# A Novel Bispecific Diabody Targeting both Vascular Endothelial Growth Factor Receptor 2 and Epidermal Growth Factor Receptor for Enhanced Antitumor Activity

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*Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor 2 (VEGFR2) are receptor tyrosine kinases known to play critical roles in the development and progression of tumors. Based on the cross-talk between EGFR and VEGFR2 signal pathways, we designed and produced a bispecific diabody (bDAb) targeting both EGFR and VEGFR2 simultaneously. The bispecific molecule (EK-02) demonstrated that it could bind to HUVEC (VEGFR2 high-expressing) and A431 (EGFR overexpressing) cells. Additionally, similar to the parental antibodies, it was able to inhibit proliferation and migration, and induced apoptosis in these cells (HUVECs and A431), demonstrating that it had retained the functional properties of its parental antibodies. Furthermore, the efficacy of EK-02 was evaluated using the human colon adenocarcinoma cell line HT29 (VEGFR2 and EGFR coexpressing). In vitro assay showed that EK-02 could bind to HT29 cells, restrain cell growth and migration, and induce apoptosis with enhanced efficacy compared to both parental antibodies. Further, it inhibited the neovascularization and tumor formation on an HT29 cell bearing chicken chorioallantoic membrane (CAM) tumor model in vivo. In conclusion, these data suggest that the novel bDAb (EK-02) has antiangiogenesis and antitumor capacity both in vitro and in vivo, and can possibly be used as cotargeted therapy for the treatment of EGFR and VEGFR2 overexpressing tumors.* © 2016 American Institute of Chemical Engineers *Biotechnol. Prog.*, 32:294–302, 2016

*Keywords: epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR2), bispecific diabody (bDAb), single-chain fragment (scFv), antiangiogenesis, antitumor*

## Introduction

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) of the ErbB family, is overexpressed in most solid cancers, including carcinomas of the head and neck, breast, colon, prostate, lung, ovaries, and sinonasal squamous cell.[1,2] EGFR overexpression or constitutive activation is common in human tumors and is associated with increased tumor proliferation, survival, and dissemination making EGFR an attractive target for anticancer therapy.[3] On the other hand, vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR in human), is a key receptor of vascular endothelial growth factor (VEGF) involved in tumor-induced angiogenesis as well as in the proliferation and survival of tumor cells.[4–6] Hence, the inhibition of VEGFR2 activity and its downstream signaling are important strategies for cancer therapy.

Increasing evidences suggest that there could be the extensive degree of cross-talk between EGFR and VEGF(R) pathways.[6] Two most potent inducers of the ligand VEGF are the epidermal growth factor (EGF) and transforming growth factor (TGF)-$\alpha$ which happen to be ligands of EGFR.[7,8] Tumor-derived VEGF is upregulated by the activation of the EGFR pathway and, conversely, (over)activation of VEGF expression independent of EGFR signaling is thought to contribute to resistance in EGFR inhibition.[9] Studies elsewhere demonstrated that Combining EGFR and VEGFR antagonists could increase antitumor effect, and combining EGFR and VEGFR targeted therapy showed significant therapeutic potential.[9,10] The extensive degree of EGFR–VEGFR

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pathway cross-talk, therefore, underlines a potential joint targeting for antibody-based therapy.[6]

Bispecific antibodies (BsAb) capable of simultaneously binding to two different epitopes have been exploited as both cancer immunodiagnostics and cancer therapeutics, and have shown promises in clinical trials in cancer therapeutic and diagnostic applications.[11] One of the most promising BsAb formats, bispecific diabody (bDAb), comprising two noncovalently associated cross-over single chain fragment (scFv) polypeptides, is the most straightforward approach to BsAb production.[12,13]

In this study, we developed a bDAb (EK-02) using an anti-EGFR scFv[14] and an anti-VEGFR2 scFv.[15] We hypothesized that our bDAb will demonstrate more potent antitumor activity than the individual scFv (E10 or AK404R) alone and therefore could potentially be used as cotargeted therapy for EGFR and VEGFR2 overexpressing tumors.

## Materials and Methods

### *Cell lines and scFvs*

The adherent human epidermoid carcinoma cell A431 purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences and the adherent human colon cancer cell line HT29 preserved in our lab were cultured in DMEM and McCoy's 5A medium containing 10% (v) FBS respectively. Cell culture media and supplements were purchased from Life Technologies. The adherent human umbilical vein endothelial cells HUVEC obtained from ScienCell Research Laboratories and maintained in endothelial culture medium (ECM, ScienCell) were used with less than five passages. The anti-EGFR scFv (E10) and the anti-VEGFR2 scFv (AK404R) biopanning from phage display library were generated in our laboratory.[14,15]

### *Molecular design and construction of the bDAb EK-02*

The bispecific molecule EK-02 was constructed by fusing the anti-EGFR scFv E10 and the anti-VEGFR2 scFv AK404R in a "cross-over" manner ($V_{LE}$-$V_{HK}$ and $V_{LK}$-$V_{HE}$) with two $G_4S$ (G: glycine, S: serine, -: $G_4S$) flexible linkers by overlap PCR. $V_{HK}$ and $V_{LK}$ are single variable heavy and light chains of AK404R, whereas $V_{HE}$ and $V_{LE}$ represent the single variable heavy and light chains of E10, respectively. The PCR-amplified product coding for EK-02 was subcloned into the *Eco*RI and *Xho*I site of pHEN2 to generate recombinant plasmid pHEN2-EK02. Subsequently, *Escherichia coli* HB2151 was transformed with the recombinant vector pHEN2-EK02. Positive clones were then identified by colony PCR and DNA sequencing.

### *Expression and purification of the bDAb*

For large-scale production of EK-02, one confirmed positive clone was fermented in 2 × TY medium, induced with 0.8 mM of IPTG for 18 h at 25°C. Subsequently, periplasmic soluble protein was collected using an osmotic shock method[16] and further purified by Nickel affinity column as previously described.[14] The product was assayed by western blotting with primary (His-Tag Mouse monoclonal antibody, Millipore) and secondary antibodies (HRP conjugated Goat Anti-Mouse IgG, Millipore).

### *Surface plasmon resonance*

Surface plasmon resonance (SPR) measurements were carried out for binding kinetic analysis with a Biacore X100 (GE Healthcare) instrument following the manufacturer's instructions. NTA sensor chip immobilized with EK-02 was used to analyze the interaction between EK-02 and KDR3 ($VEGFR_{165}$). KDR3 was injected at concentrations 6.25, 12.5, 25, 50, 100, and 200 nM/L. On the other hand, another NTA sensor chip immobilized with EK-02 was used to evaluate the binding kinetics between EK-02 and EGFR. In contrast to KDR3, the EGFR was injected at concentrations 3.125, 6.25, 12.5, 25, 50, and 100 nM/L. Sensorgrams were subsequently obtained at each concentration and evaluated with the BIA Evaluation 2.0 program and the kinetic constant for association ($k_{on}$), dissociation ($k_{off}$), and equilibrium ($K_D$) calculated with 1:1 binding model. $K_D$ was calculated from the ratio of rate constants $k_{off}/k_{on}$.

### *Flow cytometry assay*

The ability of EK-02 binding to native receptors VEGFR2 and EGFR expressed on HUVEC, A431 and HT29 was assessed by flow cytometry. Respective cells ($2.5 \times 10^4$ cells per sample) were suspended in PBS containing 2% FBS and incubated with/without the indicated antibody at 4°C for 1 h. Cells were then incubated with mouse anti-His-tag mAb (Millipore) for 30 min, followed by FITC-conjugated rabbit anti-mouse mAb (Millipore) for another 30 min. Finally, the cells were washed and analyzed with a BD FACS flow cytometer.

### *Cell proliferation assay*

Cells were seeded into separate 96-well plates at 5,000–10,000 cells/well and cultured overnight. Subsequently, different concentrations of the antibodies were added and incubated at 37°C for 72 h. The untreated groups were used as a vehicle of control. Following the 72 h incubation, cell viability was quantified with MTT assay. The OD value of each well was measured at 570 and 630 nm and the inhibitory rates expressed as percentages of the vehicle control (100%).

### *Wound healing assay*

Cells of concentration $1.6 \times 10^5$ cells/well were seeded into a 12-well plate and then starved with serum-free medium for 12 h. The monolayer cells were scratched with a 1 mL pipette tip and incubated with indicated antibody (100 nM) for 24 h. The combination group was treated with 100 nM E10 plus 100 nM AK404R. The resultant images were then captured with OLYMPUS fluorescence microscope. The scratch widths were measured by employing the Image-Pro-Plus Program. The wound healing ability was defined by the ratios of the decreased scratch width after 24 h, relative to the initial scratch width, and calculated as follows:

$$\text{Rate of wound healing } (\%) = 100 \times \frac{\text{Initial scratch width - Final scratch width}}{\text{Initial scratch width}}.$$

### *Apoptosis assay*

Cells were seeded into separate 6-well plates at $2 \times 10^5$cells/well, and incubated at 37°C for 20 h. The culture

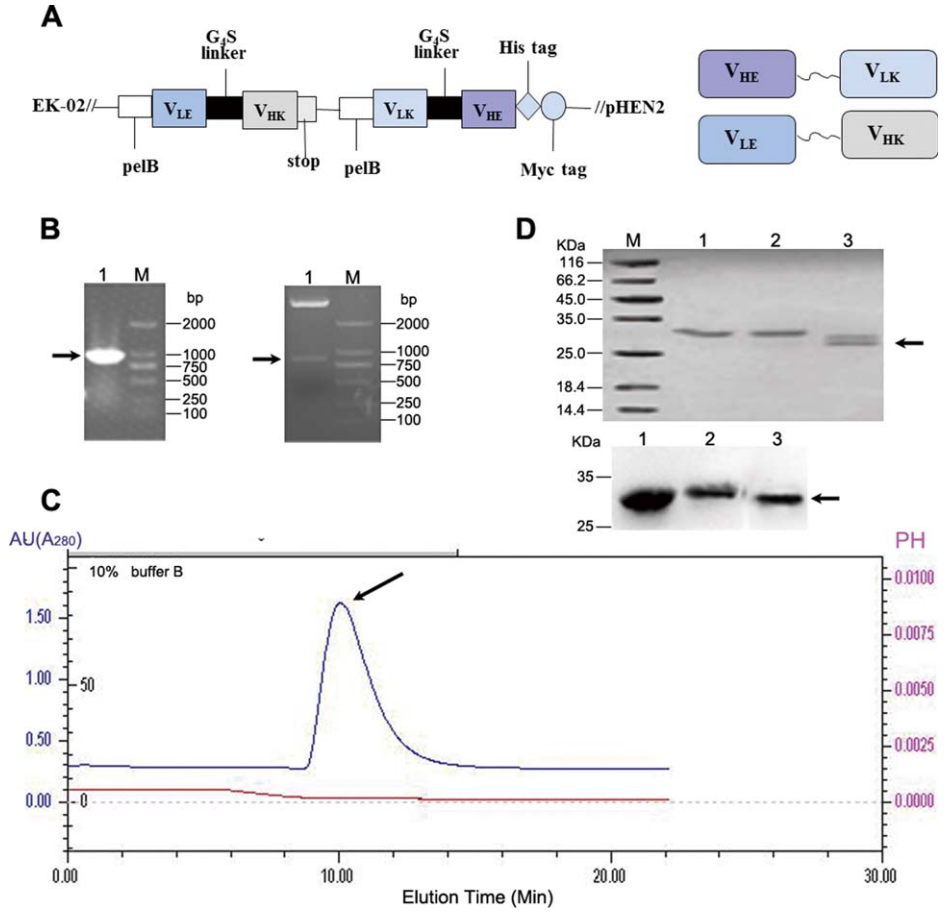


**Figure 1. The construction and expression of the anti-VEGFR2 × anti-EGFR bispecific diabody (bDAb) EK-02.**

**(A)** Schematic diagram of the construction of EK-02. $V_{HK}$ and $V_{LK}$ are single variable heavy and light chains of anti-VEGFR2 scFv (AK404R), whereas $V_{HE}$ and $V_{LE}$ represent single variable heavy and light chains of anti-EGFR scFv (E10) respectively; G: Glycine; S: serine. **(B)** Construction of the pHEN2-EK02 plasmid. (B-left) PCR amplification of the EK-02 gene from transformed HB2151 by single colonies PCR analysis; (B-right) Double digestion of the recombinant plasmid with restriction endonuclease *Xho*I and *Eco*RI: Lane M: DNA marker (bp); Lane 1: the EK-02 gene. **(C)** The purification of EK-02 by gradient imidazole elution on Ni Sepharose column. The column was equilibrated with binding buffer, and run at a flow rate of 0.5 mL/min. The peak represents the diabody protein. **(D)** Coomassie-stained SDS-PAGE (D-top panel) and Western Blotting analysis (D-bottom panel) of the purified EK-02 and the individual scFvs. Lane1: E10; Lane2: AK404R; Lane 3: EK-02; Lane M: protein marker (kDa).

medium was replaced with fresh medium containing 100 nM of the indicated antibody and incubated at 37°C for further 48 h. The parental combination was incubated with 100 nM E10 plus 100 nM AK404R. Cells were stained using an Apoptosis Annexin V–FITC Kit (Invitrogen) following the manufacturer's instructions and then analyzed by flow cytometer assay. The populations of apoptotic cells (Annexin-$V^+$/propidium $iodide^-$) and late apoptotic cells (Annexin-$V^+$/propidium iodide $positive^+$) were calculated as a percentage of total cells.

### *HT29 cell bearing chicken chorioallantoic membrane (CAM) tumor model*

Groups of fertilized chick embryos (Nanjing Medical Device Factory) were preincubated at 37°C for 8 days in 60% humidity. A square window of 1 $cm^2$ was then opened aseptically in the flat pole of the egg shell and carefully punctured the underlying CAM. HT29 cells ($4 \times 10^8$) were then seeded onto the avascular area on the CAM. The shell windows were subsequently sealed and the eggs were placed in the incubator in an upright position. Three days after tumor inoculation, filter-paper disks loaded with PBS (50 $\mu$L), AK404R (10 $\mu$M, 50 $\mu$L), E10 (10 $\mu$M, 50 $\mu$L), the combination therapy (10 $\mu$M AK404R plus 10 $\mu$M E10, 50 $\mu$L) and EK-02 (10 $\mu$M, 50 $\mu$L) were placed on the tumor-implanted area of the CAM separately in separate setups. The embryos were incubated under the same conditions for further 48 h, and then the samples were immediately fixed in 1 mL fixative (methanol- to-acetone, 1:1) for 15 min. The HT29 transplanted tumor and vascular zones were then photographed. Subsequently, the antiangiogenesis abilities of antibody on the CAMs were quantified by counting the number of blood vessel branches around the transplanted tumor, and the inhibition of tumor growth evaluated by measuring the volume of the transplanted tumors as against the negative control (PBS treated CAM). Measurements of HT29-CAM transplants were based on the average of each group (6 CAMs/group) and calculated according to the *t* test for $P < 0.05$.

### *Statistical analysis*

The data of the study were analyzed using Excel and SPSS 17.0 software. Results are presented as the mean ± SD from at least three independent experiments. The *t* test was

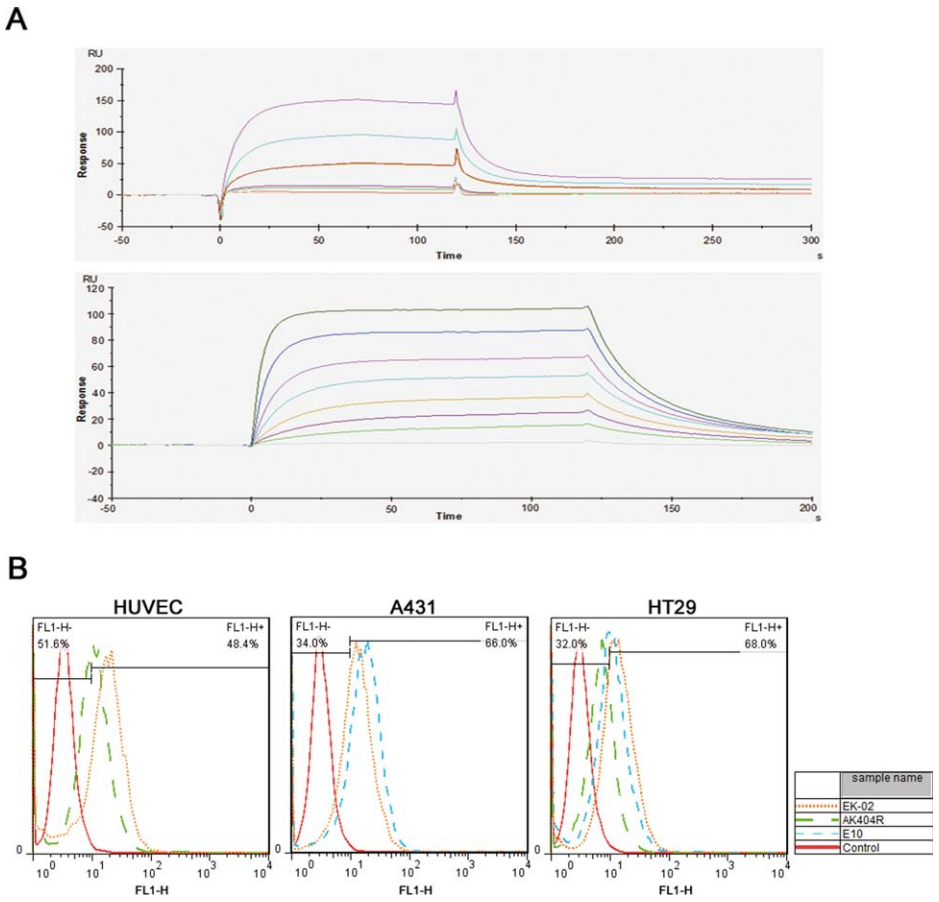


**Figure 2. EK-02 bound to either recombinant or native VEGFR2and EGFR with high binding capacity.**

**(A)** The binding kinetics analysis of EK-02 against VEGFR2/EGFR by Biacore. **(A) Top panel**: Set of sensorgrams of EK-02 binding with $VEGFR_{165}$ (6.25, 12.5, 25, 50, 100, and 200 nM/L), with calculated equilibrium dissociation constant ($K_D$) value of $3.49 \times 10^{-8}$ M. **Bottom panel**: Set of sensorgrams of EK-02 binding with EGFR (3.125, 6.25, 12.5, 25, 50, and 100 nM/L), with $K_D$ value of $1.15 \times 10^{-8}$ M. The concentration of recombinant protein increases from bottom to up. **(B)** Flow cytometry analysis showed that EK-02 binds to VEGFR2 overexpressing HUVECs, EGFR overexpressing A431 cells, and VEGFR2 and EGFR coexpressing HT29 cells.

**Table 1. Kinetic Constants of Antibodies to EGFR or VEGFR2 by BIAcore Analysis**

| Binding | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (M) |
|---|---|---|---|
| **EK-02–EGFR** | $2.95 \times 10^6$ | $3.38 \times 10^{-2}$ | $1.15 \times 10^{-8}$ |
| **EK-02–VEGFR2** | $4.50 \times 10^4$ | $1.57 \times 10^{-3}$ | $3.49 \times 10^{-8}$ |
| **E10–EGFR** | $5.93 \times 10^4$ | $1.12 \times 10^{-3}$ | $1.89 \times 10^{-8}$ |
| **AK404R–VEGFR2** | $3.97 \times 10^3$ | $1.60 \times 10^{-4}$ | $4.03 \times 10^{-8}$ |

The data on binding kinetics of E10–EGFR was determined by Jin (unpublished data) and the binding kinetics of AK404R–VEGFR2 is reported by Zhang and Li.[15] The data on sensorgrams of E10–EGFR and AK404R–VEGFR2 are not shown.

used to compare the inhibitory rates of different samples in MTT assay, wound healing assay, and so on. A $p$ value of $<0.05$ was considered statistically significant. All figures were generated with GraphPad Prism 5 software program.

## Results

### *Construction, expression, and purification of the bDAb EK-02*

EK-02 was generated by fusing fragments encoding the $V_L$ and the $V_H$ genes of the anti-EGFR scFv E10 and the anti-VEGFR2 scFv AK404R in "cross-over" manner by overlap PCR and subcloned into pHEN2 (Figure 1A). The presence of the pelB leader sequence was to direct the expressed EK-02 into the periplasm of the bacteria for easy isolation and purification. The use of the flexible linker G4S (15 bp) was to keep the individual fragments of the EK-02 bDAb active and flexible for optimum functional activity. His (18 bp) and c-Myc (39 bp) tags were directly added at the C-terminus of $V_H$E fragment for purification and characterization purposes. Colony PCR analysis of the transformed HB2151 and double digestion of the pHEN2-EK02 with *Xho*I and *EcoR*I indicated that the recombinant bDAb was successfully subcloned into pHEN2 and also the HB2151 was transformed with the recombinant pHEN2-EK02 (Figure 1B).

Periplasmic protein EK-02 was obtained by osmotic shock method and then successfully purified by nickel column chromatography with purity above 90% (Figure 1C).The expression and purification of the EK-02 bDAb and the scFvs were evaluated by 15% SDS–PAGE and Western Blotting analyses (Figure 1D). The final yield of EK-02 was 800 $\mu$g/L.

### *EK-02 binds to VEGFR2/EGFR*

The binding kinetics between EK-02 and VEGFR2/EGFR was determined with SPR technology (Figure 2A). The data indicated that the affinity ($K_D$) of EK-02 to EGFR/VEGFR2

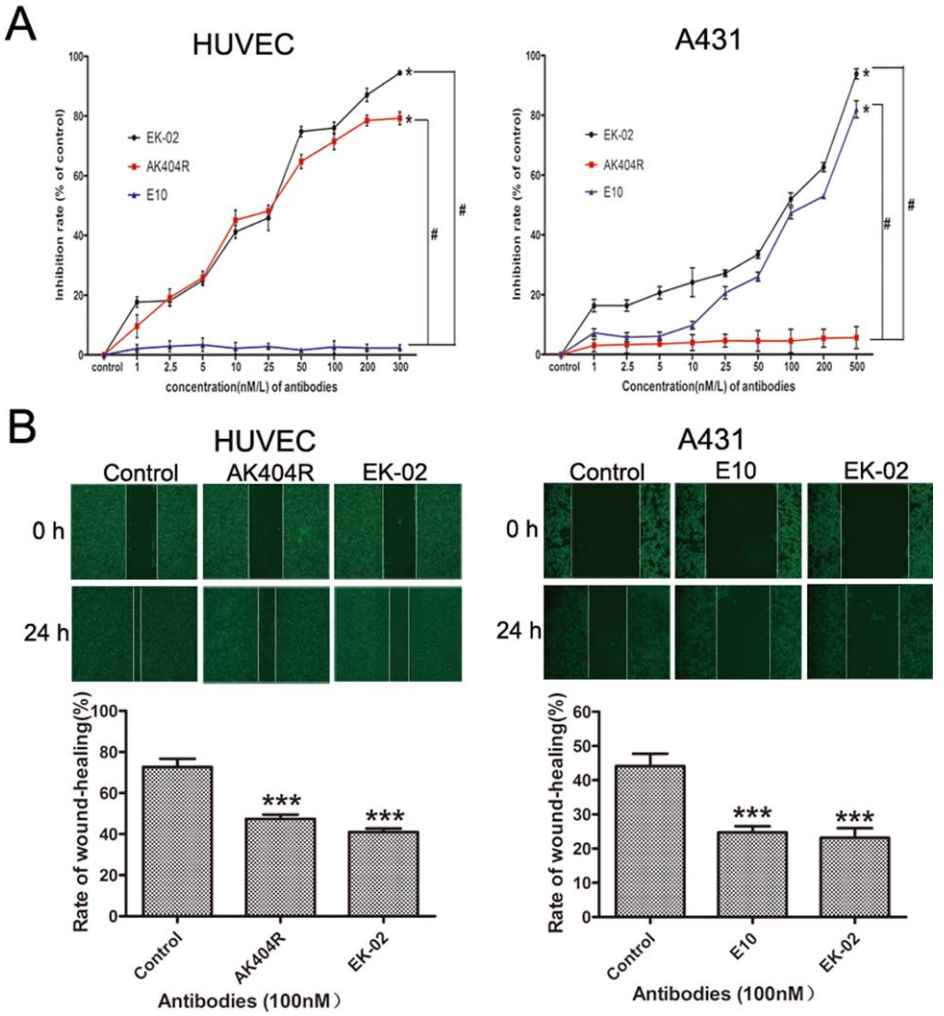


**Figure 3. EK-02 inhibited HUVEC/A431cell growth and migration.**

**(A)** MTT assay showed that EK-02 significantly restrained the proliferation of HUVEC and A431 cells in a dose-dependent manner (values represented means ± SD, $n = 3$, $*p < 0.01$ versus untreated control, $\#p < 0.01$ versus negative control as indicated). **(B)** Scratch-wound healing assay revealed that EK-02 reduced the migration of HUVEC and A431 cells after 24 h treatment. The top panels represent respective photomicrographs of three independent experiments and the bottom panels are quantitative analysis of those wound healing assays (values represented as means ± SD, $***p < 0.001$ versus untreated control).

is similar to E10 and AK404R (**Table** 1). The SPR assay demonstrated that the association rate increased with increasing concentration (from bottom to up). The whole kinetic process can be described as quick association and slow dissociation similar to other antibody–antigen kinetic process.

HUVECs (VEGFR2 high-expressing), A431 (EGFR overexpressing), and HT29 (VEGFR2 and EGFR coexpressing) were used in the flow cytometry assay to evaluate the ability of EK-02 binding to native antigens EGFR and VEGFR2 (Figure 2B). Both EK-02 and AK404R demonstrated relatively high binding signals to VEGFR2 expressing HUVECs as against the control (treated with milk), with the binding rates of 48.4 and 51.2%, respectively. EK-02 (binding rate 66.0%) demonstrated relatively high binding capacity to EGFR expressing A431 cells just like the parental scFv E10 (binding rate 72.5%), as against the control. These data clearly demonstrated that EK-02 could bind to VEGFR2/EGFR expressed cells, and therefore had retained the binding properties of the respective parental scFvs. Furthermore, EK-02, AK404R, and E10 bound to HT29 cells with binding rate 68.0, 25.1, and 50.1%, respectively. These data suggested that EK-02 bound to VEGFR2 and EGFR coexpressed cells with much higher binding capacity than the parental the scFvs, cotargeting VEGFR2 and EGFR simultaneously. It can be concluded that EK-02 bind to recombinant and native EGFR and VEGFR2 with high binding capacity, given credence to the fact that the bDAb EK-02 was successfully constructed, expressed, and purified, and that it had retained the binding properties of the parental scFvs (AK404R and E10), cotargeting VEGFR2 and EGFR.

*EK-02 Inhibits Cell Growth and Migration and Induces Cell Apoptosis in HUVEC/A431 Cells.* In the MTT assay (Figure 3A), EK-02 demonstrated significant dose-dependent inhibition on the proliferation of VEGFR2-expressing HUVEC as against the negative control (E10 treated), showing the same restraining tendency as the anti-VEGFR2 scFv (AK404R) on HUVECs. As expected, there were no obvious inhibitory effects on HUVECs by E10. Additionally, EK-02 significant suppressed the proliferation of EGFR-expressing A431 cells as against the negative control (AK404R treated), exhibiting similar dose-dependent inhibitory ability as the parental scFv E10 on A431 cells.

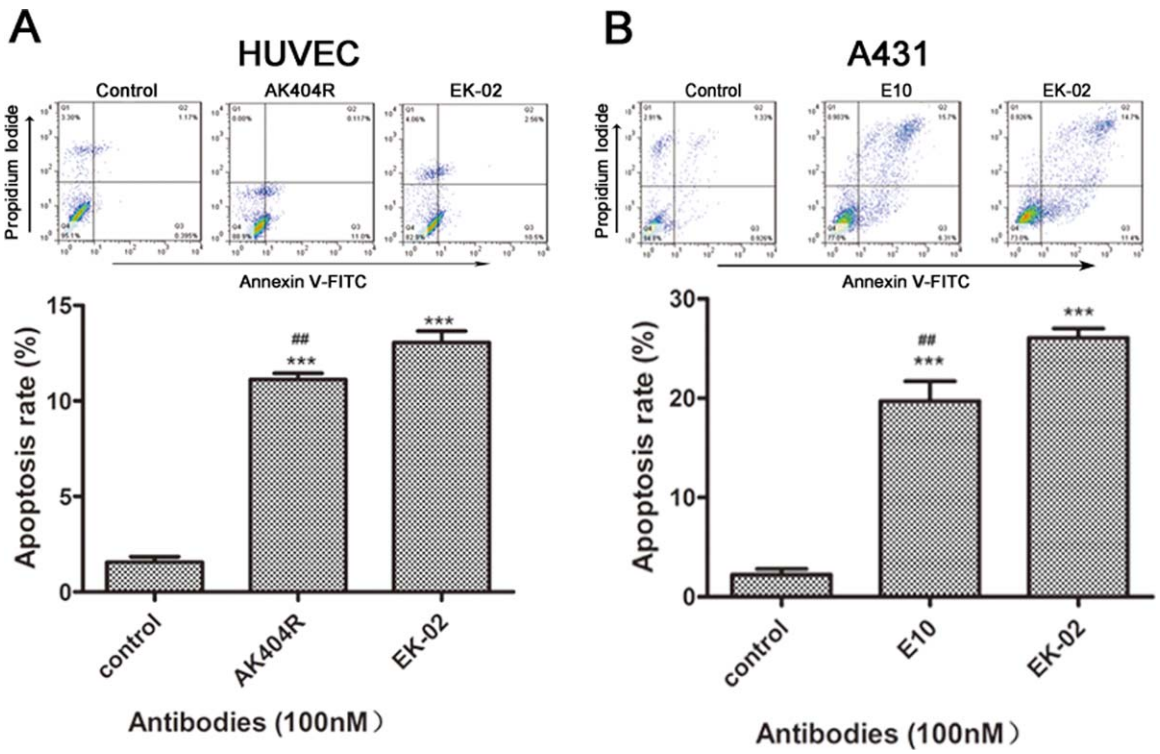


**Figure 4. The EK-02-induced apoptosis in HUVECs and A431 cells.**

EK-02 demonstrated relatively higher apoptotic potential than its parental scFvs in both the cell lines after 48 h treatment. Dot plots showed the representative data of three independent experiments and the right panels are quantitative analysis of those apoptosis assays (***$p < 0.001$ versus untreated control, ##$p < 0.01$ versus EK-02 treated group).

Wound healing assay was carried out to assess the effect of EK-02 on the migration of HEVECs and A431 cells (Figure 3B). EK-02 restrained the migration of both cell lines just like the individual scFvs (AK404R and E10). The width of the gap of EK-02 untreated control group narrowed more drastically than that of the EK-02-treated group in both cell lines (HUVECs and A431).

In the FITC-Annexin V/PI double staining assay (Figure 4), EK-02 demonstrated significant apoptotic induction potential in both HUVECs and A431 cells. Dot plots showed a representative data of the three independent experiments. The percentage of apoptotic cells of representative dot plots for HUVCEs were (Figure 4A) blank control (1.56%), 100 nmol/L AK404R (11.12%), and 100 nmol/L EK-02 (13.06%); whereas those for A431 were (Figure 4B) blank control (2.2%), 100 nM/L E10 (22.01%), and 100 nM/L EK-02 (26.10%). These data indicated that the apoptosis-inducing activity of EK-02 was more potent than the individual scFvs (AK404R or E10 alone).

The *in vitro* assay with HUVECs (VEGFR2 expressed) and A431 cells (EGFR expressed) have demonstrated that we have produced an anti-VEGFR2 × anti-EGFR bDAb, which had retained the functional properties of its parental antibodies, and therefore needed to further test its antitumor potential with VEGFR2 and EGFR coexpressed cells (HT29 cells).

*EK-02 Exhibits Enhanced Inhibitory Activity in VEGFR2 and EGFR Coexpressing HT29 Cells.* The flow cytometry assay showed that EK-02 could bind to VEGFR2 and EGFR coexpressed HT29 cells with much higher binding capacity than the parental scFvs. Here HT29 cells were used for cotargeting evaluation assays. EK-02 was more efficient in the inhibition of HT29 cell growth and migration than AK404R or E10 alone, demonstrating superior inhibitory capacity than both parental scFvs in cotargeting the VEGFR–EGFR pathway (Figure 5A,B). In HT29 apoptosis assay (Figure 5C), the populations of apoptotic cells in representative dot plots were blank control (0.041%), AK404R (10.33%), E10 (15.10%), EK-02 (21.72%), and the combined (AK404R plus E10) treated group (29.0%). These data were consistent with MTT and would healing assays. Similar to the combination therapy, EK-02 obviously demonstrated enhanced efficacy in the induction apoptosis, leading to more apoptotic cell populations than AK404R or E10 alone, which corroborated the potency of the bDAb EK-02 in targeting both VEGFR and EGFR pathways simultaneously.

### *HT29 cell bearing CAM tumor model*

CAM tumor model can potentially be applied in preclinical evaluation of anticancer agents.[17,18] Here HT29 cell bearing CAM tumor model was employed to evaluate the antiangiogenesis and antineoplastic activities of EK-02. The inhibitory effects of EK-02, the combination therapy (AK404R plus E10), E10, and AK404R on CAM (antiangiogenesis) which were quantified in relation to the negative control (PBS treated group) were 71.76%, 59.13%, 36.21%, and 49.83%, respectively (Figure 6A,B). The inhibitory effects of EK-02, the combination therapy, E10, and AK404R on the growth of HT29 tumor transplanted on CAM were 75.41%, 63.39%, 46.45%, and 29.51%, respectively (Figure 6C,D). Taken together, EK-02 demonstrated significant inhibitory effect on angiogenesis and tumor growth on CAM, showing more potent than the individual scFvs (AK404R or E10 alone).

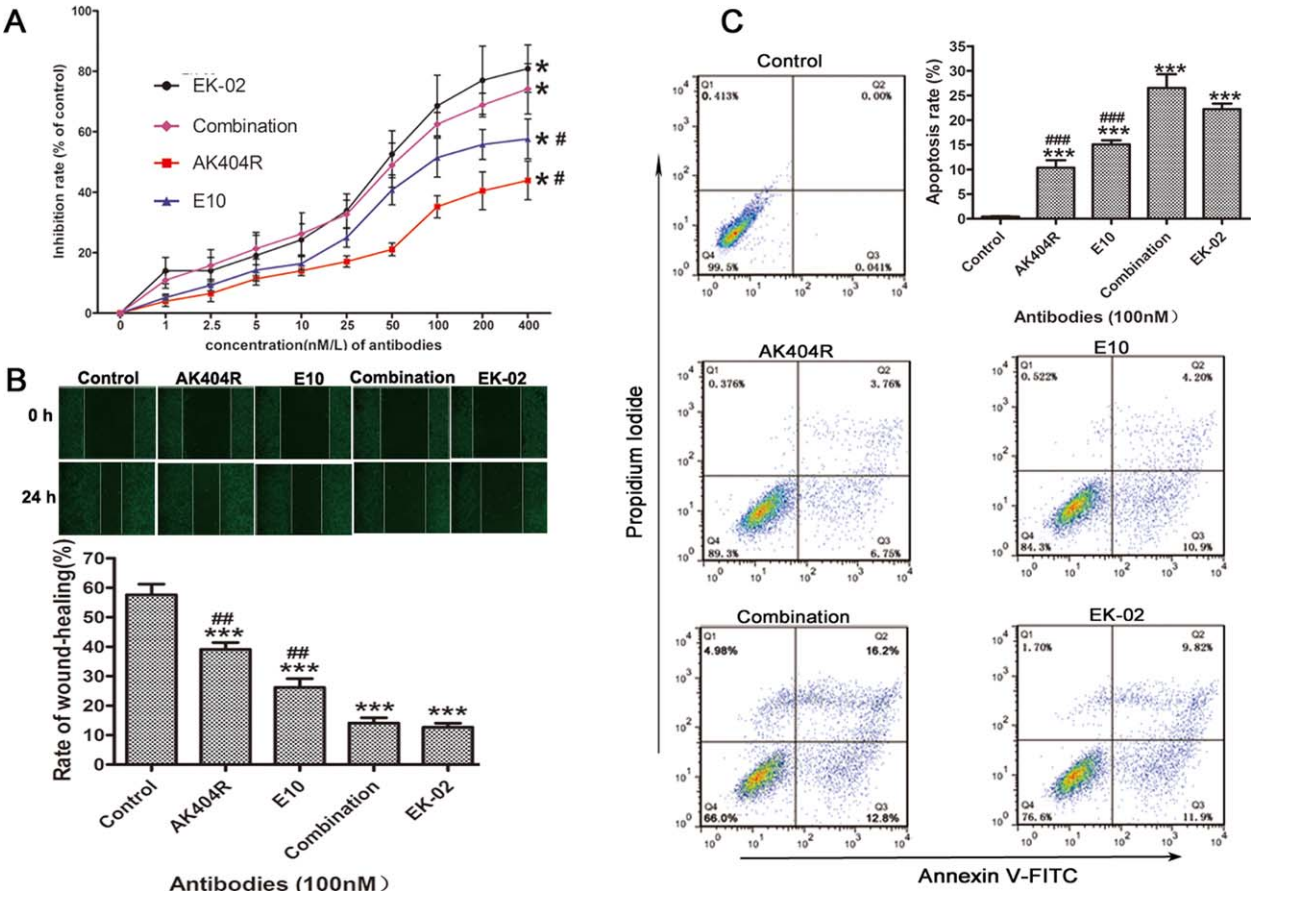


**Figure 5. EK-02 suppressed cell growth and migration and induces apoptosis in VEGFR2 and EGFR coexpressed HT29 cell with enhanced activity.**

(A) MTT assay revealed that the EK-02 demonstrated superior inhibitory activity than both parental scFvs after 72 h treatment (values represented means ± SD, $n = 3$, *$p < 0.01$ versus untreated control, #$p < 0.01$ versus EK-02-treated group). **(B)** In the wound healing assay, EK-02 suppressed HT29 cell migration with enhanced inhibitory effect as against both parental scFvs (values represented means ± SD, ***$p < 0.001$ versus untreated control, ##$p < 0.01$ versus EK-02-treated group) **(C)** In HT29 cell assays, EK-02 exhibited relatively higher apoptotic potential than its parental scFvs after 48 h treatment (***$p < 0.001$ versus untreated control, ###$p < 0.001$ versus 100 nM/L EK-02-treated group).

## Discussion and Conclusions

Inhibition of EGFR or VEGFR2 signaling is one of the effective strategies that could be adopted to eliminate tumors overexpressing EGFR or VEGFR2, and combining EGFR and VEGFR2 antagonists could increase the antitumor effect.[9,10] This implies that an EGFR and VEGFR2 cotargeted therapy could be a potential strategy. A bDAb could simultaneously bind to target antigens.[13] Similar to other bispecific constructs, bDAb could potentially be more efficacious and less costly to develop due to their structure and mode of action.[19] In this study, a bDAb named EK-02 targeting both EGFR and VEGFR2 was generated and evaluated.

The variable domain orientation (i.e., $V_H$–linker–$V_L$ or $V_L$–linker–$V_H$) in bDAb construction impacts the expression, antigen-binding activity, and protein stability of the resulting bDAb.[13] More than half dozen bDAb constructed by Lu et al., the "$V_{L1}$–$V_{H2}$/$V_{L2}$–$V_{H1}$" orientation/arrangement has always yielded a bDAb with good retention of dual antigen-binding activity. This orientation may therefore represent the preferred format for bDAb construction.[13] In addition, the variable domain arrangement also has significant effects on the antigen-binding activity of the resulting bDAb.[13] The antigen-binding activity of bDAb is influenced by the correct assembly/folding of each cognate VL/VH domains, which depends on both the intrinsic properties of the interacting variable domains as well as the extrinsic contributions from the partnering variable domains.[13] Requirement of a free N- or C-terminus of a component scFv may also influence the antigen-binding activity.[20] However, there is no universal variable domain arrangement for all bDAb constructions. Therefore, an optimal arrangement needs to be selected for a particular bDAb. The arrangement of the variable domain in our bDAb EK-02 followed the arrangement pattern of the bispecific single-chain diabody (scDb) that we previously designed and constructed in the "$V_{LE}$—$G_4S$—$V_{HK}$—$G_4SGGRASG_4S$—$V_{LK}$—$G_4S$—$V_{HE}$" arrangement pattern with AK404R and E10,[21] which showed significant binding capacity to EGFR and VEGFR2, maintaining the antigen-binding activity of both parental antibodies (AK404R and E10). Thus, our current bDAb (EK-02) was also constructed in the arrangement ($V_{LE}$–$V_{HK}$ and $V_{LK}$–$V_{HE}$), and was subsequently confirmed by BIAcore analysis and flow cytometry assay to have retained the binding properties of the parental scFvs (Figure 2), suggesting that the bDAb EK-02 variable domain orientation/arrangement was effective. A flexible linker used to connect the "cross-over" two domains of each chain, was too short to allow pairing between domains on the same chain, permitting interchain, but not intrachain, pairing of the variable domains to recreate the two antigen-

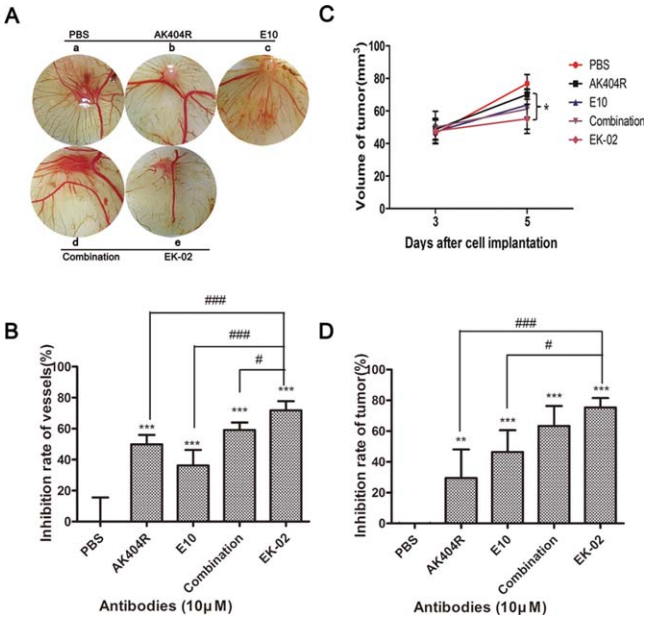


**Figure 6. HT29 cell bearing CAM tumor model.**

**(A)** Representative photographs of CAM assay. **(B)** Statistical analysis of the CAM assay revealed that EK-02 and individual scFvs demonstrated inhibition efficacy on tumor-induced angiogenesis. **(C)** The volume of HT29 transplanted tumor on CAM measured after treating with EK-02 and individual scFvs for 48 h. **(D)** EK-02 inhibited both angiogenesis and tumor growth on CAM, which was more potent than the individual scFvs (values represented means ± SD, $n = 6$, * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$, versus PBS-treated control, # $p < 0.05$, ### $p < 0.001$ versus EK-02-treated group).

binding sites.[13] The $G_4S$ linker was to keep the individual fragments of EK-02 at active positions for optimum functional activity. His and Myc tags were added for easy purification and characterization.

EK-02 was expressed in the periplasmic region of *E. coli*. The decision to express the EK-02 in prokaryotic host was informed by the fact that prokaryotic expression is inexpensive and less time-consuming.[22] Moreover, recombinant proteins can easily be extracted by a simple osmotic shock method, coupled with the fact that periplasmic extracts are rarely contaminated by bacterial proteins, suggesting easier purification.[22]

Our previous anti-EGFR/VEGFR2 bispecific scDb was produced in *E. coli* HB2151 host as a monomeric bispecific molecule by connecting the two diabody-forming polypeptide chains with an additional 15 amino acid residues linker ($G_4SGGRASG_4S$). However, we found that the scDb was unstable and characterized with production too low to meet the research requirement. Hence, we discontinued the study on this single-chain diabody format and focused our attention on the current construct bDAb. Additionally, we have constructed an IgG-like bispecific antibody (named Bi-Ab), cotargeting VEGFR2 and EGFR which was expressed in *Pichia pastoris* X-33 cells. The novel Bi-Ab with enhanced antitumor activity compared to the parental antibodies (alone or in combination), showed promising therapeutic potential, and therefore can be used against tumors overexpressing both EGFR and VEGFR2 (Chen, unpublished).

Employing binding kinetics analysis and flow cytometry assay, the EK-02 demonstrated that it could bind to either recombinant or native VEGFR2 and EGFR with high binding capacity, given credence to the fact that, the EK-02 bDAb was successfully constructed, expressed, and purified with the individual scFvs (AK404R and E10) retaining their binding properties. Restraining the proliferation and migration of VEGFR2/EGFR overexpressing tumor cells by EK-02, demonstrated its ability to block the EGF-EGFR and VEGF-VEGFR2 pathways simultaneously to induce apoptosis and inhibit angiogenesis in such tumor cells.[6]

In our quest to confirm the apoptotic inducing potential of EK-02, HUVECs (which are normal cells), A431, and HT29 (tumor cells) were treated with EK-02 for 48 h. And as expected, EK-02 proved to be potent apoptosis inducer in all the three cell lines. However, the rate of apoptosis in the normal HUVECs was relatively lower compared to the tumor cell lines (A431 and HT29). This disparity could be due to the differences in antigen expression level, as shown by the flow cytometry assays. More importantly, EK-02 demonstrated relatively higher apoptotic potential than its parental scFvs in all the three cell lines.

The *in vivo* investigation on the antitumor activity of EK-02 on HT29 cell bearing CAM tumor model was consistent with the *in vitro* studies. As anticipated, the anti-VEGFR2 scFv (AK404R) exhibited more potent antiangiogenic activity than the anti-EGFR scFv (E10). This is because its antigen, VEGFR2, is the key target in cancer angiogenesis through the VEGFR2–VEGF signaling pathway.[23] However, both EK-02 and the combination therapy (AK404R plus E10) presented more potent antiangiogenic activity than AK404R or E10 alone. This could possibly be due to the synergistic effects of the two scFvs.[10,24] Interestingly, EK-02 demonstrated slightly potent antiangiogenic activity than the combination therapy. The antitumor potential of EK-02 was further confirmed by evaluating its capacity to restrain the proliferation of HT29 tumor cells transplanted on CAM (*in vivo*). Similar to the combination therapy, EK-02 proved more efficacious compared to the individual scFvs, which was consistent with the *in vitro* antitumor activity. EGFR and VEGFR2 contribute to the activation of MAPK and AKT signaling in tumor angiogenesis.[25,26] In cancer cells coexpressed EGFR and VEGFR2, the MAPK and AKT signals are regulated by both EGFR and VEGFR2.[27,28] However, it is probable that Erk1/2 and AKT still remain activated when EGF/EGFR or VEGF/VEGFR signal pathway is blocked alone.[29] In such situation, the best way out is to block both EGF/EGFR and VEGF/VEGFR signaling pathways simultaneously. This could explain why the bDAb EK-02 and the combination therapy were more potent than individual scFvs. Thus, the bDAb EK-02 and combination therapy inhibited phosphorylation of AKT and Erk1/2 more thoroughly than AK404R or E10 alone. Also, EK-02 being slightly potent than the combination therapy could be due to more effective inhibition of MAPK signal pathway by EK-02 compared to the combination therapy. In view of the fact that this study focused on the construction, characterization, and preliminary antitumor activity of the bDAb EK-02, the next phase of this study is to confirm the activity pathways through Western blot analysis and evaluate its potency *in vivo*, using animal models.

In conclusion, we have constructed, expressed, and purified a novel bDAb co-targeting EGFR and VEGFR2. The novel bDAb EK-02 has demonstrated that it can produce enhanced antiangiogenic and antitumor activities by cotargeting EGFR and VEGFR2 simultaneously, compared to the individual scFvs (AK404R and E10 alone). Hence, it is a potential antitumor or antiangiogenic agent, which could be developed further for clinical use.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Literature Cited

1. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *N Engl J Med*. 2008; 358:1160–1174.
2. Metro G, Finocchiaro G, Cappuzzo F. Anti-cancer therapy with EGFR inhibitors: factors of prognostic and predictive significance. *Ann Oncol*. 2006; 17:42–45.
3. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol*. 2009; 21:177–184.
4. Nussenbaum F, Herman IM. Tumor angiogenesis: insights and innovations. *J Oncol*. 2010; 2010:132641
5. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A, Jurgensmeier JM, Womack C. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. *Clin Cancer Res*. 2010; 16:3548–3561.
6. Larsen AK, Ouaret D, El Ouadrani K, Petitprez A. Targeting EGFR and VEGF(R) pathway cross-talk in tumor survival and angiogenesis. *Pharmacol Ther*. 2011; 131:80–90.
7. Riedel F, Gotte K, Li M, Hormann K, Grandis JR. EGFR antisense treatment of human HNSCC cell lines down-regulates VEGF expression and endothelial cell migration. *Int J Oncol*. 2002; 21:11–16.
8. Ferrara N. The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res Treatment*. 1995; 36:127–137.
9. Tabernero J. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. *Mol Cancer Res*. 2007; 5:203–220.
10. Tonra JR, Deevi DS, Corcoran E, et al. Synergistic antitumor effects of combined epidermal growth factor receptor and vascular endothelial growth factor receptor-2 targeted therapy. *Clin Cancer Res*. 2006; 12:2197–2207.
11. Byrne H, Conroy PJ, Whisstock JC, O'Kennedy RJ. A tale of two specificities: bispecific antibodies for therapeutic and diagnostic applications. *Trends Biotechnol*. 2013; 31:621–632.
12. Holliger P, Winter G. Diabodies: small bispecific antibody fragments. *Cancer Immunol Immunotherap CII*. 1997; 45:128–130.
13. Lu D, Jimenez X, Witte L, Zhu Z. The effect of variable domain orientation and arrangement on the antigen-binding activity of a recombinant human bispecific diabody. *Biochem Biophys Res Commun*. 2004; 318:507–513.
14. Zhou Y, Zhang J, Jin H, Chen Z, Wu Q, Li W, Yue M, Luo C, Wang M. Prokaryotic expression and refolding of EGFR extracellular domain and generation of phage display human scFv against EGFR. *Biomed Pharmacotherap Biomed Pharmacotherap*. 2013; 67:737–743.
15. Zhang J, Li H, Wang X, Qi H, Miao X, Zhang T, Chen G, Wang M. Phage-derived fully human antibody scFv fragment directed against human vascular endothelial growth factor receptor 2 blocked its interaction with VEGF. *Biotechnol Progr*. 2012; 28:981–989.
16. Cao W, Li H, Zhang J, Li D, Acheampong DO, Chen Z, Wang M. Periplasmic expression optimization of VEGFR2 D3 adopting response surface methodology: antiangiogenic activity study. *Protein Express Purif*. 2013; 90:55–66.
17. Kunzi-Rapp K, Genze F, Kufer R, Reich E, Hautmann RE, Gschwend JE. Chorioallantoic membrane assay: vascularized 3-dimensional cell culture system for human prostate cancer cells as an animal substitute model. *J Urol*. 2001; 166:1502–1507.
18. Dani SU, Espindola R. A model system for testing gene vectors using murine tumor cells on the chorioallantoic membrane of the chick embryo. *Genet Mol Res*. 2002; 1:167–175.
19. Dhimolea E, Reichert JM. World Bispecific Antibody Summit, September 27–28, 2011, Boston, MA. *MABS* 2012; 4:4–13.
20. Kipriyanov SM, Moldenhauer G, Braunagel M, Reusch U, Cochlovius B, Le Gall F, Kouprianova OA, Von der Lieth CW, Little M. Effect of domain order on the activity of bacterially produced bispecific single-chain Fv antibodies. *J Mol Biol*. 2003; 330:99–111.
21. Zhou YQ, Zhang J, Jin HZ, He Y, Wang T, Wang M. Construction and expression of an anti-EGFR/anti-KDR bispecific single-chain diabody. *Yao Xue Xue Bao = Acta Pharmaceutica Sinica*. 2012; 47:1317–1322.
22. Arbabi-Ghahroudi M, Tanha J, MacKenzie R. Prokaryotic expression of antibodies. *Cancer Metastas Revs*. 2005; 24:501–519.
23. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol*. 2005; 23:1011–1027.
24. Jung YD, Mansfield PF, Akagi M, Takeda A, Liu W, Bucana CD, Hicklin DJ, Ellis LM. Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. *Eur J Cancer*. 2002; 38:1133–1140.
25. Xie W, Li D, Zhang J, Li Z, Acheampong DO, He Y, Wang Y, Chen Z, Wang M. Generation and characterization of a novel human IgG1 antibody against vascular endothelial growth factor receptor 2. *Cancer Immunol Immunotherap CII*. 2014; 63: 877–888.
26. Pal HC, Sharma S, Strickland LR, Agarwal J, Athar M, Elmets CA, Afaq F. Delphinidin reduces cell proliferation and induces apoptosis of non-small-cell lung cancer cells by targeting EGFR/VEGFR2 signaling pathways. *PloS One*. 2013; 8:e77270
27. Yang F, Tang X, Riquelme E, Behrens C, Nilsson MB, Giri U, Varella-Garcia M, Byers LA, Lin HY, Wang J, Raso MG, Girard L, Coombes K, Lee JJ, Herbst RS, Minna JD, Heymach JV, Wistuba, II. Increased VEGFR-2 gene copy is associated with chemoresistance and shorter survival in patients with non-small-cell lung carcinoma who receive adjuvant chemotherapy. *Cancer Res*. 2011; 71:5512–5521.
28. Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol*. 2006; 24:2666–2672.
29. Humtsoe JO, Kramer RH. Differential epidermal growth factor receptor signaling regulates anchorage-independent growth by modulation of the PI3K/AKT pathway. *Oncogene*. 2010; 29: 1214–1226.

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