A murine–human chimeric IgG antibody against vascular endothelial growth factor receptor 2 inhibits angiogenesis in vitro

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Abstract Vascular endothelial growth factor receptor 2 (VEGFR2) has been reported to play an important role in angiogenesis and tumorigenesis. A murine anti-VEGFR2 mAb (A8H1) has been established in a previous study. To reduce the incompatibility of the murine mAb for human use, the chimeric anti-VEGFR2-IgG was developed by genetic recombination of the variable regions of the A8H1 antibody and the constant regions of human IgG, and was expressed in Sp2/0 cells transfected with the two recombinant vectors containing the heavy chain and the light chain regions. After screening, clone 2F12 was selected and was found to stably secrete the murine–human chimeric anti-VEGFR2-IgG (coded 2F12). This chimeric IgG maintained the specificity and the affinity of the parental murine antibody against VEGFR2, and effectively identified VEGFR2 expressed on the surface of HUVECs and BEL-7402 cells. Furthermore, the 2F12 antibody demonstrated

inhibition of angiogenesis in vitro, such as proliferation, migration, invasion and tube formation of HUVECs. This murine–human chimeric IgG may be considered for further development as an anti-angiogenesis and anti-tumor agent.

Keywords Vascular endothelial growth factors receptor 2 · Murine–human chimeric IgG · Anti-angiogenesis · Genetic recombination

Abbreviations

VEGF2	Vascular endothelial growth factor receptor 2
mAb	Monoclonal antibody
HUVECs	Human umbilical vein-derived endothelial cells
K _d	Dissociation constant

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Introduction

Angiogenesis is a tightly regulated multiple-step process that results in the formation of new blood vessels from preexisting vasculature (Ferrara and Kerbel 2005). Accumulating experimental and clinical studies have implicated uncontrolled angiogenesis as a major contributor in tumor growth and metastasis (Mojzis et al. 2008; Lai et al. 2012). A clear correlation between tumor growth and angiogenesis has been identified. Without vascularization, solid tumors only

grow to 1–2 mm in diameter (Naumov et al. 2006). Therefore, tumor growth and metastasis are angiogenesis-dependent, and microvascular endothelial cells (ECs) recruited by tumors have become an important secondary target in cancer therapy (Jin et al. 2012). In contrast to conventional therapies that require targeting individual tumor cells, local interruption of tumor vasculature may adversely affect all tumor cells that are dependent on the vasculature for nourishment. In addition, targeting ECs which support tumor growth is particularly promising because these cells are genetically stable and, therefore, less likely to accumulate mutations that would allow them to develop drug resistance (Kerbel and Folkman 2002).

Compelling evidence suggests that vascular endothelial growth factor (VEGF), a primary stimulant of angiogenesis, can bind and activate its receptors, VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2, or kinase insert domain-containing receptor, KDR) (Lakshmikanthan et al. 2011), both of which are relatively endothelial cell-specific transmembrane tyrosine kinases. VEGF-mediated signaling pathways can mediate endothelial proliferation, migration, morphogenesis, and differentiation (Sharma et al. 2011). Interaction of VEGF with its receptors, especially with VEGFR2, plays a critical role in angiogenesis under both physiological and pathological conditions (Huang et al. 2011; Ugarte-Berzal et al. 2010). VEGFR2 is frequently up-regulated in a number of clinically important human diseases including cancer (Xia et al. 2006; Charpidou et al. 2011). As a result, angiogenesis inhibitors have been developed to target VEGFR2 on vascular ECs and block angiogenesis that is required for tumor growth. These inhibitors include antibodies (Wicki et al. 2012; Kodack et al. 2012), immunotoxins (Behdani et al. 2013), ribozymes (Tao et al. 2007), soluble receptors (Li et al. 2012), and small molecule tyrosine kinase inhibitors (Shen et al. 2012). Some of them have demonstrated significant therapeutic efficacy both in preclinical animal models and in clinical trials in patients with cancer. Taken together, these findings indicate that VEGFR2 represents a potential target for therapeutic intervention of human diseases such as cancer in which pathological angiogenesis is involved (Terme et al. 2013; Mazor et al. 2013).

Monoclonal antibodies (mAbs), owing to their high specificity towards a given target, represent a unique class of novel therapeutics as angiogenesis inhibitors,

especially in tumor therapy (Yao et al. 2011). Compared with small molecule inhibitors against VEGFR2 which normally have off-target effects thus influencing other related tyrosine kinase receptors that are required for the functional regulation of normal cells, the anti-VEGFR2 mAbs may provide higher specificity towards vascular endothelial cells in tumors, and therefore, have a better safety profile. Furthermore, neutralizing mAbs to VEGFR2 will not only block the angiogenic activity of VEGF, but also block that of the other growth factors exerting angiogenic effects via the receptor, including VEGF-C, VEGF-D and VEGF-E. As a result, the neutralizing mAbs can effectively inhibit the pathological angiogenesis. Extensive studies have focused on the development of anti-VEGFR2 mAbs. For example, it is demonstrated that a chimeric anti-VEGFR2 mAb (IMC-1C11, ImClone Systems Corporation, NJ, USA) is capable of blocking VEGF-VEGFR2 interactions and inhibiting VEGF-stimulated receptor activation and mitogenesis of human endothelial cells (Lam et al. 2007). In addition, Ramucirumab (IMC-1121B, ImClone Systems Corporation, NJ, USA) is a fully human IgG1 mAb that binds to the extracellular domain of VEGFR2, thereby blocking VEGF-VEGFR2 interactions. Phase I and II studies of Ramucirumab have been completed, and Phase III clinical trials are underway (Spratlin et al. 2010; Franklin et al. 2011). Taken together, these studies demonstrate that the strategy using VEGFR2-targeting mAbs is a promising therapeutic approach for tumor suppression.

We have previously reported the generation of a murine mAb (A8H1) against VEGFR2 (Huang et al. 2010). In this study, we describe the creation of the murine–human chimeric antibody by genetic recombination of the variable regions of the A8H1 antibody and the constant regions of human IgG. Furthermore, the anti-angiogenesis effect of this chimeric mAb was evaluated *in vitro*.

Materials and methods

Cell lines and agents

The hepatocellular carcinoma cell line (BEL-7402) and the human umbilical vein-derived endothelial cell (HUVEC), both positive for VEGFR2 expression, and mouse fibroblast cell line (NIH3T3) were obtained

from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in DMEM medium (GIBCO, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS) (GIBCO) and maintained at 37 °C in a humidified 5 % CO₂ incubator. The murine A8H1 hybridoma was maintained in our lab as described previously (Huang et al. 2010). A murine–human chimeric anti-anthrax IgG (TEX-IgG) was used as a control mAb which was developed by us (manuscript submitted).

Construction of the vectors for the expression of chimeric anti-VEGFR2 mAb

Total RNA was extracted from the A8H1 hybridoma by the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols. V_H and V_L genes with corresponding signal sequences were amplified by PCR with degenerate primers as described (Roque-Navarro et al. 2003). Amplification conditions consisted of 2 min at 94 °C for Taq activation followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at gradient temperatures from 52.9 to 62.2 °C (52.9, 55.1, 56.7, 58.6, 60.2 and 62.2 °C) for 30 s and elongation at 72 °C for 30 s. PCR products of V_H and V_L were purified and then cloned into pAH4604 at *EcoR* V/*Nhe* I and into pAG4622 at *EcoR* V/*Sal* I, respectively. The recombinant vectors pAH4604/A8H1-V_H and pAG4622/A8H1-V_L were sequenced using an ABI 3700-capillary electrophoresis DNA sequencer. Sequences were further analyzed using the VBASE2 database (<http://www.vbase2.org/>).

Screening of murine–human chimeric anti-VEGFR2 mAb

The pAH4604/A8H1-V_H and pAG4622/A8H1-V_L were linearized by the restriction endonuclease *Pvu* I and co-transfected into the murine myeloma Sp2/0 cells by electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). After 48 h, the selective medium (OPTI-MEM I medium (GIBCO) containing 20 % FBS (GIBCO), 1 mg/mL L-histidinol for heavy chain selection, and 1 µg/mL mycophenolic acid (MPA) plus 250 µg/mL xanthine (all from Sigma-Aldrich, St.

Louis, MO, USA) for light chain selection) was added. Following a two-week selection, cells were subcloned into 96-well plates (Corning Inc., Corning, NY, USA). Supernatants containing the mAbs were screened by a specific three-round ELISA.

Briefly, EIA plates were coated with 5 µg/mL goat anti-human IgG (Fab specific) (Sigma-Aldrich). After blocking with 3 % bovine serum albumin (BSA) in PBS, 100 µL of supernatant containing monoclonal IgG was equally divided into two wells (50 µL per well), and the plates were incubated for 2 h at room temperature. Supernatant of untransfected Sp2/0 cells, the selective medium and 3 % BSA in PBS served as negative controls while the TEX-IgG was used as a positive control. After washing, 50 µL of either alkaline phosphatase (AP)-conjugated goat anti-human IgG (Fc specific) or AP-conjugated goat anti-human kappa light chain (Sigma-Aldrich) were added and incubated for a further 1 h at room temperature for enzyme reaction using the p-Nitrophenyl Phosphate (pNPP) Liquid Substrate System (Sigma-Aldrich). The absorbance was read at 405 nm. The double-positive clones for both anti-kappa and anti-Fc detection were then verified by a VEGFR2-specific ELISA. Briefly, EIA plates were coated with 2 µg/mL of purified human VEGFR2 protein (Cell Sciences, Canton, MA, USA). Supernatants of the clones double-positive for both anti-kappa and anti-Fc detection were applied, followed by the addition of the AP-conjugated goat anti-human IgG (Fc specific) (Sigma-Aldrich) and further incubation for 1 h at room temperature for enzyme reaction using the p-Nitrophenyl Phosphate (pNPP) Liquid Substrate System. The absorbance was read at 405 nm. One of the triple-positive clones with the highest absorbance was chosen for further evaluation.

Purification of murine–human chimeric anti-VEGFR2-IgG

The selected clone was cultured at a large scale in the Hybridoma-SFM medium (GIBCO) supplemented with 5 % ultra-low IgG FBS. The chimeric anti-VEGFR2-IgG was purified by affinity chromatography using a Protein G HP column (GE Healthcare, Piscataway, NJ, USA). The purity of the chimeric anti-VEGFR2-IgG was analyzed by SDS-PAGE (10 %) with Coomassie Blue staining.

Stability of chimeric antibody secretion

The cells secreting the chimeric anti-VEGFR2-IgG were cultured in 10 % FBS/OPTI-MEM I medium (GIBCO) without L-histidinol and MPA (both from Sigma-Aldrich) to test the stability of antibody expression. Cells were transferred every 3–4 days after reaching 90 % confluence. The cells and culture supernatant were collected for 30 passages. To test the stability of the genetic recombination, total RNA from cells of the 30th passage was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To avoid possible genomic DNA contamination, all RNA samples were treated with RNase-free DNase (Promega, Madison, WI, USA). RT-PCR was then carried out while the primer sets and conditions (annealing at 60.2 °C) were used as mentioned above. To test the stability of secretion, all supernatants of 30 passages were tested by the VEGFR2-specific ELISA as described above.

Affinity determination of the chimeric anti-VEGFR2-IgG

The affinity of the chimeric anti-VEGFR2-IgG was calculated by non-competitive ELISA (Loomans et al. 1995). EIA plate was coated at 4 °C overnight with purified VEGFR2 protein either at 4 or 2 µg/mL. After the plate was blocked, serial dilutions of the chimeric anti-VEGFR2-IgG were added (3 replicated wells for each dilution). The concentration of the chimeric anti-VEGFR2-IgG and the absorbance at 405 nm were plotted to two hyperbolic curves by the GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The dissociation constant (K_d) was then calculated using the SPSS statistical software version 13.0 (SPSS Inc., Chicago, IL, USA).

Western blot

HUVECs, BEL-7402 or NIH3T3 cells were lysed in cold RIPA buffer (100 mM Tris HCl, 300 mM NaCl, 2 % NP40 and 0.5 % sodium deoxycholate) supplemented with a proteinase inhibitor cocktail (the cOmplete proteinase inhibitor cocktail; Roche, Indianapolis, IN, USA). Protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay kit according to the manufacturer's instruction (Pierce, Rockford, IL, USA). Typically, 50 µg of protein from

whole-cell lysate were separated by 10 % SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with PBS containing 5 % non-fat milk overnight at 4 °C, incubated with the chimeric anti-VEGFR2-IgG at 20 µg/mL or rabbit polyclonal anti-β-actin antibody (Boster Corp., Wuhan, China) for 1.5 h at room temperature, washed in PBS with 0.05 % Tween 20 (0.05 % PBST), and reacted with HRP-conjugated goat anti-human IgG (Fc specific) (Sigma-Aldrich) or HRP-conjugated goat anti-rabbit IgG (Boster Corp., Wuhan, China), for an additional 1 h at room temperature. The reaction was detected by ECL Western Blot Substrate (Pierce) per the manufacturer's instruction. The ratio of VEGFR2 to β-actin was determined by scanning densitometry using the Quantity One software (version 4.6.3, Bio-Rad).

Cell ELISA assay

HUVECs or BEL-7402 cells harvested in the logarithmic growth phase were seeded at a cell density of 2×10^4 cells/well in 96-well plates. After cells were incubated overnight, the medium was aspirated. Then the cells were washed three times with ice-cold PBS, and fixed with 4 % paraformaldehyde in PBS for 20 min at room temperature. After discarding the fixation solution and drying completely, the plates were blocked with 1 % non-fat milk (Bio-Rad) for 2 h, followed by the addition of 50 µL of the chimeric anti-VEGFR2-IgG or TEX-IgG with gradient antibody concentrations (80, 40, 20, 10, 5, 1.25 and 0.31 µg/mL) in triplicate wells. After 2 h, the antibody solution was removed, and the wells were washed six times with 0.05 % PBST, followed by the addition of goat anti-human IgG coupled to horseradish peroxidase (HRP) at a dilution of 1:2,000 to each well which was next incubated for 1 h at room temperature. After washing for six times with 0.05 % PBST, TMB solution of 50 µL/well was added. After 30 min, color development was stopped by 1 M H_2SO_4 , and the optical absorbance was measured at 450 nm.

In vitro immunofluorescence assay

Briefly, HUVECs or BEL-7402 cells were grown in the 96-well plates at a cell density of 5×10^3 cells/well overnight, washed three times with ice-cold PBS, and fixed with 4 % paraformaldehyde in PBS for

20 min at room temperature. After blocking with 1 % non-fat milk for 2 h, 50 µL of the chimeric anti-VEGFR2-IgG, TEX-IgG or the murine anti-VEGFR2-IgG (A8H1) at an antibody concentration of 80 µg/mL was added into the wells. Following 4 h incubation, the wells with the chimeric anti-VEGFR2-IgG or TEX-IgG added were washed 3 times with 0.05 % PBST, and the fluorescein isothiocyanate (FITC) or rhodamine conjugated goat anti-human IgG antibodies (Abnova, Walnut, CA, USA) were added at a dilution of 1: 16. Meanwhile, the wells with A8H1 antibody added were treated with FITC- or rhodamine-conjugated goat anti-murine IgG antibody (LifeSpan BioSciences, Seattle, WA, USA). The cells were incubated for 1 h at room temperature. Finally the cells were washed as described above and examined with fluorescence microscopy (Olympus, Tokyo, Japan).

Proliferation assay

Cell proliferation was determined using the MTT assay (Chen et al. 2012a). HUVECs, BEL-7402 or NIH3T3 cells (3×10^3 cells/well) were seeded in 96-well plates and exposed to various concentrations of the chimeric anti-VEGFR2-IgG (0, 25, 50, 100, 200 and 400 µg/mL) at 37 °C. The cell viability was observed after incubation for 24, 48, and 72 h (BEL-7402 cells were observed until 96 h of treatment). At 4 h before each observation time point, 10 µL of MTT solution (5 mg/mL) was added to each well. The cells were further incubated for 4 h at 37 °C followed by the removal of medium from the wells. Finally, 150 µL DMSO was added to each well, and the absorbance was measured at 570 nm. All experiments were performed in triplicate.

Migration assay

A modified Boyden chamber assay was used (Glinskii et al. 2012). HUVECs (2×10^4 cells/well) were plated in serum-free medium with the chimeric anti-VEGFR2-IgG at 25, 50, 100, 200 or 400 µg/mL on cell culture inserts with a pore size of 8.0 µm (BD Biosciences, San Jose, CA, USA) and allowed to migrate at 37 °C. Minimum essential medium (MEM) (GIBCO) containing 2.5 % FBS in the lower chamber served as the chemoattractant. All experiments were performed in triplicate. After 12 h of incubation, cells

on the upper side of the filter were removed with cotton swabs, and the migrated cells on the lower side of the filter were fixed and stained with gentian violet (Sigma-Aldrich). Next, the cells migrated were observed and photographed under a light microscope. The average number of migrated cells was calculated from five random microscopic fields. The percentage of migrated cells inhibited by the chimeric anti-VEGFR2-IgG was calculated based on cell migration in control wells.

Cell invasion assay

The invasion assay was carried out as described previously, with some modifications (Zhang et al. 2011). Briefly, the filter of the Transwell plate (Millipore) was coated with Matrigel (BD Biosciences). After Matrigel polymerization, the bottom chambers were filled with MEM containing 2.5 % FBS, and the top chambers were seeded with 100 µL serum-free medium containing the chimeric anti-VEGFR2-IgG at various concentrations (0, 25, 50, 100, 200 and 400 µg/mL) and HUVECs (2×10^4 cells/well). Cells were allowed to migrate for 20 h. Non-invaded cells were scraped with a cotton swab, and invaded cells were fixed. The cells were quantified by manual counting and photographed under a light microscope. The percentage of invaded cells inhibited by the chimeric anti-VEGFR2-IgG was calculated based on cell migration in control wells.

In vitro tube formation assay

In this assay, two study protocols were employed. In the first protocol, the tube formation assay was performed on Matrigel as described previously (Selbonne et al. 2012; Adya et al. 2012). The Matrigel was placed at 4 °C for 24 h prior to the experiment, and then the Matrigel (70 µL/well) was added to 96-well plates and was left for solidification for 1 h at 37 °C. Next, HUVECs were seeded at 1×10^4 cells/well on the Matrigel and exposed to various concentrations of the chimeric anti-VEGFR2-IgG (0, 25, 50, 100, 200 and 400 µg/mL) in DMEM containing 10 % FBS. After 2 h and 4 h of incubation, randomly chosen fields were photographed under a microscope at 100× magnification.

In the second protocol, HUVECs at 1×10^4 cells/well were seeded in 96-well plates 12 h prior to the

assay. Next, following removal of the medium by aspiration, a confluent monolayer of HUVECs was coated with Matrigel (1: 2 dilution with serum-free medium) containing the chimeric anti-VEGFR2-IgG at concentrations of 25, 50, 100, 200, or 400 $\mu\text{g/mL}$. All procedures were performed at 4 °C. After incubation for 2 h at 37 °C to solidify Matrigel, 50 μL of DMEM with the chimeric anti-VEGFR2-IgG (25, 50, 100, 200 and 400 $\mu\text{g/mL}$) were added to the corresponding wells with an identical antibody concentration. After 24, 48 and 120 h, randomly chosen fields from 3 wells/condition were photographed under a microscope at 200 $\times$ magnification.

Statistical analysis

All quantitative data were described as means $\pm$ standard deviation, and analyzed by Variance Analysis and SNK-*q* test of the SPSS statistical software. Differences were considered significant at $P < 0.05$ (two-tailed).

Results

Construction of the vectors for the expression of chimeric anti-VEGFR2 mAb

V_H and V_L genes with the corresponding signal sequences of the murine A8H1 antibody, both about 420 bp, were amplified by RT-PCR at different annealing temperatures (Fig. 1a, b). Two recombinant vectors, pAH4604/A8H1- V_H and pAG4622/A8H1- V_L , were constructed successfully according to sequencing analysis and could be re-cut by *EcoR* *V/Nhe* I or *EcoR* *V/Sal* I (Fig. 1c), confirming the integrity of the vectors.

Screening and purification of the chimeric antibody 2F12

Following transfection Sp2/0 cells were subcloned and 4 positive monoclones (1D6, 1D8, 3B3 and 2F12) were identified by triple-ELISA screening (Table 1).

Fig. 1 Generation and purification of the chimeric anti-VEGFR2-IgG. PCR product of V_H (a) and V_L (b) with signal sequences at different annealing temperatures, from lane 1 to 6 for annealing at 52.9, 55.1, 56.7, 58.6, 60.2 and 62.2 °C. c Recombinant vectors pAH4604/A8H1- V_H re-cut by *EcoR* V and *Nhe* I and pAG4622/A8H1- V_L re-cut by *EcoR* V and *Sal* I. d Coomassie Blue staining demonstrated both heavy chain (about 55 kDa) and light chain (about 25 kDa) of the chimeric anti-VEGFR2-IgG (2F12) were purified from the supernatant of Sp2/0 cells transfected by two vectors. M indicates for marker (Fig. 1a–d)

Table 1 Four positive clones were identified after triple-ELISA screening

Antigen	Primary antibody									Secondary antibody (AP-conjugated)
	1D6	1D8	3B3	2F12	TEX-IgG	Control 1	Control 2	Control 3	Control 4	
Anti-human IgG (Fab-specific)	1.687	1.639	1.613	1.658	1.693	0.339	0.343	0.027	0.007	Anti-human IgG (Fc specific)
Anti-human IgG (Fab-specific)	0.873	0.932	0.909	0.982	0.934	0.366	0.218	0.012	0.001	Anti-human kappa light chain
Purified VEGFR2 protein	0.603	0.749	0.671	0.938	0.025	0.068	0.045	0.034	0.018	Anti-human IgG (Fc specific)

Clone 2F12 showing the strongest reaction was selected for further evaluation

Control 1 Supernatant of untransfected Sp2/0 cells, *Control 2* selective medium, *Control 3* 3 % BSA in PBS, *Control 4* PBS

One of the clones, 2F12, with the strongest affinity to the antigen, was cultured at a large scale and the supernatant was purified using a protein G column. Polyacrylamide gel electrophoresis (10 %) followed by Coomassie Blue staining showed equal expression of the heavy chain (about 55 kDa) and the light chain (about 25 kDa) of the antibody, as shown in Fig. 1d. The purity of the chimeric anti-VEGFR2-IgG (coded 2F12) was above 95 % after purification. According to the sequences of recombinant vectors, pAH4604/A8H1-V_H and pAG4622/A8H1-V_L, the isotype of chimeric antibody clone 2F12 secreted was IgG1/kappa. The detailed DNA sequences of the 2F12 antibody are shown in Supplementary Fig. 1.

Stability of chimeric antibody secretion

As shown in Fig. 2a, using the primer sets and conditions as mentioned in the methods, the expected PCR products of V_H and V_L genes with corresponding signal sequences, both about 420 bp, were obtained from clone 2F12 which is still capable of secreting the chimeric anti-VEGFR2-IgG at the 30th passage following culture without the selective pressure by L-histidinol and MPA. As a positive control, identical PCR products were also obtained from the A8H1 hybridoma secreting murine anti-VEGFR2 antibody. No such products were obtained in the control Sp2/0 cells without transfection. The sequencing results confirmed that the V_H and V_L genes inserted were correct.

By ELISA, all supernatants from the cells of clone 2F12 cultured without the selective pressure during 30 passages were shown to be positive against the VEGFR2 protein (Fig. 2b). A further detailed analysis

revealed that no significant declines were observed in antibody secretion by clone 2F12 during the passages.

Affinity determination of the 2F12 antibody

The 2F12 antibody could bind VEGFR2 protein (either at 4 or 2 µg/mL) in a dose-dependent manner. The concentration of mAbs and the absorbance at 405 nm were plotted to two hyperbolic curves using GraphPad Prism software. Using SPSS statistical software, the K_d of the 2F12 antibody was calculated to be 8.998×10^{-9} mol/L, compared with 6.952×10^{-9} mol/L of the A8H1 antibody, using the SPSS statistical software (Fig. 2c). The result showed that the affinity of the chimeric 2F12 antibody remained similar to its parental murine one.

Western blot

Western blot results revealed that the 2F12 antibody could recognize the VEGFR2 protein in HUVECs, BEL-7402 and NIH3T3 cells as its parental murine A8H1 antibody. The level of VEGFR2 protein expression was highest in HUVECs, moderate in BEL-7402 cells and lowest in NIH3T3 cells, according to scanning densitometry (Fig. 3a).

Cell ELISA assay and in vitro immunofluorescence assay

Cell ELISA analysis revealed that the 2F12 antibody could specifically bind the VEGFR2 on HUVEC and BEL-7402 cell surface in a dose-dependent manner, while the control antibody TEX-IgG with different antigen specificity could not bind to these two cell

Fig. 2 Stability and affinity determination of the 2F12 antibody. **a** Cells of clone 2F12 were cultured in 10 %FBS/OPTI-MEM I medium without L-histidinol or MPA. After 30 passages, the total RNA from cells of the 30th passage was extracted, and the V_H and V_L fragment with signal sequences of the 2F12 antibody were amplified by RT-PCR. A8H1 hybridoma and Sp2/0 cells were also treated as controls. M indicates for DNA marker. **b** The ELISA result of supernatants from clone 2F12 of all 30 passages against VEGFR2 protein. Purified human/

murine chimeric anti-VEGFR2-IgG (2F12) was used as positive control and supernatant of untransfected Sp2/0 cells was used as negative control. **c** The affinity determination of the 2F12 antibody by non-competitive ELISA. The 2F12 antibody could bind VEGFR2 protein (either at 4 or 2 μg/mL) in a dose-dependent manner. The concentration of the 2F12 antibody and the absorbance at 405 nm were plotted to two hyperbolic curves by GraphPad Prism software version 5.0

lines even at a higher antibody concentration (Fig. 3b, c). Taken together, these results demonstrated that the 2F12 antibody could specifically bind to the VEGFR2-positive cells as its parental murine A8H1 antibody.

The 2F12 antibody could specifically bind HUVECs and BEL-7402 cells with VEGFR2 expression as evidenced by the immunofluorescence microscopic

observation (Fig. 4). Following incubation with the 2F12 antibody, BEL-7402 cells treated with FITC-conjugated anti-human-IgG exhibited green fluorescence, and HUVECs treated with rhodamine-conjugated anti-human IgG exhibited red fluorescence around the cell surface and in the cytoplasm. Following incubation with the A8H1 antibody, HUVECs

Fig. 3 Binding efficacy of the 2F12 antibody on VEGFR2. **a** Western blot showed that the 2F12 antibody could recognize the VEGFR2 protein of (230 kDa) in HUVECs, BEL-7402 and NIH3T3 cells. Compared with β-actin expression, the level of VEGFR2 protein expression was highest in HUVECs, modest in BEL-7402 cells and lowest in NIH3T3 cells, according to the scanned Western Blot using the Quantity One software. HUVECs (**b**) and BEL-7402 (**c**) cells detected by cell ELISA. HUVECs and BEL-7402 cells were incubated with the 2F12 antibody or the TEX-IgG at concentration ranging from 0.31 to 80 μg/mL. The 2F12 antibody could bind BEL-7402 cells and HUVECs, but the TEX-IgG could not

treated with FITC-conjugated anti-murine-IgG exhibited green fluorescence, and BEL-7402 cells treated with rhodamine-conjugated anti-murine IgG exhibited red fluorescence around the cell surface and in the cytoplasm. In contrast, no fluorescent signal was

detected in these two cell lines treated with TEX-IgG. These results implied that this chimeric antibody could specifically bind to the VEGFR2 protein on the cell surface as its parental A8H1 antibody.

Proliferation assay

As shown in Fig. 5a, b, it was demonstrated that the 2F12 antibody at various concentrations could significantly inhibit the proliferation of HUVECs in a dose-dependent manner. The absorbance values, which corresponded to the cell density, for wells treated with the 2F12 antibody at various concentrations were significantly lower than that for the control wells at each time point ($P < 0.05$). The 2F12 antibody exhibited anti-proliferation effect on HUVECs following treatment for 24 h. The percentage of viable cells dropped to $(51.07 \pm 8.64) \%$ of control following treatment with 25 μg/mL of the 2F12 antibody, and the percentages further went down to $37.85 \pm 6.90 \%$ of control when 400 μg/mL of the antibody was used. The inhibition effect was most pronounced at 48 h after treatment and the percentages of viable cells were (32.23 ± 2.35) and $(25.33 \pm 4.94) \%$ when the antibody was added at 25 and 400 μg/mL, respectively.

As shown in Fig. 5b, the 2F12 antibody of 400 μg/mL could significantly inhibit the proliferation of BEL-7402 cells when compared with the control ($P < 0.05$). The anti-proliferation effect of 2F12 was weaker on BEL-7402 cells than on HUVECs at each time point. The percentages of viable BEL-7402 cells were higher than those of HUVECs, even the observe time prolonged to 96 h after 2F12 treatment.

In contrast to the above two cell lines, there was no significant difference ($P > 0.05$) in the proliferation of NIH3T3 cells treated with the 2F12 antibody at various concentrations (Fig. 5c).

Migration and cell invasion assay

The 2F12 antibody could antagonize the migration and invasion of HUVECs, as the number of cells that migrated to and invaded the lower side of the filter was significantly reduced in the wells treated with the 2F12 antibody than in the control wells in a dose-dependent manner (Fig. 6a). After 2F12 treatment for 12 h, the inhibition rates of migration of HUVECs through the Matrigel were 13.04, 19.56, 43.91, 71.74 and 79.57 %

Fig. 4 Binding efficacy of the 2F12 antibody on HUVECs and BEL-7402 detected by immunofluorescence assay. The binding efficacy of the 2F12 antibody was assessed by immunofluorescence observation. HUVECs and BEL-7402 cells, incubated with the 2F12 antibody followed by either FITC-conjugated goat anti-human IgG antibody (*green*) or rhodamine-conjugated goat anti-human IgG antibody (*red*), showed fluorescence on the

membrane. HUVECs, incubated with the A8H1 antibody followed by either FITC-conjugated goat anti-murine IgG antibody (*green*) or rhodamine-conjugated goat anti-murine IgG antibody (*red*), showed membrane fluorescence. The cell nuclei were stained with DAPI (*blue*). No signal was observed in HUVECs incubated with the TEX-IgG incubated with the 2F12 antibody. (Color figure online)

Fig. 5 Anti-proliferation of the 2F12 antibody on HUVECs detected by the MTT method. **a** The absorbance values of cells treated with the 2F12 antibody at different doses at 24, 48 and 72 h. The 2F12 antibody at different doses could significantly inhibit the cell proliferation of HUVECs. There were significant differences in the absorbance values between control wells and wells treated with the 2F12 antibody at different doses at each time point ($P < 0.05$). The cell viability rate was calculated after the 2F12 incubation at 24, 48 and 72 h. After the 2F12 treatment of 24 h, the cell viability was (51.07 ± 8.64) , (46.47 ± 7.51) , (43.73 ± 6.67) , (40.92 ± 5.82) and (37.85 ± 6.90) % of control, respectively, in cells treated with the 2F12 antibody with

different concentration (25–400 µg/mL). The inhibition effect was most obvious at 48 h treatment of the 2F12 antibody, and the cell viability was (32.23 ± 2.35) %, (34.14 ± 5.03) %, (30.18 ± 5.30) %, (26.71 ± 5.82) %, (25.33 ± 6.90) % of control, respectively, in cells treated with the 2F12 antibody at different concentration (25–400 µg/mL). **b** The 2F12 antibody at 400 µg/mL could significantly inhibit the cell proliferation of BEL-7402 cells, when compared with the control ($P < 0.05$). The inhibition effect was most obvious under the 400 µg/mL 2F12 treatment of 96 h, and the cell viability was (56.24 ± 5.06) % of control. **c** The 2F12 antibody at different doses has no significant inhibition effect on NIH3T3 cells

at 25, 50, 100, 200 and 400 µg/mL doses, respectively (Fig. 6b). Similarly, the 2F12 treatment for 18 h inhibited the number of HUVECs invading through the Matrigel by 8.28, 24.14, 58.62, 74.48 and 81.38 % at doses of 25, 50, 100, 200 and 400 µg/mL,

respectively (Fig. 6c). There were significant differences in the number of migrating and invading cells between wells of 100, 200 and 400 µg/mL of the 2F12 treatment and control ($P < 0.05$). When the 2F12 at 400 µg/mL was added, the number of HUVECs having

Fig. 6 Migration and invasion of HUVECs treated with the 2F12 antibody in vitro. **a** For migration and invasion analysis of cells on the lower side of the filter were fixed, stained with gentian violet, and observed by microscopy ($\times 200$). The percentage of cells inhibited by the 2F12 antibody was expressed on the basis of control wells: **(b)** migration cell

migrated and invaded to the membrane was smallest, (20.43 ± 8.56) and (18.62 ± 7.34) % of control, respectively.

In vitro tube formation assay

The control HUVECs plated on Matrigel became elongated, exhibited extended protrusions, and were

number, and **(c)** invasion cell number. The 2F12 antibody could reduce migration and invasion of HUVECs. With the increased doses of the 2F12 antibody, the number of HUVECs having migrated to and invaded the membrane of the Boyden Chambers was decreased, compared to control without the 2F12 treatment

aligned along the perimeter of the enclosed spaces after 4 h of culture. In contrast, the presence of the 2F12 antibody significantly reduced the tubular network formation of HUVECs in a dose-dependent manner (Fig. 7). After 24 h of culture, the control HUVECs exhibited the anastomosing tubes with multicentric junctions, while the phenomenon was not obvious in cells treated with the 2F12 antibody

Fig. 7 The effect of the 2F12 antibody on the tube formation of HUVECs in vitro. Control HUVECs without 2F12 treatment aligned to form lumen-like structures and anastomosing tubes with multicentric junctions after 4 h culture. Cells treated with the 2F12 antibody of different concentrations, formed fewer tubes and weaker anastomoses. And the effect of inhibiting tube formation was stronger with the increased concentration of the

2F12 antibody. With time prolonged, control HUVECs without 2F12 treatment exhibited more obvious lumen-like structures and anastomosing tubes with multicentric junctions. While cells treated with the 2F12 antibody of different concentrations, formed fewer tubes and weaker anastomoses. The effect of inhibiting tube formation of the 2F12 antibody was in a dose-dependent manner

(Fig. 7). With extended incubation time, HUVECs in control medium were aligned to form more pronounced lumen-like structures and the anastomosing tubes with multicentric junctions. In contrast, the 2F12 antibody inhibited the formation of such tubes and anastomoses. The inhibitory effect became stronger with increased antibody concentrations. Taken together, these results demonstrated that the 2F12 antibody could effectively inhibit the tube formation of HUVECs which exhibited fewer lumen-like structures and anastomosing tubes in a dose- and time-dependent manner.

Discussion

In the present study, we screened and developed a cell line derived from Sp2/0 cells transfected with recombinant vectors containing the V_H and V_L of murine anti-VEGFR2 antibody (A8H1), which could stably secrete a murine–human chimeric anti-VEGFR2-IgG antibody (2F12). The affinity of the chimeric antibody 2F12 was similar to that of the parental A8H1 antibody, and the 2F12 antibody could specifically bind to VEGFR2 on the cell surface. Furthermore, the 2F12 antibody could suppress key steps involved in angiogenesis, including proliferation, migration, invasion and tubulogenesis in endothelial cells.

The mAb shows high specificity for a given target and represents a unique class of novel therapeutic agents as an angiogenesis inhibitor. However, many mAb-based clinical trials in the 1980s and early 1990s failed to show efficacy, mainly because of several intrinsic properties associated with rodent-derived mAbs, such as the recognition and rejection of the murine antibodies by the human host which can generate an human anti-mouse antibody (HAMA) response (Ott et al. 2012). Ideally, fully human mAbs are desired for application in humans, which, unfortunately, is ethically impractical. In addition, the lack of a human cell line that has high fusion efficiency and maintains stable secretion of mAbs has hindered the development of human–human hybridomas. Consequently, molecular modifications of murine mAbs including chimeric and humanized mAbs are therefore carried out to reduce the immunogenicity, but to retain similar affinity and stability of the murine parental antibodies, so as to facilitate clinical applications (Salles et al. 2012).

One of the methods to develop the chimeric antibody is using eukaryotic expression plasmid(s) in mammalian cells. The two plasmids, pAH4604 and pAG4622, contain intron(s) between the variable region and constant region(s), which complies with antibody secretion *in vivo*. Linearization of both vectors by *Pvu* I, which cuts once in the ampicillin-resistance gene, might not be able to increase transfection efficiency. However, it raised the chances of the integration of the vectors into the host cell genome without disrupting the inserted antibody genes or other elements required for expression in mammalian cells. The selective markers in these two vectors are the histidinol dehydrogenase (*His D*) gene in pAH4604 and the xanthine-guanine phosphoribosyl transferase (XGPRT, also known as GPT) gene in pAG4622. The selective medium contained L-histidinol for *His D* screening and MPA together with xanthine for XGPRT screening. A stably transfected cell line that secreted anti-VEGFR2-IgG was obtained. By RT-PCR assay, identical sequences of the V_H and V_L regions of murine A8H1 antibody could be amplified from 2F12 cells even after 30 culture passages. By ELISA assay, the 2F12 cells that were maintained in medium without L-histidinol and MPA could secrete anti-VEGFR2-IgG stably during passages, and there was no significant change in antibody secretion among all the 30 passages.

The 2F12 antibody showed a high binding activity with a recombinant human VEGFR2 protein by ELISA, and had a similar affinity as its parental A8H1 antibody according to the affinity determination. The 2F12 antibody could specifically bind to HUVECs and BEL-7402 cells with VEGFR2 expression by cell ELISA and immunofluorescence assay. All these results demonstrate that the antibody engineering process did not change the specificity of the chimeric mAb 2F12 compared with its parental murine anti-VEGFR2 A8H1 antibody. The murine A8H1 antibody was designed to target VEGFR2 extracellular Ig domain 3 which is the VEGFs binding region (Roskoski 2008). The binding of antibody and VEGFR2 may attenuate the interaction between VEGFs and VEGFR2, and may partly block downstream pathways. The chimeric 2F12 antibody has the same specificity and is supposed to target the same epitope of VEGFR2 and to affect the pathways as the murine precursor. The Fc region was consequently converted from murine IgG2a to human IgG1. Fc region of human IgG1 can enhance

Fc γ RIII-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), and can be the most suitable for therapeutic use against pathogen and cancer cells (Sawada et al. 2011). The effect of ADCC and CDC of the 2F12 antibody on hepatocellular carcinoma cells needs to be further confirmed.

Tumor angiogenesis is a complex interrelated multistep process. Inhibition of some steps of these processes may lead to the disruption of angiogenesis and can be exploited as a potential antitumor therapy. The effect of anti-angiogenesis could be achieved by inhibiting the proliferation, migration and invasion of vascular endothelial cells. In our results, the 2F12 antibody could inhibit the proliferation, migration, invasion and tube formation of HUVECs in vitro. As the A8H1 antibody has the ability to bind to NIH3T3 cells (in the nuclei) according to a previous study (Huang et al. 2010), we assessed whether the 2F12 antibody had an effect on NIH3T3 cells in current study. The result showed that the 2F12 antibody had no significant influence on the proliferation of NIH3T3 cells which has sparing VEGFR2 expression in the nuclei while no VEGFR2 expression on the surface. Furthermore, we observed that the 2F12 antibody had much weaker inhibition effect on BEL-7402 cells than on HUVECs. This phenomenon may be partly because the VEGFR2 expression was lower in hepatocellular carcinoma cells like BEL-7402 cells than in HUVECs (Chen et al. 2012b). This phenomenon may also be partly explained by the fact that the main subtype of VEGF receptor on the surface of BEL-7402 cells is VEGFR1 rather than VEGFR2. A recent study has found that 82.8 % of the cultured BEL-7402 cells expressed VEGFR1, while only 13.1 % of the cells expressed VEGFR2 (Zhang et al. 2012). Above all, it is suggested that the chimeric antibody 2F12 has an efficient anti-angiogenesis effect through the VEGFR2 on the surface of HUVECs, and has the potential to suppress tumor growth by its anti-angiogenesis effect.

One of the methods to reveal anti-angiogenesis is the tube formation assay performed on Matrigel. Vascular endothelial cells can be seeded on the Matrigel to observe the tube formation. In this method, the phenomenon can only be observed within 24 h. In contrast, we seeded the cells under the Matrigel mimicking a 3D culture. With the nutrients in the Matrigel and the top liquid culture medium, cells can grow three-dimensionally in the Matrigel, overcoming

the contact inhibition in the plane culture. Thus, changes in cell morphology and anti-angiogenesis effects can be observed even after 120 h. In addition, some tumor cells are observed to highly express the VEGFR2 protein on the cell surface, such as breast cancer cells (Lee et al. 2013) and non-small-cell lung cancer cells (Chen et al. 2013). It is possible to further increase the efficacy of the chimeric anti-VEGFR2-IgG antibody by conjugating the antibody with anti-tumor agents, as the conjugated antibody can not only bind and inhibit the mesenchyma of vascular endothelial cells for stopping the nutrient supply to tumor cells, but also target and kill the parenchyma of cancer cells directly. Further experiments might also focus on how the VEGFR2 signaling pathway is affected by the 2F12 antibody, like the phosphorylation of VEGFR2 and regulation of downstream pathways, and on whether the 2F12 antibody can inhibit the tube formation of HUVECs in vivo, although it had this effect in vitro.

For in vitro study, 400 μ g/ml of mAb used is relative high. The reasons were considered as follows. First, it was found that the dissociation constant of the 2F12 antibody was 8.998×10^{-9} mol/L, higher than 6.952×10^{-9} mol/L of the A8H1 antibody. This means that the 2F12 antibody has a little lower affinity to VEGFR2 than the A8H1 antibody. So a relative high concentration of 2F12 was used for the in vitro study. Second, the 2F12 antibody might have a little lower inhibition effect of angiogenesis in vitro than the A8H1 antibody. Hence, we chose a wide range of concentrations of the 2F12 antibody for evaluation. Third, even at 25 μ g/mL, the 2F12 antibody significantly inhibited the proliferation of HUVECs, and at 100 μ g/mL, 2F12 antibody could significantly inhibit the migration and invasion of HUVECs. Furthermore, the 2F12 antibody could obviously inhibit the formation of such tubes and anastomoses, and the inhibitory effect became stronger with antibody concentrations increased. As a result, it is definite that the 2F12 antibody has an inhibition effect on angiogenesis in vitro at a wide range of antibody concentrations. Of course, the optimal inhibition concentration is desirable and will be further investigated in vitro and in vivo.

In conclusion, we report here the development of a chimeric anti-VEGFR2-IgG antibody (2F12) generated by antibody engineering using the variable regions of the murine A8H1 antibody. It is

demonstrated that the 2F12 antibody can specifically bind VEGFR2 on HUVECs and inhibit HUVECs in vitro. The results highly suggest that the 2F12 antibody might be a potential candidate for the anti-angiogenesis and anti-tumor therapy, and more detailed mechanisms should be explored in further studies.

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References

- Adya R, Tan BK, Chen J, Rande HS (2012) Protective actions of globular and full-length adiponectin on human endothelial cells: novel insights into adiponectin-induced angiogenesis. *J Vasc Res* 49:534–543
- Behdani M, Zeinali S, Karimipour M, Khanahmad H, Schoonooghe S, Aslemarzi A, Seyed N, Moazami-Godarzi R, Baniahmad F, Habibi-Anbouhi M, Hassanzadeh-Ghassabeh G, Muyldermans S (2013) Development of VEGFR2-specific Nanobody *Pseudomonas* exotoxin A conjugated to provide efficient inhibition of tumor cell growth. *N Biotechnol* 30:205–209
- Charpidou A, Gkiozos I, Konstantinou M, Eleftheraki A, Demertzis P, Harrington K, Polyzos A, Syrigos KN (2011) Bronchial washing levels of vascular endothelial growth factor receptor-2 (VEGFR2) correlate with overall survival in NSCLC patients. *Cancer Lett* 304:144–153
- Chen R, Zhang D, Mao Y, Zhu J, Ming H, Wen J, Ma J, Cao Q, Lin H, Tang Q, Liang J, Feng Z (2012a) A human Fab-based immunoconjugate specific for the LMP1 extracellular domain inhibits nasopharyngeal carcinoma growth in vitro and in vivo. *Mol Cancer Ther* 11:594–603
- Chen ZY, Shi M, Peng LX, Wei W, Li XJ, Guo ZX, Li SH, Zhong C, Qian CN, Guo RP (2012b) Dovitinib preferentially targets endothelial cells rather than cancer cells for the inhibition of hepatocellular carcinoma growth and metastasis. *J Transl Med* 10:245
- Chen S, Liu X, Gong W, Yang H, Luo D, Zuo X, Li W, Wu P, Liu L, Xu Q, Ji A (2013) Combination therapy with VEGFR2 and EGFR siRNA enhances the antitumor effect of cisplatin in non-small cell lung cancer xenografts. *Oncol Rep* 29:260–268
- Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. *Nature* 438:967–974
- Franklin MC, Navarro EC, Wang Y, Patel S, Singh P, Zhang Y, Persaud K, Bari A, Griffith H, Shen L, Balderes P, Kussie P (2011) The structural basis for the function of two anti-VEGF receptor 2 antibodies. *Structure* 19:1097–1107
- Glinkii OV, Sud S, Mossine VV, Mawhinney TP, Anthony DC, Glinki GV, Pienta KJ, Glinki VV (2012) Inhibition of prostate cancer bone metastasis by synthetic TF antigen mimic/galectin-3 inhibitor lactulose-L-leucine. *Neoplasia* 14:65–73
- Huang J, Tan Y, Tang Q, Liu X, Guan X, Feng Z, Zhu J (2010) A high-affinity human/mouse cross-reactive monoclonal antibody, specific for VEGFR-2 linear and conformational epitopes. *Cytotechnology* 62:61–71
- Huang J, Zhu H, Wang X, Tang Q, Huang H, Wu K, Zhu J, Feng Z, Shi G (2011) The patterns and expression of KDR in normal tissues of human internal organs. *J Mol Histol* 42:597–603
- Jin F, Brockmeier U, Otterbach F, Metzen E (2012) New insight into the SDF-1/CXCR4 axis in a breast carcinoma model: hypoxia-induced endothelial SDF-1 and tumor cell CXCR4 are required for tumor cell intravasation. *Mol Cancer Res* 10:1021–1031
- Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2:727–739
- Kodack DP, Chung E, Yamashita H, Incio J, Duyverman AM, Song Y, Farrar CT, Huang Y, Ager E, Kamoun W, Goel S, Snuderl M, Lussiez A, Hiddingh L, Mahmood S, Tannous BA, Eichler AF, Fukumura D, Engelman JA, Jain RK (2012) Combined targeting of HER2 and VEGFR2 for effective treatment of HER2-amplified breast cancer brain metastases. *Proc Natl Acad Sci USA* 109:E3119–E3127
- Lai SL, Cheah SC, Wong PF, Noor SM, Mustafa MR (2012) *In vitro* and *in vivo* anti-angiogenic activities of panduratin A. *PLoS ONE* 7:e38103
- Lakshmikanthar S, Sobczak M, Chun C, Henschel A, Dargatz J, Ramchandran R, Chrzanoska-Wodnicka M (2011) Rap1 promotes VEGFR2 activation and angiogenesis by a mechanism involving integrin $\alpha v \beta_3$. *Blood* 118:2015–2026
- Lam T, Hetherington JW, Greenman J, Little S, Maraveyas A (2007) Metronomic chemotherapy dosing-schedules with estramustine and temozolomide act synergistically with anti-VEGFR-2 antibody to cause inhibition of human umbilical venous endothelial cell growth. *Acta Oncol* 46:1169–1177
- Lee E, Koskimaki JE, Pandey NB, Popel AS (2013) Inhibition of lymphangiogenesis and angiogenesis in breast tumor xenografts and lymph nodes by a peptide derived from transmembrane protein 45A. *Neoplasia* 15:112–124
- Li ZJ, Zhu H, Ma BY, Zhao F, Mao SH, Liu TG, He JP, Deng LC, Yi C, Huang Y (2012) Inhibitory effect of *Bifidobacterium infantis*-mediated sKDR prokaryotic expression system on angiogenesis and growth of Lewis lung cancer in mice. *BMC Cancer* 12:155
- Loomans EE, Roelen AJ, Van Damme HS, Bloemers HP, Gribnau TC, Schielen WJ (1995) Assessment of the functional affinity constant of monoclonal antibodies using an improved enzyme-linked immunosorbent assay. *J Immunol Methods* 184:207–217
- Mazor R, Alsaigh T, Shaked H, Altshuler AE, Pocock ES, Kistler EB, Karin M, Schmid-Schönbein GW (2013) Matrix metalloproteinase-1 mediated upregulation of vascular endothelial growth factor-2 in endothelial cells. *J Biol Chem* 288:598–607
- Mojzis J, Varinska L, Mojzisova G, Kostova I, Mirossay L (2008) Antiangiogenic effects of flavonoids and chalcones. *Pharmacol Res* 57:259–265
- Naumov GN, Akslen LA, Folkman J (2006) Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. *Cell Cycle* 5:1779–1787
- Ott MG, Marmé F, Moldenhauer G, Lindhofer H, Hennig M, Spannagl R, Essing MM, Linke R, Seimetz D (2012)

- Humoral response to catumaxomab correlates with clinical outcome: results of the pivotal phase II/III study in patients with malignant ascites. *Int J Cancer* 130:2195–2203
- Roque-Navarro L, Mateo C, Lombardero J, Mustelier G, Fernández A, Sosa K, Morrison SL, Pérez R (2003) Humanization of predicted T-cell epitopes reduces the immunogenicity of chimeric antibodies: new evidence supporting a simple method. *Hybrid Hybridomics* 22:245–257
- Roskoski R Jr (2008) VEGF receptor protein-tyrosine kinases: structure and regulation. *Biochem Biophys Res Commun* 375:287–291
- Salles G, Morschhauser F, Lamy T, Milpied N, Thieblemont C, Tilly H, Bieska G, Asikanius E, Carlile D, Birkett J, Pisa P, Cartron G (2012) Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. *Blood* 119:5126–5132
- Sawada R, Sun SM, Wu X, Hong F, Ragupathi G, Livingston PO, Scholz WW (2011) Human monoclonal antibodies to sialyl-Lewis^x (CA19.9) with potent CDC, ADCC, and antitumor activity. *Clin Cancer Res* 17:1024–1032
- Selbonne S, Azibani F, Iatmanen S, Boulaftali Y, Richard B, Jandrot-Perrus M, Bouton MC, Arocas V (2012) *In vitro* and *in vivo* antiangiogenic properties of the serpin protease nexin-1. *Mol Cell Biol* 32:1496–1505
- Sharma PS, Sharma R, Tyagi T (2011) VEGF/VEGFR pathway inhibitors as anti-angiogenic agents: present and future. *Curr Cancer Drug Targets* 11:624–653
- Shen G, Li Y, Du T, Shi G, Dai L, Chen X, Zheng R, Li W, Su X, Zhang S, Wei Y, Yang S, Deng H (2012) SKLB1002, a novel inhibitor of VEGF receptor 2 signaling, induces vascular normalization to improve systemically administered chemotherapy efficacy. *Neoplasia* 59:486–493
- Spratlin JL, Cohen RB, Eadens M, Gore L, Camidge DR, Diab S, Leong S, O'Bryant C, Chow LQ, Serkova NJ, Meropol NJ, Lewis NL, Chiorean EG, Fox F, Youssoufian H, Rowinsky EK, Eckhardt SG (2010) Phase I pharmacologic and biologic study of ramucirumab (IMC-1121B), a fully human immunoglobulin G1 monoclonal antibody targeting the vascular endothelial growth factor receptor-2. *J Clin Oncol* 28:780–787
- Tao Y, Kim J, Yin Y, Zafar I, Falk S, He Z, Faubel S, Schrier RW, Edelstein CL (2007) VEGF receptor inhibition slows the progression of polycystic kidney disease. *Kidney Int* 72:1358–1366
- Terme M, Pernot S, Marcheteau E, Sandoval F, Benhamouda N, Colussi O, Dubreuil O, Carpentier AF, Tartour E, Taieb J (2013) VEGFA-VEGF receptor pathway blockade inhibits tumor-induced regulatory T cell proliferation in colorectal cancer. *Cancer Res* 73:539–549
- Ugarte-Berzal E, Redondo-Muñoz J, Eroles P, Del Cerro MH, García-Marco JA, Terol MJ, García-Pardo A (2010) VEGF/VEGFR2 interaction down-regulates matrix metalloproteinase-9 via STAT1 activation and inhibits B chronic lymphocytic leukemia cell migration. *Blood* 115: 846–849
- Wicki A, Rochlitz C, Orleth A, Ritschard R, Albrecht I, Herrmann R, Christofori G, Mamot C (2012) Targeting tumor-associated endothelial cells: anti-VEGFR2 immunoliposomes mediate tumor vessel disruption and inhibit tumor growth. *Clin Cancer Res* 18:454–464
- Xia G, Kumar SR, Hawes D, Cai J, Hassanieh L, Groshen S, Zhu S, Masood R, Quinn DI, Broek D, Stein JP, Gill PS (2006) Expression and significance of vascular endothelial growth factor receptor 2 in bladder cancer. *J Urol* 175:1245–1252
- Yao J, Wu X, Zhuang G, Kasman IM, Vogt T, Phan V, Shibuya M, Ferrara N, Bais C (2011) Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PIGF antibodies efficacy. *Proc Natl Acad Sci USA* 108: 11590–11595
- Zhang S, Cao Z, Tian H, Shen G, Ma Y, Xie H, Liu Y, Zhao C, Deng S, Yang Y, Zheng R, Li W, Zhang N, Liu S, Wang W, Dai L, Shi S, Cheng L, Pan Y, Feng S, Zhao X, Deng H, Yang S, Wei Y (2011) SKLB1002, a novel potent inhibitor of VEGF receptor 2 signaling, inhibits angiogenesis and tumor growth *in vivo*. *Clin Cancer Res* 17:4439–4450
- Zhang L, Wang JN, Tang JM, Kong X, Yang JY, Zheng F, Guo LY, Huang YZ, Zhang L, Tian L, Cao SF, Tuo CH, Guo HL, Chen SY (2012) VEGF is essential for the growth and migration of human hepatocellular carcinoma cells. *Mol Biol Rep* 39:5085–5093