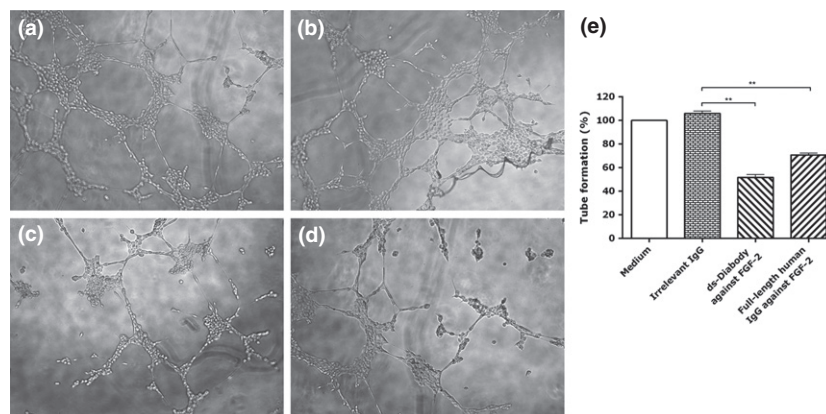
Inhibition of the ds-Diabody against fibroblast growth factor-2 on the angiogenesis and lymphangiogenesis in tumors. The FGF-2 is one of the most important angiogenic and lymphangiogenic factors, and could promote tumor angiogenesis and lymphangiogensis.[31–34] The anti-FGF-2 ds-Diabody also showed inhibition of tumor angiogenesis and lymphangiogensis in mice. The immunohistochemistry results showed that the microvessels stained with anti-CD31 antibody and lymphatic vessels stained with anti-LYVE1 antibody were significantly reduced (Fig. 11). The average number of microvessels was approximately 25.00 in the anti-FGF-2 ds-Diabody group and 65.67 in the PBS group. The average number of lymphatic vessels was approximately 7.33 in the anti-FGF-2 group and 25.00 in the PBS group. The results demonstrated that the ds-

Fig. 7. The inhibitory effect of the ds-Diabody against fibroblast growth factor-2 (FGF-2) on the invasion of MCF-7 cells. (a) MCF-7 cells were treated with DMEM serum-free medium. (b) MCF-7 cells were treated with the irrelevant IgG. (c) MCF-7 cells were treated with the ds-Diabody against FGF-2. (d) MCF-7 cells were treated with the full-length human antibody against FGF-2. (e) The quantitative analysis of MCF-7 invasion. The invasion cell numbers in the control group of medium was set as 100. The results are presented as the means ± SD. *$P < 0.05$; **$P < 0.01$.
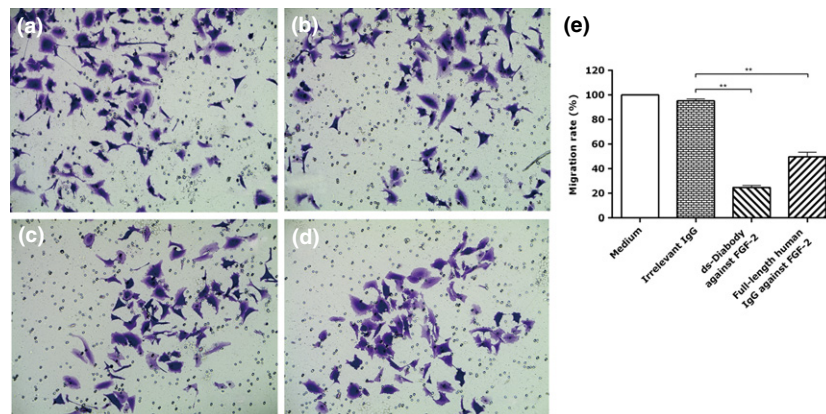
Fig. 8. The inhibitory effect of the ds-Diabody against fibroblast growth factor-2 (FGF-2) on tube formation of HUVEC. (a) HUVEC were only in serum-free M199 medium. (b) HUVEC were treated with the irrelevant IgG. (c) HUVEC were treated with the ds-Diabody against FGF-2. (d) HUVEC were treated with the full-length human antibody against FGF-2. (e) The quantitative analysis of HUVEC tube formation. The tube formation in the control group of medium was set as 100. The results are presented as the means ± SD. *$P < 0.05$; **$P < 0.01$.

Fig. 9. The inhibitory effect of the ds-Diabody against fibroblast growth factor-2 (FGF-2) on HUVEC migration. The HUVEC (5×10^4 cells/insert in the medium of M199 with 15 ng/mL FGF-2) were seeded into upper chamber. (a) HUVEC were in serum-free M199 medium only. (b) HUVEC were treated with the irrelevant IgG. (c) HUVEC were treated with the ds-Diabody against FGF-2. (d) HUVEC were treated with the full-length human antibody against FGF-2. (e) The quantitative analysis of HUVEC migration. The migration cell numbers in the control group of medium was set as 100. The results are presented as the means ± SD. *$P < 0.05$; **$P < 0.01$.
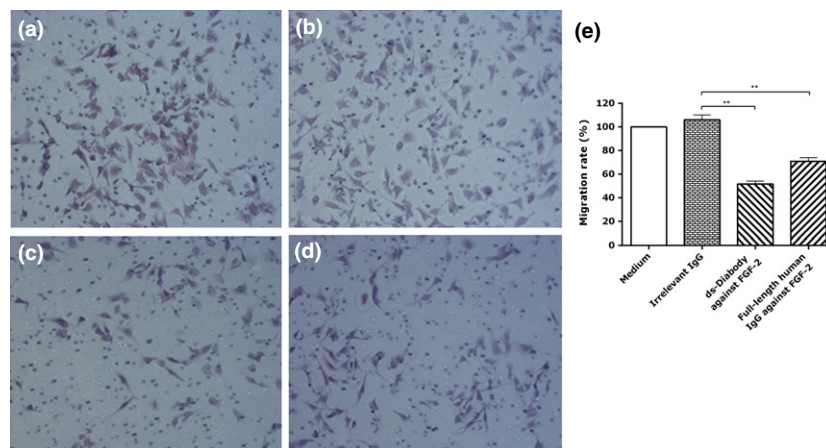
Diabody against FGF-2 was able to suppress angiogenesis and lymphangiogenesis *in vivo*.

Discussion

Fibroblast growth factor-2 is an important angiogenic factor for breast cancer growth, progression and metastasis. Blockade of FGF-2 signaling not only attenuated the tumor angiogenesis induced by FGF-2 and VEGF-A, but also inhibited the tumor lymphangiogenesis.[35] Therefore, targeting FGF-2 may be an effective method for inhibiting tumor growth, invasion and metastasis. In this study, we constructed a ds-Diabody against

FGF-2, which showed remarkable anti-tumor and anti-angiogenic effects on human breast cancer (MCF-7) in mice.

Diabody is one of the smallest bivalent antibodies, at approximately one-third the size of IgG, which may improve its penetration into tumor tissue.[22] In order to increase the stability of the Diabody against FGF-2, we constructed a ds-Diabody against FGF-2 by introducing the disulphide bonds between the VH and VL domains through site directed mutation. The cysteine bridge between the scFv fragments helps to drive the correct pairing of the two chains in diabody and could also help to maintain the integrity of the binding site and improve the serum stability.[23,36–38] Bera *et al.*[36] reports
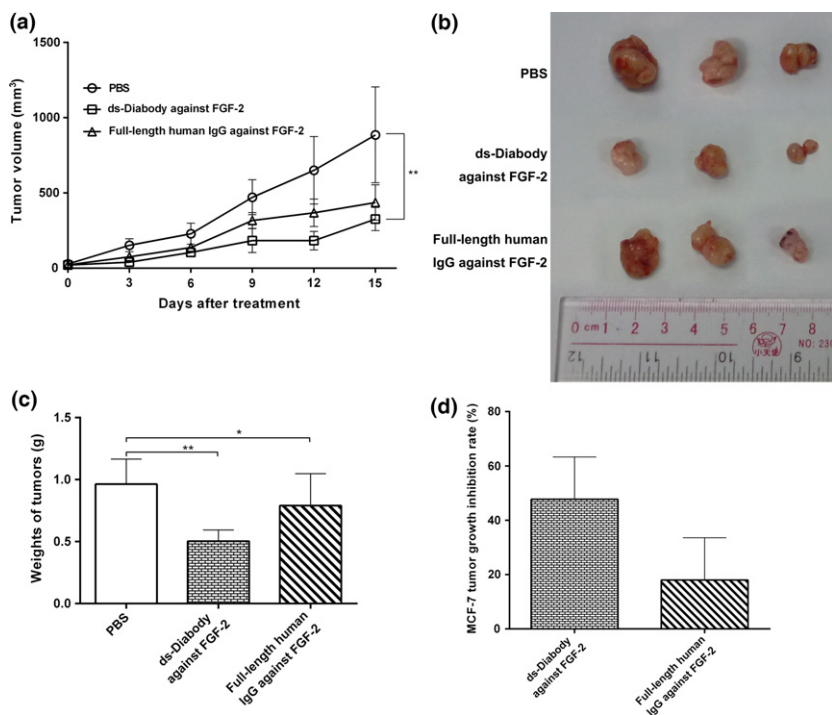
Fig. 10. The inhibitory effect of the ds-Diabody against fibroblast growth factor-2 (FGF-2) on MCF-7 tumor growth. (a) Tumor volumes in different groups. The breast cancer cells (MCF-7, 1×10^6 cells) were injected into the shoulders of BALB/c nude mice ($n = 6$). After the tumor was palpable (≥ 5 mm in diameter), the mice were treated with the ds-Diabody against FGF-2 (10 mg/kg in 100 μL PBS) six times at 3-day intervals, and tumor volume (mm^3) was measured at different time-points after treatment. (b) Tumors in different groups. (c) Quantitative analysis of the tumor weight. (d) The growth inhibition rate of tumors in different groups. The results are presented as the means ± SD (error bars) from six animals. *$P < 0.05$; **$P < 0.01$.
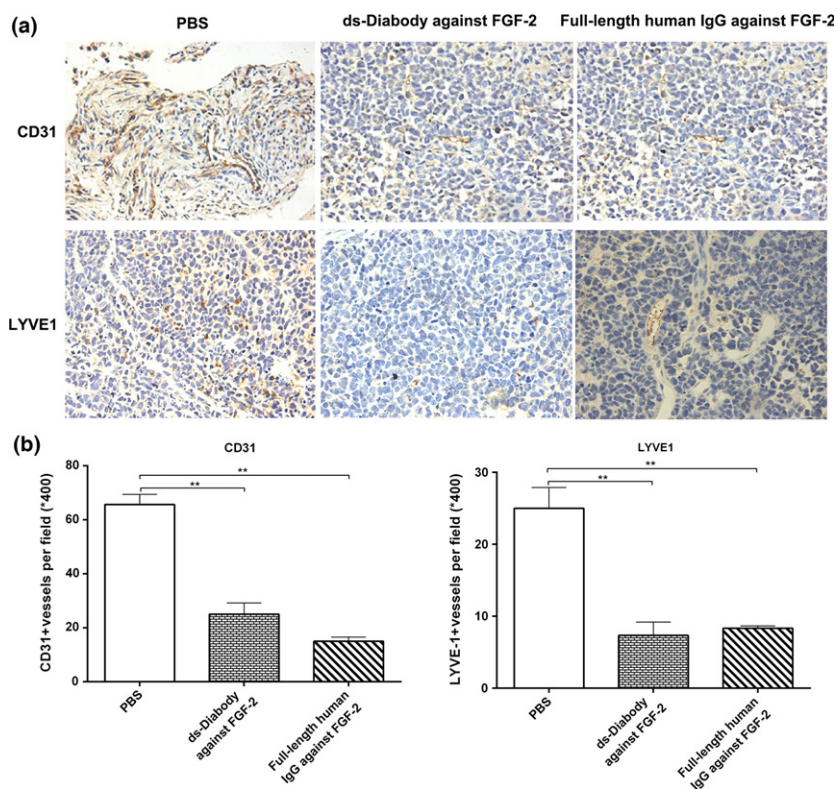
Fig. 11. Immunohistochemical analysis of microvessels and lymphatic vessels in tumor tissues. (a) Paraffin sections of MCF-7 tumors were stained for vascular endothelial cells with anti-CD31 antibody and lymphatic endothelial cells with anti-LYVE1 antibody, respectively. (b) Microvessel density and lymphatic vessel density were quantified. The number of blood vessels and lymphatic vessels at five high-power fields ($\times 400$) per section were counted; results are showed as the means ± SD (error bars). *$P < 0.05$; ***$P < 0.01$.

that a bivalent disulfide-stabilized Fv could bind to erbB2 with improved antigen-binding activity compared to Fv. Duan et al.[39] constructed a disulfide-stabilized scFv against RV (ds-FV57); the stability of ds-FV57 was notably improved, and its neutralizing potency against RV infection was enhanced *in vitro*. Our results indicated that the disulphide crosslinking could prevent dissociation of the dimer and lead to improved antigen-binding specificity and serum stability.

Stability is a critical factor contributing to the antitumor activity of antibody fragments. It is important that antibody fragments are stable at 37°C in human serum so that they will retain activity for a prolonged period after injection into

patients. Li *et al.*[24] constructed an anti-CD3 × anti-CD19 ds-Diabody, which could retain more than 90% antigen-binding capacity after 72 h. Liu *et al.*[40] reports on an anti-Pgp × anti-CD3 ds-Diabody that can effectively inhibit the growth of multidrug-resistant human tumors and retain approximately 80% antigen-binding capacity even after 7 days. Our results showed that the anti-FGF-2 ds-Diabody retained almost 100% antigen-binding capacity after 96 h and approximately 89% for 240 h at 37°C. In mice, the anti-FGF-2 ds-Diabody remained relatively stable in the treatment period. The residual antibody activity of the ds-Diabody against FGF-2 could maintain approximately 30–40%.

Compared to traditional antibodies, the ds-Diabody can be produced relatively easily in *Pichia pastoris* at high yields.[41] *Pichia pastoris* can be cultivated on cheap mineral media, and can be grown to high cell densities rapidly, and, therefore, its use could reduce production costs.[25,42] Besides, the ds-Diabody could be correctly folded in *Pichia pastoris* and effectively secreted.[42] In this study, the yield of ds-Diabody against FGF-2 was 30–50 mg per liter of cell culture. The molecular weight of the ds-Diabdoy was approximately 35 kDa under reducing conditions and 60 kDa under non-reducing conditions, which demonstrated that the disulphide bond involved the formation of the bivalent ds-Diabody against FGF-2. The results demonstrated that the ds-Diabody against FGF-2 could be effectively secreted in the expression supernatant of *Pichia pastoris* with a correct conformation.

The signaling pathways of MAPK/ERK and PI3K/Akt play essential roles in a variety of cellular processes, including cell proliferation and migration in tumor cells.[43] In our study, the ds-Diabody against FGF-2 significantly inhibited the FGF-2-mediated proliferation of MCF-7 cells. This inhibitory effect might be caused by the neutralization of FGF-2 and result in the blocking of the signal pathway of MAPK and Akt.

FGF-2 is over-expressed in breast cancer.[44] Anti-FGF-2 antibody neutralized the autocrine and paracrine FGF-2, blocked the signal pathways of MAPK/ERK and PI3K/Akt and resulted in the suppression of tumor angiogenesis and lymphogenesis, and then led to the inhibition of tumor growth and metastasis.[10–16] In the present study, the anti-FGF-2 ds-Diabody not only inhibited the invasion and migration of MCF-7 cells and tube formation of HUVEC *in vitro*, but also inhibited the angiogenesis and lymphogenesis and tumor growth *in vivo*.

In conclusion, an anti-FGF-2 ds-Diabody was successfully constructed and expressed in *Pichia pastoris*; the introduction of the cysteine residues and the formation of disulphide bonds in the ds-Diabody against FGF-2 did not influence its antigen-binding activity and did improve its stability. The results revealed that the ds-Diabody against FGF-2 could remarkably inhibit the growth of breast cancer cells both *in vitro* and *in vivo*. Furthermore, the combination of the ds-Diabody against FGF-2 and Avastin may provide a better anti-tumor effect and reduce the amount of chemotherapy drugs required as well as the associated side effects.

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers used for the construction of the ds-Diabody against FGF-2.