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Development of humanized rabbit monoclonal antibodies against vascular endothelial growth factor receptor 2 with potential antitumor effects

Yanlan Yu [a,*], Pierre Lee [b], Yaohuang Ke [b], Yongke Zhang [b], Jungang Chen [c], Jihong Dai [c], Mingzhen Li [c], Weimin Zhu [b], Guo-Liang Yu [b]

[a] Department of Urology, Sir Run-Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, China
[b] Epitomics, Inc., Burlingame, CA, USA
[c] Hangzhou Yikang Biotech, Inc., Hangzhou, China

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) plays a critical role in physiologic and pathologic angiogenesis through its receptors especially through VEGFR2. The lack of cross-reactivity of monoclonal antibodies with human VEGFR2/mouse Flk-1 is a major obstacle in preclinical developments. In this study, using a unique hybridoma technique, we generated a panel of 30 neutralization anti-VEGFR2 rabbit monoclonal antibodies (RabMAbs) either blocking VEGF/VEGFR2 interaction or inhibiting VEGF-stimulated VEGFR2 tyrosine kinase phosphorylation. Among 18 RabMAbs with human/mouse VEGFR2 cross-reactivity, we humanized one lead candidate RabMAb by Mutational Lineage Guided (MLG) method and further demonstrated its potent inhibition of tumor growth in xenograft mouse model. Our study suggests that RabMAbs are highly relevant for therapeutic applications.

1. Introduction

Vascular endothelial growth factor-A (VEGF-A) is one of the most widely studied angiogetic factors, which plays a critical role in tumor growth, maintenance and metastasis [1]. VEGF exerts its effects on endothelial cells through binding to two high-affinity receptors, VEGFR1 and VEGFR2 (KDR in human or Flk-1 in mouse) [2]. Of these two receptors, VEGFR2 appears to be the main receptor mediating the conformational change for phosphorylation of tyrosine residues and for transduction of a series of kinases which subsequently propagate intracellular signaling leading to proliferation, migration, differentiation, tube formation and increased vascular permeability [3]. Endothelial cells in the tumor vasculature exhibit a high level of VEGFR2 expression, about 3 to 5-fold higher than in the normal vasculature [4]. In endothelial cells, the VEGF-A expressed by tumor cells and activated by stroma cells up-regulates VEGFR2 gene expression via a positive feedback mechanism [5]. It has also been reported that the over-expression of VEGFR2 on tumor cells is induced by hypoxia or genetic alterations, suggesting that the autocrine VEGF-A/VEGFR2 loop plays important roles in the tumor progression [6,7]. Therefore, VEGFR2 becomes an attractive target for both antiangiogenesis and anti-tumor therapy.

There are a number of compounds targeting angiogenesis factors or tyrosine kinases currently under various stages of clinical development. Compared with small molecular inhibitors, therapeutic antibodies may provide higher specificity towards tumors, and therefore, better safety profile. Although an anti-VEGF blocking antibody, bevacizumab (AVASTIN) approved by the FDA to treat metastatic colorectal cancer and nonsquamous, non-small-cell lung cancer [8,9], the potential of antibodies targeting VEGFR2 has not been fully explored. To our knowledge, Ramucirumab (IMC-1121B), is the only fully humanized therapeutic antibody specifically developed against VEGFR2 currently in clinical trials [10]. The lack of cross-reactivity of monoclonal antibodies (mAbs) directed to human VEGFR2 with the mouse antigen has been a major obstacle in their preclinical development [11]. In preclinical studies, human tumor xenograft mouse models are the major testing system to evaluate efficacy of these potential therapeutic antibodies. Newly formed blood vessels in the transplanted tumor originate from the host, but conventional mouse antihuman mAbs typically do not react with cell surface receptors displayed by mouse endothelial cells [12].

In the previous study, using a unique hybridoma and Mutational Lineage Guided (MLG) Humanization technology of rabbit monoclonal antibodies(RabMAbs), we were able to obtain mAbs

* Corresponding author. Address: Department of Urology, Sir Run-Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, China.
E-mail address: yanlanyu@gmail.com (Y. Yu).

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recognizing both human and mouse antigens, and successfully developed a fully humanized VEGF monoclonal antibody [13]. The antibody has showed better anti-tumor activity in animal studies than currently available drug in the market and will be in clinical trials. In this study, we generated a panel of anti-VEGFR2 rabbit monoclonal antibodies blocking the interaction of VEGF-A and VEGFR2 (KDR). More than 50% of these antibodies cross react with murine Flk-1. We selected one of the anti-VEGFR2 RabMAbs and then evaluated its efficacy and potency in tumor xenograft mice models. Guided by sequence and lineage analysis of variable regions of the neutralizing RabMAbs, we humanized the non-human residues in the frameworks as well as in the CDR regions. The humanized RabMAb retained their parental biological properties and potent inhibition of the growth of lung cancer xenografts in mice. This study also provides proof of principle on the feasibility of developing humanized RabMAbs as human therapeutics.

2. Materials and methods

2.1. Cell lines and proteins

A majority of the cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA). 293/KDR cell line stably expressing human VEGFR-2 was purchased from SibTech, Inc (Brookfield, CT). The 293/KDR cells and NCI-H460 were maintained in culture with DMEM medium, supplemented with 10% FBS at 37 °C in 5% CO_2. The rabbit IgG Fc-VEGFR2 fusion protein expression constructs were prepared by cloning VEGFR2 (ECD) DNA fragment into rabbit IgG Fc built-in pTT5 vector (National Research Council Canada) at *Bgl* II and *Bam*H I sites. Recombinant human VEGFR1, VEGFR3 and mouse VEGFR2 were purchased from R&D System (Mineapolis, MN). Human VEGF-A_{165} was purchased from Shanghai Primegene Bio-Tech Co. (Shanghai, China).

2.2. Generation of rabbit monoclonal anti-VEGFR2 antibodies

Rabbit anti-VEGFR2 monoclonal antibodies were generated by Epitomics RabMAb technology (Epitomics Inc., Burlingame, CA). Briefly, New Zealand white rabbits were immunized subcutaneously with recombinant VEGFR2-Fc in TiterMax™ Gold adjuvant (Sigma–Aldrich Corp., St. Louis, MO). Splenocytes were harvested from the immunized rabbit and fused with rabbit hybridoma cells using PEG4000 (Sigma Chemical, St. Louis, MO) and selected by HAT (hypoxanthine, aminopterin and thymidine) according to the conventional methodology [14]. Hybridomas producing anti-VEGFR2 RabMAbs were screened for specific binding and blocking activity in ELISA-based binding and blocking VEGF-VEGFR2 interaction assays. Positive hybridomas were subcloned and expanded for future uses.

2.3. Recombination of rabbit monoclonal anti-VEGFR2 antibodies

Selected hybridoma cells were lysed and the total RNA was extracted by using the Qiagen TurboCapture mRNA kits. L chain and the variable region of H (VH) chain of candidate clones were amplified by PCR with rabbit H and L chain primers and cloned into pTT5 mammalian expression vector. The L and H chain plasmids were cotransfected into 293-6E cells to express recombinant RabMAb and supernatants were harvested 5 days after post-transfection. The recombinant RabMAb were purified through Protein A columns (GE Healthcare Bio-Sciences, Piscataway, NJ). All data presented in this paper are results using recombinant RabMAbs.

2.4. Anti-VEGFR2 RabMAbs binding specificities and cross-reactivities

For binding specificities of anti-VEGFR2 RabMAbs, Fc-VEGFR2 and other VEGF receptors including VEGFR1, VEGFR3, mouse Flk-1 and Fc conjugated non-relevant antigen were coated on an ELISA plate at 2 μg/ml in coating buffer and blocked with 1% BSA and 0.05% Tween-20 for 1 h at room temperature. The bound antibody was detected with a goat anti-human IgG-alkaline phosphatase antibody (Pierce, Rockford, IL).

2.5. Anti-VEGFR2 RabMAbs binding to KDR/293 cells

293/KDR or 293T cells (4×10^4 cells/well) were seeded and incubated at 37 °C in 5% CO_2 overnight. All the following procedures were done at 4 °C. After blocked with 3% BSA/DMEM, anti-VEGFR2 RabMAbs premixed with 1 μg Fc-KDR were added into the cells and incubated for 30 min. The plates were washed with blocking buffer three times and then incubated for another half an hour with a goat anti-rabbit IgG-HRP antibody (Amersham Pharmacia Biotech, Piscataway, NJ). After added peroxidase substrate, the absorbance at 450 nm was detected.

2.6. Receptor–ligand blocking assay and IC50 evaluation

A serial dilution of anti-VEGFR2 antibodies was mixed with 5 μg/ml recombinant FC-KDR for 1.5 h in 96-well transfer plates. The mixture was then transferred to 96-well microplates precoated with 2 μg/ml VEGF-A and incubated for additional 1 h. After washed with TBST two times, 1 μg/ml monoclonal mouse anti-human VEGFR2 antibody (R and D, Mineapolis, MN) was added to the wells and incubated for 1.5 h, followed by adding goat anti-mouse IgG-AP antibody and incubating for 1 h. *p*-nitrophenyl phosphate substrate for alkaline phosphatase was added to the wells for color development. The absorbance at 405 nm was read for quantification. IC50, the antibody concentration required for 50% inhibition of KDR binding to VEGF, was calculated. All the experiments were performed in triplicate.

2.7. Inhibition of VEGF-stimulated VEGFR2 phosphorylation

293/KDR cells (0.8×10^6 cells/well) were seeded into 6-well culture dishes and grown to 80% confluence. Cells were washed twice in PBS and starved in DMEM containing 0.2%FBS for at least 4 h. The cells were first incubated with various amount of antibodies at 37 °C for 30 min, followed by stimulation with 20 ng/ml VEGF-A at 37 °C for 3 min. After washed with ice-cold PBS three times, the cells were lysed with lysis buffer (Cell signaling, Danvers, MA) on ice. After the centrifugation at 10,000×*g* for 20 min at 4 °C, the supernatants were collected and the protein concentrations were determined by BCA Kit (Pierce, Rockford, IL). Equal amounts of cell lysates were resolved by a 4–20% SDS–PAGE and the proteins were transferred to a PVDF membrane (Millipore, Billerica, MA). The PVDF membranes were incubated with anti-phospho-tyrosine (Y99) (Cell signaling, Danvers, MA), or total VEGFR2 (Epitomics, Burlingame, CA). The specific protein signals were visualized on X-ray film after incubation of blotted membranes with horseradish peroxidase–conjugated secondary antibodies followed by ECL (Pierce, Rockford, IL). Semi-quantitative analysis of western blots by densitometry was carried out using ImageQuant software.

2.8. Mutational-Lineage-Guided humanization of RabMAbs

Amino acid sequences of the variable region of heavy chain (VH) and the variable of K light chain (VK) from this collection of RabMAb sequences were aligned to form a phylogenetic tree. Related

antibodies were grouped into one linage. The sequence alignment and phylogenetic analysis were performed by using ClustalX software. Based on the characteristics of rabbit antibody, Mutational-Lineage-Guided (MLG) method was used as described in our previous study[13]. The VH and light chains VK sequences of RabMAbs were blasted against human germline VH and VK database. To express the humanized antibodies, the humanized VK fragment was cloned into human CK built-in pTT5 vector at *Hind* III and *Nhe* I and the humanized VH into human IgG1 CH built-in pTT5 vector at *Hind* III and *Bsi*W I site. The expression construct containing VK and VH was obtained by substituting the signal peptide and the residues at the terminal of VK and VH with the human's by PCR.

2.9. Anti-VEGFR2 antibodies binding affinities

Binding affinities of antibodies were measured by BIAcore3000 (BIAcore, Inc., Uppsala, Sweden). Carboxymethylad dextran biosensor chips (CM5) were activated with *N*-ethyl-*N*'-dimethylaminopropyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). FC-VEGFR2 was immobilized on the chips. Serial dilution of antibodies was injected at a flow rate at 40 μl/min. Association rates (K_{on}) and dissociation rates (K_{off}) were calculated using one-to-one Langumuir binding model. The equilibrium dissociation constant (*K*d) was derived as the ration K_{off}/K_{on}.

2.10. In vivo tumor xenograft experiment

All animals were used in accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiment of Zhejiang University. NCI-H460 cells were cultured until 80% confluent and then harvested at a concentration of 2.5×10^7 cells/ml. Xenografts were established in 6–8 weeks old female BALB/c nude mice by dorsal flank subcutaneous injection of 5×10^6 cells/mouse (Zhejiang Chinese Medical University, Hangzhou, China). When tumors reached a volume between $50 \sim 200$ mm^3, a cohort was randomly selected ($n = 8$). Antibodies were administered intraperitoneally at the dose of 5 mg/kg, 2.5 mg/kg or 0.6 mg/kg. Tumor sizes and bodyweights were recorded every 2–3 days. Perpendicular tumor diameters were measured using a Vernier scale caliper. Tumor volumes were calculated according to the following equation: volume = length $\times$ width^2 $\times$ 1/2. All mice were euthanized three weeks after the initiation of treatment and tumors were removed and weighed. Tumor growth inhibition was calculated by the formula: tumor growth inhibition ratio = (1−average tumor weight in treatment group/average tumor weight in control group) $\times$ 100%.

2.11. Immunohistochemistry

Tissues obtained were fixed in 10% formalin and embedded in paraffin. The embedded tissues were subsequently cut into 4 mm sections and placed on glass slides. Tissue sections were deparaffinized, rehydrated, and quenched for endogenous peroxidase activity. The microvessel density (MVD) in tumor xenografts was determined by a modified method described previously [15]. Briefly, murine vessels in tumor xenografts were immunostained with rabbit anti-mouse CD34 antibody (Zhongshan Goldenbridge Biotechology Co., Ltd., Beijing, China). Tumor microvessel elements were scored five fields per tumor at 400 $\times$ magnification by two blinded observers. The average vessel count in five areas was taken as the MVD.

2.12. Statistical analysis

All results were expressed as mean ± standard deviation, unless otherwise indicated. Data and graphs were analyzed with Graphpad Prism software (Graphpad Software Inc., La Jola, CA). Statistical significance between two groups was determined by two-tailed Student's *t* test and ANOVA was used for multiple comparisons. A *P* value of <0.05 was considered as statistically significant.

3. Results

3.1. The binding specificities of Anti-VEGFR2 RabMAbs

A panel of 30 antibodies was identified by blocking human VEGF binding to VEGFR-2. The binding specificities of the anti-VEGFR2 RabMAbs were first determined on immobilized Fc-KDR, mouse Flk-1, VEGFR1, VEGFR3 and non-relevant antigen Fc-OX40L. There were 18 out of the total 30 RabMAbs bound to both Fc-KDR and mouse Flk-1, whereas none of them cross-reacted with either VEGFR1 or VEGFR3 (Table 1). Results for 8 representative antibodies are shown in Fig. 1A. The binding of Anti-VEGFR2 RabMAbs to KDR/293 cells was also determined by cell ELISA. All the RabMAbs bound to KDR/293 cells except for four clones (EBK413, EBK414, EBK417 and EBK418). There were 7 clones (EBK423, EBK424, EBK425, EBK427, EBK430, EBK436 and EBK442) showed very strong binding activity (Table 1). The binding was specific to KDR transfected 293T cells but not untransfected 293 cells. The RabMAbs binding to 293/KDR cells can be blocked efficiently with Fc-KDR (Fig. 1B).

3.2. Anti-VEGFR2 RabMAbs blocking VEGF/VEGFR2 interaction

The neutralization of recombinant anti-VEGFR2 RabMAbs was first confirmed by receptor–ligand ELISA assay. All of the 30 Recombinant RabMabs maintained their blockade of VEGF/VEGFR2 interaction with various IC50 values between $0.103 \sim 10.499$ nM (Table 1). Fig. 1C showed that eight representative positive clones blocked VEGF binding to VEGFR2 in a dose dependent manner.

The biological activity of anti-VEGFR2 RabMAbs was then evaluated in a VEGF-stimulated VEGFR2 phosphorylation assay by using 293/KDR cells. 5 μg/ml was efficient enough for most of the anti-VEGFR2 RabMAbs to have visible inhibition on VEGFR2 phosphorylation, in the presence of VEGF (Table 1, Fig. 1D). Ten clones showed strong inhibitions at a concentration of 5 μg/ml such as EBK413, EBK423 and EBK436 showed in Fig. 1D.

3.3. Humanization of anti-VEGFR2 RabMAb by Mutational Lineage Guided (MLG) method

Amino acid sequences of the variable region of heavy chain (VH) and the variable of K light chain (VK) of *anti-VEGFR2* RabMAbs were aligned to form a phylogenetic tree (Fig. 2A). The phylogenetic tree of VH was overlapped with the phylogenetic tree of VK, suggesting that antibodies in a lineage group are derived from a single progenitor B cell.

Based on the characterizations of the RabMAbs, EBK436 was selected for further humanization by using MLG method. The benefit of MLG humanization was most evident for humanizing RabMAb EBK436 VH chain. The EBK436 related lineage group was identified by analysis of all anti-VEGFR2 RabMAbs that blocked VEGF-A binding to VEGFR2. In this lineage, all clones have the same lengths and similar sequences of VH and VK CDRs in addition to an identical VH CDR3. As shown in Fig. 2B, we identified the residues that in the CDRs and in the frameworks by aligning the sequences with that of a human antibody by blasted against human germline VH and

Table 1
Summary of 30 recombinant anti-KDR RabMAbs bioactivities.

Anti-VEGFR2 RabMAb #	Live cell binding*	Mouse Flk-1 crossreactivity	Blocking VEGF/VEGFR2 interaction IC50 (nM)*	Block VEGFR2 phosphorylation
EBK404	+	+	6.614 ± 0.645	+
EBK405	+	–	5.511 ± 0.883	+
EBK406	+	+	3.422 ± 1.062	+
EBK413	–	–	8.424 ± 5.226	++
EBK414	–	+	0.346 ± 0.478	+
EBK415	+	–	2.027 ± 0.503	+
EBK417	–	–	4.338 ± 1.133	+
EBK418	–	–	0.954 ± 0.675	++
EBK423	++	–	2.772 ± 1.265	++
EBK424	++	–	2.058 ± 0.711	++
EBK425	+	–	0.103 ± 0.133	++
EBK427	++	+	4.099 ± 0.812	–
EBK430	++	+	3.466 ± 0.394	+
EBK436	++	+	3.008 ± 0.045	++
EBK440	+	–	5.176 ± 2.069	–
EBK442	++	+	1.795 ± 2.23	–
EBK443	+	–	1.461 ± 0.509	++
EBK450	+	+	5.315 ± 0.673	–
EBK454	+	+	10.499 ± 0.832	–
EBK456	+	+	3.347 ± 0.971	+
EBK457	+	+	4.018 ± 1.666	++
EBK468	+	+	6.555 ± 0.312	+
EBK469	+	+	2.774 ± 0.461	–
EBK471	+	+	4.625 ± 1.104	+
EBK477	+	+	6.912 ± 2.786	+
EBK481	+	+	5.206 ± 5.725	+
EBK483	+	+	5.22 ± 2.678	+
EBK491	+	–	5.547 ± 0.905	+
EBK492	+	–	6.687 ± 0.438	++
EBK493	+	–	3.689 ± 2.367	++
EBK495	+	+	5.752 ± 1.583	–

*'–', antigen binding signal <3X of background reading or inhibition <20% baseline signal between 3–10 × of background reading or inhibition between 20–50% baseline signal; '++', antigen binding signal >10X of background reading or >50% baseline signal.

VK database. 3–66 (VH3) and I02 (VK) were selected as the template for EBK436 humanization. In addition to framework residues, two residues in CDR1 and three residues in CDR2 were humanized. These residues would have been kept without MLG. CDR3 has no overlap with the human germline sequences thus no modification was made. EBK436 VK chain was humanized in the similar manner. Humanized EBK436 (hEBK436) is overall 92% identical to a human germline antibody. VH and VK in the frameworks are 92% and 94% respectively identical to human germline frameworks.

3.4. Humanized anti-VEGFR2 EBK436 retained biological activities of parental antibodies in vitro

We confirmed the bioactivities of humanized EBK436 (hEBK436) *in vitro* using the same screening methods as above and showed its similar effects to RabMAb (Fig. 3A). The dose-inhibition profile is almost indistinguishable between the humanized and parental RabMAbs.

The binding kinetics was determined by surface plasmon resonance on a BIAcore instrument. hEBK436 bound to immobilized KDR with *K*d of 0.04 nM, which is 2.5 higher than its parent RabMAb (0.1 nM, Sup. 1).

3.5. Inhibition of anti-VEGFR2 antibodies in NCI-H460 xenograft mice

We first compared the effects of RabMAb EBK436, hEBK436 in NCI-H460 bearing mice with 5 mg/kg/dose. hEBK436 and its parental anti-VEGFR2 RabMAb showed significant inhibition of tumor growth as compared to saline control at a dose of 5 mg/kg (Fig. 3B, $p = 0.04$ and $p = 0.0144$ *vs.* control). The tumor growth inhibitions [(C-T)/C%] by hEBK436 and EBK436 were 76.74% and 69.90% respectively. We also investigated the inhibition of tumor growth by lower doses of hEBK436 (2.5 mg/kg/dose or 0.6 mg/kg/dose). 2.5 mg/kg/dose of antibody inhibit tumor growth effectively ($p = 0.031$ *vs.* control), and the tumor growth inhibition ratio was 54.54%. However, 0.6 mg/kg/dose of hEBK436 did not show statistic difference compared to control group (Fig. 3C).

3.6. Reduction of tumor microvessel density in mice treated with anti-VEGFR2 antibodies

Histological examination of NCI-H460 tumor xenograft excised at the conclusion of study was performed to determine the microvessel density. Tumor sections stained with an antibody against vascular endothelial marker CD34 revealed that hEBK436 treatment resulted in a weaker CD34 staining and a lower density of CD34 positive blood vessels compared to control group (Fig. 3D). The reduction in tumor size and MVD suggests that the antitumor effect of hEBK436 is likely acting through inhibition of tumor angiogenesis (Fig. 3E).

4. Discussion

In this study, we describe the development of a panel of neutralizing monoclonal antibody against human VEGFR2 with murine cross-reactivity. The potential biological activities of these RabMAbs were identified by blocking the interactions of VEGF-A and VEGFR2 and VEGF-stimulated VEGFR2 phosphorylation. Furthermore, one RabMAb EBK436 was selected and humanized by MLG method. We confirmed that the humanized RabMAb retained its parental biological properties and potent anti-tumor effects in lung cancer xenografts mice.

Monoclonal antibodies are a new class of potentially therapeutic angiogenesis inhibitors. Although a variety of chimeric,

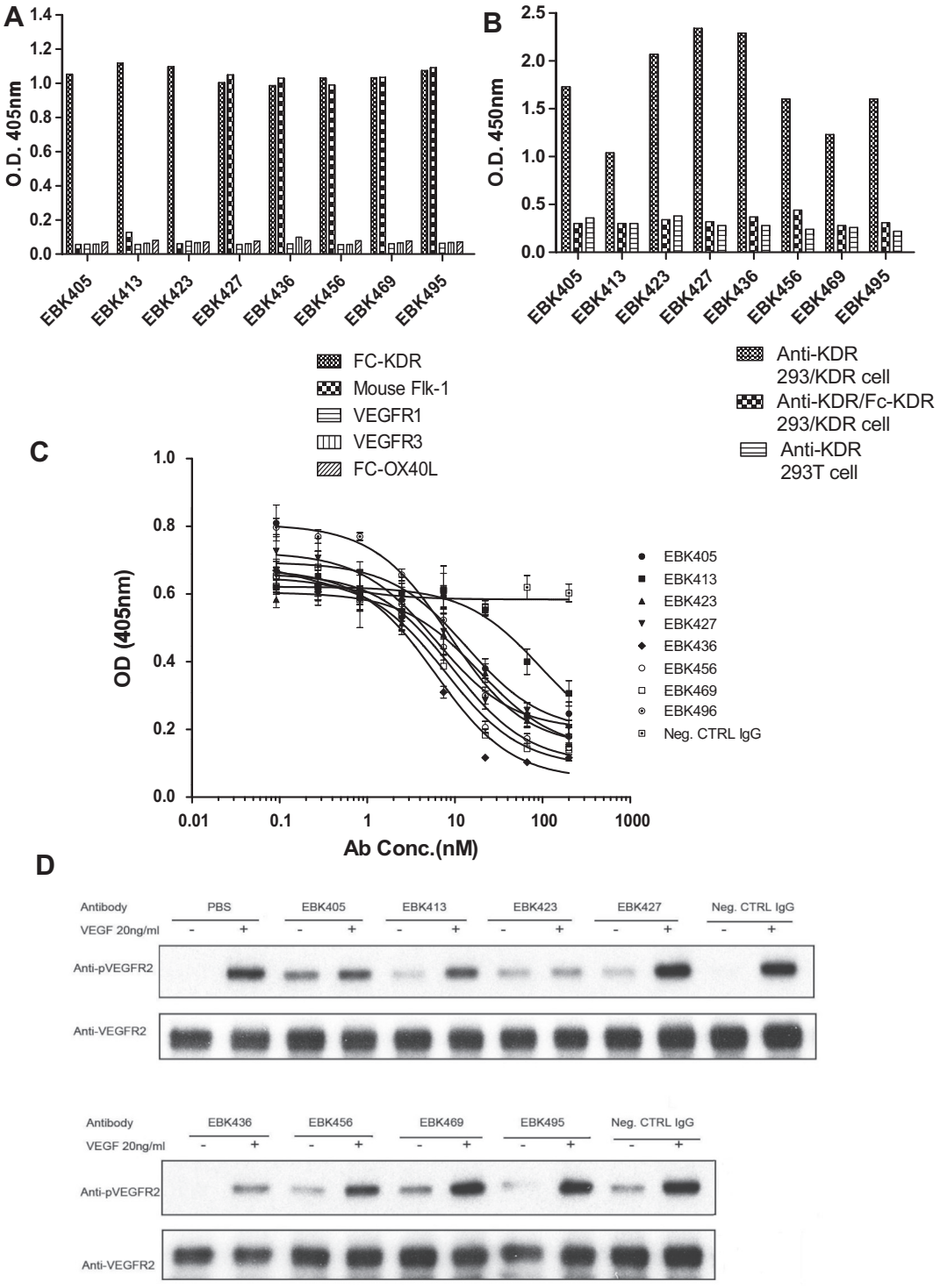

Fig. 1. Characterization of anti-VEGFR2 RabMAbs. (A) Specificity and cross-reactivity of eight representative anti-VEGFR2 RabMAbs. EBK427, EBK436, EBK456, EBK469, and EBK495 showed strong binding capability to mouse Flk-1 as well as human KDR and no detectable binding to other VEGF receptors, VEGFR1 or VEGFR3. FC-OX40L is a non-relevant antigen control. (B) Anti-VEGFR2 RabMAbs specifically bound to transfected 293/KDR cells. (C) The dose dependent inhibition of anti-VEGFR2 RabMAbs on VEGF/VEGFR2 interaction. 2.5 μg/ml Fc-KDR was incubated with various amount of anti-VEGFR2 RabMAbs or negative control rabbit IgG. Points represent the means of three replicates; Error bars represent the standard error. (D) Inhibition of VEGF-stimulated VEGFR2 Phosphorylation. VEGF stimulation resulted in significant VEGFR2 phosphorylation in 293/KDR cells (Lane 2). EBK405, EBK413, EBK423, EBK436 and EBK456 showed inhibition on VEGFR2 phosphorylation at concentrations of 5 μg/ml. Total VEGFR2 blotting was also performed as quantitative controls.

humanized, and fully human mAbs are readily produced from transgenic mice or antibody libraries [16,17], these antibody platforms have their own limitations. A major obstacle in the acceptance of peptides as drugs is usually their low stability *in vivo*, interfering with the activities of enzymes and other target proteins in the circulation and specific organs [18]. It is desirable to explore other antibody sources to increase the overall success rate of developing more effective antibody therapies.

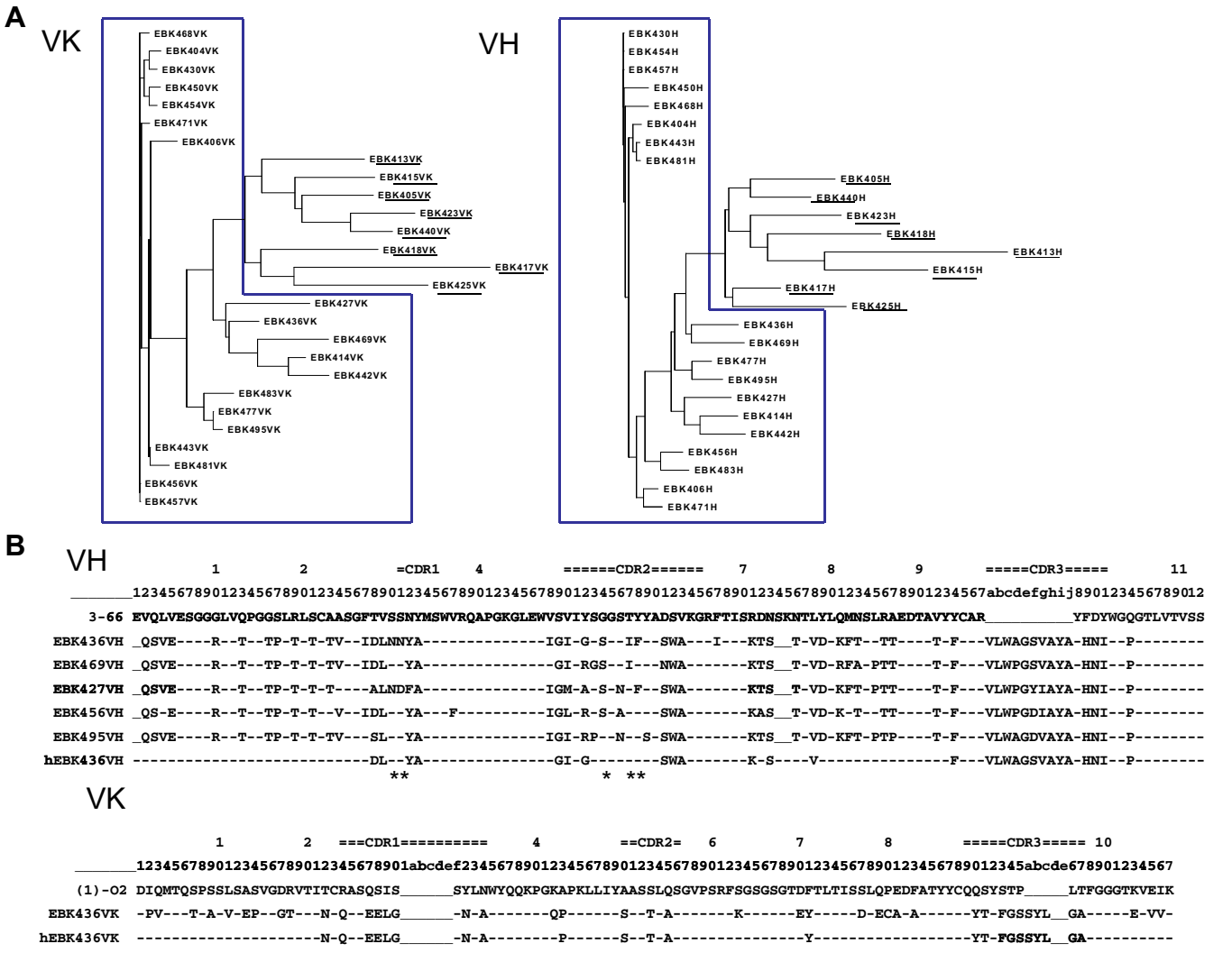

Fig. 2. Humanization of anti-VEGFR2 RabMAb EBK436. (A) Phylogenetic trees of VH and VK of anti-VEGFR2 RabMAbs. Human antigen-specific clones are underlined and the other clones recognize both human and mouse antigens. Clones in the same lineage group are boxed. (B) Protein sequence alignment of RabMAb EBK436. The closest human germlines and the humanized RabMAb, VH and VK sequence alignment of EBK436 lineage-related group (EBK436, EBK469, EBK427, EBK456, EBK495). -denotes residues are identical at the corresponding positions. *marks residues humanized in CDRs.

The application of RabMAbs in the therapeutic field has not been fully explored yet. Compared to the conventional mouse-derived mAbs, RabMAbs can recognize more epitopes and usually have a wider range of affinities. Therefore, it is an advantage of RabMAbs to get a large pool of candidates with varieties. In our present study, we identified a panel of functional anti-VEGFR2 antibodies. Most of them blocked the VEGF/VEGFR2 interactions with nM range IC50 values, but appeared not to be totally consistent with the abilities of inhibiting VEGFR2 phosphorylation in KDR/293 cells. The antibodies might recognize different epitopes of VEGFR2, which plays different extent roles in the VEGR2 phosphorylation signaling pathways. The different binding affinity of these RabMAbs to VEGFR2 molecules on the cell surface may also affect their biological activities. Antibodies generated in this study provide useful materials for further investigation.

Moreover, as we expected, in our panel of anti-VEGFR2 antibodies, 60% of them can recognize mouse Flk-1. In contrast to the mouse-derived antibodies, which are negatively selected against epitopes displayed by the mouse antigen, thus, typically lack of cross-reactivity, antibodies from rabbits have highly potential cross-reactivity with human as well as mouse antigens. The antibodies with cross-reactivity with human and mouse antigens facilitate preclinical evaluation of therapeutic antibodies in mouse models of human diseases. In this particular case, since the antibodies are aimed to against VEGFR2, majorly located on blood vessels of the host, antibodies cannot exert their neutralization effects unless they can recognize mouse Flk-1. More generally, only the antibodies with human/mouse cross-reactivity could easily reveal the undesired toxicities in the host [19].

Another advantage of RabMAbs for therapeutic use is that the rabbit antibody is more closely related to human than mouse-derived antibodies, which makes the process of antibody humanization easier. Rabbit IgG has more conserved HCDR3 region similar to human IgG [20]. We have developed a novel humanization method that incorporates the knowledge of mutational lineage termed MLG humanization. The MLG method involves humanization of both frameworks (FWs) and CDRs of the non-human antibody and thus increases the degree of humanization. Here we humanized one leading anti-VEGFR2 RabMAb and validated its efficacy in a tumor bearing mouse model. Humanized antibody had comparable inhibition on tumor growth as its parent rabbit antibody.

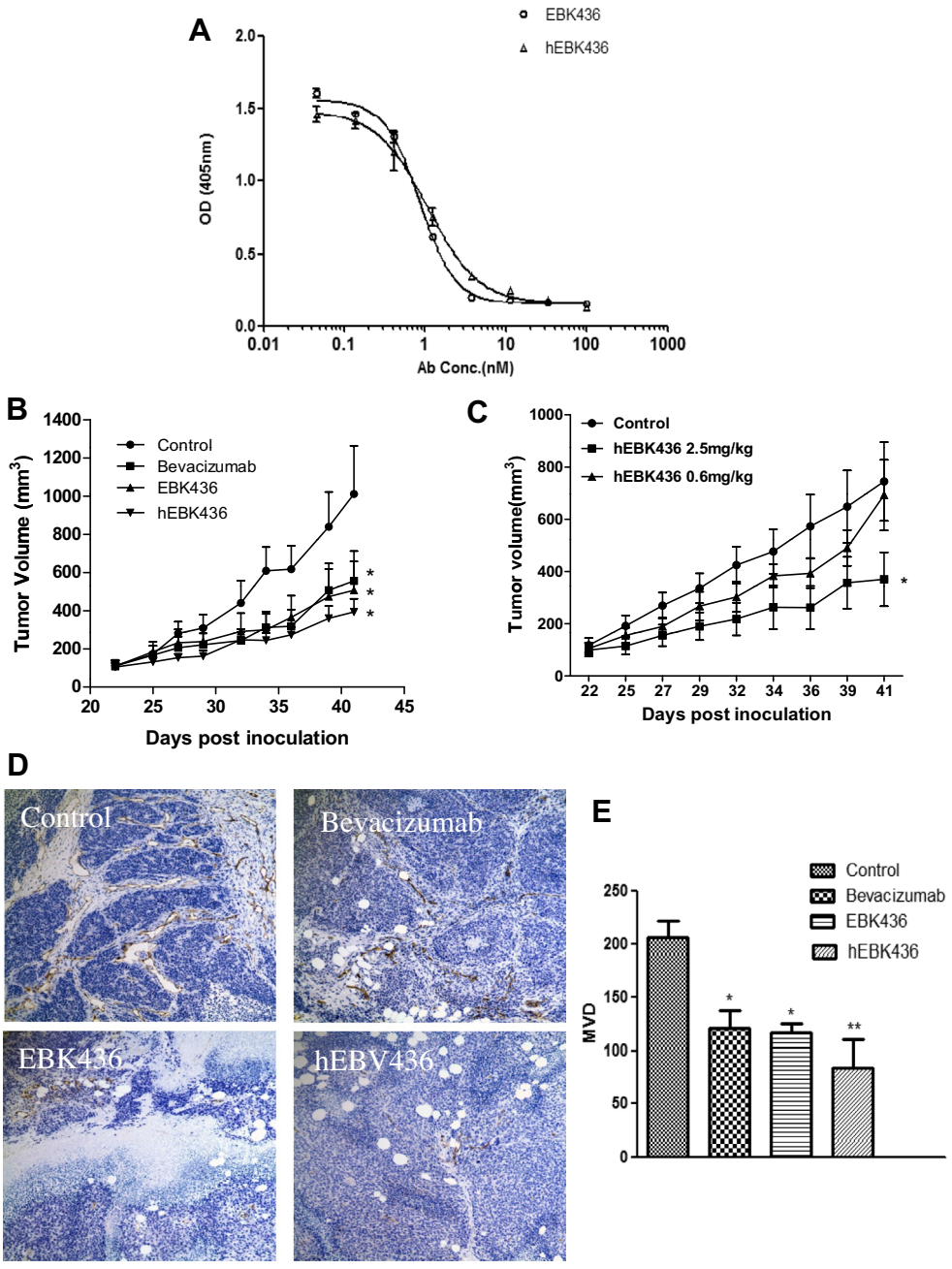

Fig. 3. Comparison of hEBK436 with RabMAb EBK436 *in vitro* and *in vivo*. (A) hEBK436 compared with its parental RabMAb EBK436 in the receptor–ligand interaction ELISA assay. (B) *In vivo* efficacy of anti-VEGFR2 antibodies in inhibiting NCI-H460 xenografts. Inhibition of tumor growth by EBK436, hEBK436 (5 mg/kg/dose) in NCI-H460 bearing mice was shown by the tumor volume (mm^3). Bevacizumab(5 mg/kg/dose) was included as positive control. Error bars represent the S.E. ($n = 8$). (C) Inhibition of tumor growth by different doses of hEBK436 (2.5 mg/kg/dose or 0.6 mg/kg/dose). (D) Representative images of NCI-H460 tumor xenograft staining with anti-CD34 anitibody ($\times$ 200). (E) MVD scoring of CD34 staining in NCI-H460 xenograft tumors. Significant difference when compared to the control group by ANOVA was denoted with $^{*}P < 0.05$ or $^{**}P < 0.01$.

Therefore, this study suggests that humanized rabbit monoclonal antibodies are highly relevant for therapeutic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.007.

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