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A novel rabbit anti-hepatocyte growth factor monoclonal neutralizing antibody inhibits tumor growth in prostate cancer cells and mouse xenografts

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ABSTRACT

The hepatocyte growth factor and its receptor c-Met are correlated with castration-resistance in prostate cancer. Although HGF has been considered as an attractive target for therapeutic antibodies, the lack of cross-reactivity of monoclonal antibodies with human/mouse HGFs is a major obstacle in preclinical developments. We generated a panel of anti-HGF RabMAbs either blocking HGF/c-Met interaction or inhibiting c-Met phosphorylation. We selected one RabMAb with mouse cross-reactivity and demonstrated that it blocked HGF-stimulated downstream activation in PC-3 and DU145 cells. Anti-HGF RabMAb inhibited not only the growth of PC-3 cells but also HGF-dependent proliferation in HUVECs. We further demonstrated the efficacy and potency of the anti-HGF RabMAb in tumor xenograft mice models. Through these *in vitro* and *in vivo* experiments, we explored a novel therapeutic antibody for advanced prostate cancer.

1. Introduction

Prostate cancer is the most common malignancy among men and the second-leading cause of cancer deaths in the United States. It is estimated that there will be more than 220,800 new cases and 27,540 deaths from prostate cancer in 2015 in the United States [1]. In developing countries, its incidence is also on the rise. Androgen deprivation therapy is the first-line treatment for advanced prostate cancer. Unfortunately, nearly all patients develop castration-resistant prostate cancer (CRPC) within 2–3 years [2]. Molecular mechanisms of the growth of CRPC including: ①androgen receptor aberrations; ②the maintenance of tumor-derived androgens; ③activation of other growth factors or signaling pathways, such as PTEN mutation [3,4]. Although a number of novel agents have sprung up for the treatment of CRPC over the last decade, interest remains in targeted therapies [5].

The hepatocyte growth factor/scatter factor (HGF/SF) is a growth factor that plays a critical role in the regulation of cell proliferation, malignant progression and angiogenesis in a wide variety of human carcinomas [6]. It is believed that HGF and its receptor are correlated with castration-resistance in prostate cancer [7–10]. HGF derived from prostate stroma significantly increases the proliferation, motility, and invasion of malignant epithelial cells. C-Met is preferentially expressed in the androgen-insensitive prostate cancer cell lines, such as PC-3 and DU-145, but not the androgen-sensitive LNCaP cell line [11,12]. HGF or c-Met expression was increased after castration or hormone therapy [7] and androgen ablation can induce c-Met expression which may contribute to the castration-resistant tumor growth [9,10]. Additionally, serum HGF levels are highly expressed in late stage and metastatic prostate cancer and HGF is related to the development of bone metastasis in prostate cancer [8]. Therefore, HGF/c-Met becomes an attractive target for the treatment of CRPC.

To date, several antagonists are being exploited to target HGF/c-Met signaling pathway, such as neutralizing antibodies to HGF [13], or small-molecule c-Met kinase inhibitors [14]. Compared to small-molecule c-Met kinase inhibitors, the development of therapeutics antibodies has been accelerated in recent years because of its higher specificity and better safety. In preclinical studies, human tumor xenograft mouse models are the major testing system to evaluate efficacy of these potential therapeutic antibodies.

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Typically, HGF is produced by stromal cells and functions as a paracrine growth factor in prostate cancer [15]. However, the conventional mouse derived antibodies are negatively selected against epitopes displayed by the mouse antigen [16,17]. The lack of cross-reactivity with the mouse antigen is a major obstacle in the applications of monoclonal antibodies (mAbs) in the preclinical development, such as rilotumumab (AMG102), a monoclonal antibody against HGF, can be only tested in autocrine HGF/c-Met-dependent human tumor xenograft models during preclinical stages [13,18], but not prostate cancer models. In the previous study, we have successfully a novel platform for efficiently generating rabbit monoclonal antibodies (RabMAbs) recognizing both human and mouse antigens and proved their better efficacy *in vivo* and *in vitro* than currently available drug in the market [19,20]. In this study, using the same technology, we generated and characterized a panel of anti-HGF RabMAbs neutralizing HGF/c-Met interaction. We selected one of the anti-HGF RabMAbs with mouse cross-reactivity and then evaluated its efficacy and potency in tumor xenograft mice models. Through these *in vitro* and *in vivo* experiments, we explored a novel therapeutic antibody for advanced prostate cancer.

2. Materials and methods

2.1. Cell lines and proteins

Human prostate cancer cell line PC-3 and DU145 were obtained from ATCC and maintained in RPMI1640 supplemented with 10% FBS at 37 °C in 5% CO_2. Human umbilical vein endothelial cells (HUVECs) from Lonza (Walkersville, MD) were maintained in Endothelial Basal Medium-2 (EGMH-2, Lonza) supplemented with 2% fetal bovine serum and growth factors (BulletKitH, Lonza). The fusion protein rabbit IgG Fc-hHGF and Fc-c-Met were provided by Yikang Bio-tech Co. (Hangzhou, China) and human HGF (hHGF) and mouse HGF (mHGF) were perchased from R&D System (Mineapolis, MN).

2.2. Generation of rabbit anti-HGF monoclonal antibodies

Anti-Human HGF RabMAbs were generated by the method described previously [19]. Briefly, New Zealand white rabbits were immunized subcutaneously with 0.2 mg Fc-hHGF. Splenocytes were harvested and fused with rabbit plasmacytoma cells 240E-W2. Hybridoma supernatants were screened by antigen binding and neutralization of HGF/c-Met interaction. Positive hybridomas cells were lysed and the total RNA was extracted by using the Qiagen TurboCapture mRNA kits (Qiagen, Inc,Vaencia, CA). L chain and the variable region of H (VH) chain were amplified by PCR with rabbit H and L chain primers and cloned into pTT5 expression vector. The L and H chain plasmids were co-transfected into 293-6E cells to express recombinant RabMAbs.

2.3. Anti-HGF RabMAbs binding to human HGF and mouse HGF ELISA

96-well plates coated with 2 μg/ml hHGF or mHGF in coating buffer overnight at 4 °C. The plates were then blocked with PBS containing 1% BSA and 0.05% Tween-20 1 h at room temperature. RabMAbs were added to wells for 1.5 h at room temperature (RT). Plates were washed twice in TBST (PBS with 0.05% Tween-20), and alkaline phosphatase-coupled goat anti-rabbit IgG (Pierce, Rockford, IL) was added for another 1.5 h. After washing three times, phosphatase substrate p-nitrophenyl phosphate substrate was added, and absorbance was measured at 405 nm.

2.4. Receptor-ligand binding assay

96-well plates coated with 5 μg/ml of Fc-c-Met overnight at 4 °C. Serial dilutions of anti-HGF RabMAbs or control rabbit IgG were pre-incubated with hHGF for 1.5 h. After 1 h of incubation, the mixture was transferred to the plates and incubated for another 1 h. HGF bound to immobilized c-Met was detected by a mouse anti-hHGF monoclonal antibody (Abcam, Cambridge, UK), followed by the addition of goat anti-mouse IgG alkaline phosphatase conjugated antibody (Fisher Scientific/Pierce Biotechnology, Rockford, IL). The data were evaluated by calculating the percentage inhibition compared with the maximal signal (control antibody) and the IC50 values were calculated.

2.5. Quantitative real time PCR (qPCR)

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcription was conducted following the instructions of the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For qPCR, 1 μl gene primers with SYBR Green PCR Master Mix (Applied Biosystems) in 20 μl reaction volume were performed. Primers were designed as: HGF, forward, 5′-CTCA-CACCCGCTGGGAGTAC-3′, reverse, 5′-TCCTTGACCTTGGATGCATTC-3′; c-Met, forward, 5′- CTGCCTGCAATCTACAAGGT-3′, reverse, 5′-ATGGTCAGCCTTGTCCCTC-3′; actin, forward, 5′-CTCCTCCTGAGCG-CAAGTACTC-3′, reverse, 5′-TCCTGCTTGCTGATCCACATC-3′. mRNA levels of tested genes were normalized to Actin according to the following formula: 2ˆ −(CT test − CT Actin), where CT is the threshold cycle. Fold of gene expression of PC-3 cells was defined as '1'.

2.6. Inhibition of HGF-stimulated c-Met phosphorylation in prostate cancer cell lines

PC3 and DU145 cells (1×10^6 cells/well) were plated in 6-well plates and starved overnight. 10 μg/ml Anti-HGF RabMAbs were pre-mixed with 50 ng/ml hHGF for 1 h and then added to the cells for 10 min. Cells were washed with cold PBS and lysed in RIPA buffer (Cell Signaling, Danvers, MA). Protein samples boiled in SDS-sample buffer were resolved on a 6% SDS-PAGE, transferred onto a nitrocellulose membrane, and then blotted with rabbit monoclonal anti-human phosphorylated c-Met (diluted in the ratio 1:1000), rabbit anti-human phosphorylated Akt1, rabbit anti-human phosphorylated ERK1/2, rabbit anti-human c-Met rabbit or rabbit anti-human GAPDH antibody (diluted in the ratio 1:1000, respectively) (Abcam, Cambridge, UK). Blots were detected using the ECL kit (Pierce, Rockford, IL).

2.7. Cell proliferation assays

Two thousand PC-3 cells or 4×10^3cells DU145 were seeded in 96-well plates and cultured in RPMI1640 supplemented with 10% FBS at 37 °C in 5% CO_2 overnight. Following this incubation, the cells were washed once with serum-free RPMI1640 and replaced with a medium containing 5% FBS and treated with or without 50 ng/ml human HGF and 10 μg/ml, 20 μg/ml, or 30 μg/ml of RabMAbs. After 72 h, cell proliferation assays were carried out using the MTS tetrazolium kit (Promega, Madison, WI). For endothelial cell proliferation assay, HUVEC (4×10^3cells/well) were cultured for 3 days in medium containing HGF (50 ng/ml) or VEGF (15 ng/ml).

2.8. PC-3 cells xenograft experiments

All animals performed in this study conformed to the recommendations in the guide for the Care and Use of Laboratory Animals

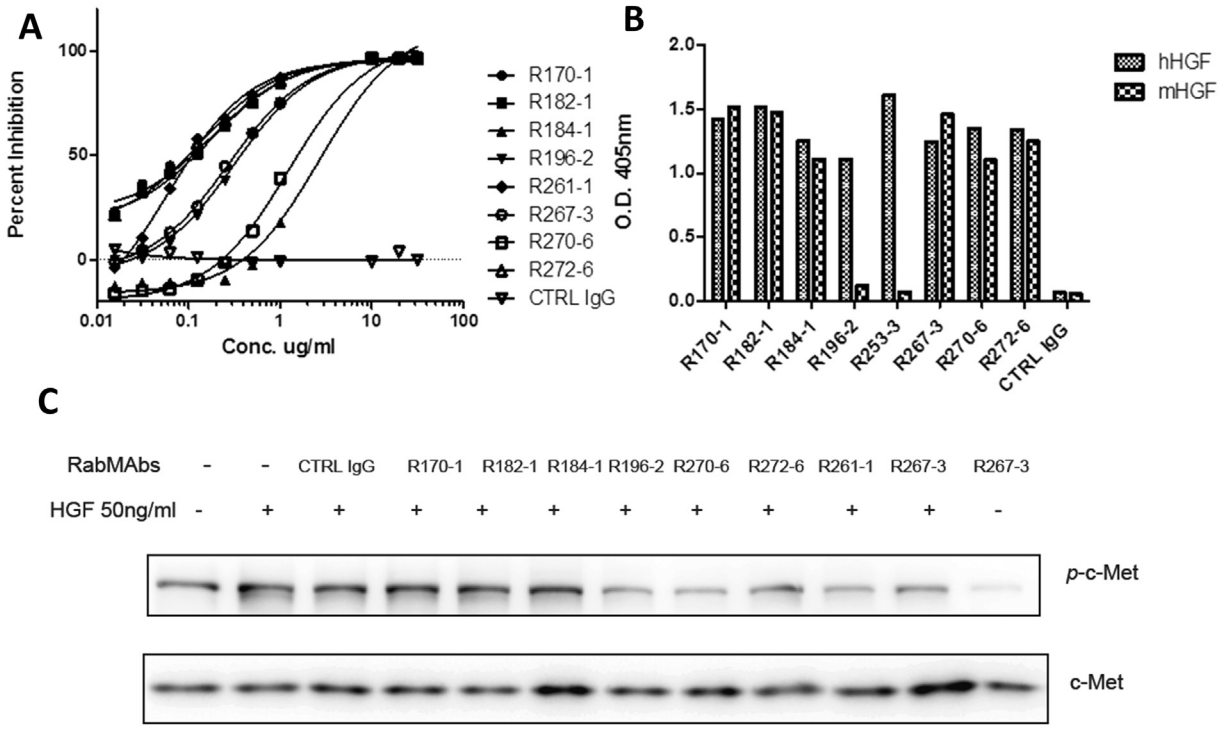

Fig. 1. Characterization of anti-HGF RabMAbs. (A) The anti-HGF RabMAbs inhibit HGF/C-Met interaction. The percentage inhibition of HGF/c-Met binding was measured of eight representative anti-HGF RabMAbs by R-L assay. A non-relevant RabMAb was included as a negative control. (B) Anti-HGF RabMAbs cross-react with mouse HGF. eight representative RabMAbs bind to human HGF and six of the eight antibodies were cross-reactive with mouse HGF. A non-relevant RabMAb was included as a negative control. (C) Anti-HGF RabMAbs neutralize HGF-mediated c-Met phosphorylation in PC-3 cells. Human HGF (50 ng/ml) and neutralizing anti-HGF antibodies (10 μg/ml) are indicated on the figure. A non-relevant RabMAb was included as a Control.

of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiment of Zhejiang University. 6–8 weeks old male BALB/c nude mice were obtained from shanghai laboratory animal center (Shanghai, China). Mice were injected s.c. in the flank with 5×10^6 PC-3 in 0.2 ml medium. When tumors reached a volume between 50 and 200 mm^3, a cohort was randomly selected (n = 8). Antibodies were administered by *i.p.* injection at the dose of 1 mg/kg or 5 mg/kg, 3 times per week and tumor measurements and body weights were recorded at the same time. Tumor volume was calculated as length × width × 1/2 in mm^3. After 3 weeks, mice were euthanized by CO_2 inhalation and tumors were collected for histologic analysis. 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) × 100%.

2.9. Histology

Tissues were fixed in 10% formalin and embedded in paraffin. The embedded tissues were subsequently cut at 4 μm intervals and placed on glass slides. Tissue sections were deparaffinized, rehydrated, and quenched for endogenous peroxidase activity. The sections were immunostained with a rabbit anti-mouse CD34 antibody (Zhongshan Goldenbridge Biotechology Co., Ltd., Beijing, China) and visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Adjacent sections were routinely stained with H&E. The microvessel density (MVD) in tumor xenografts was determined by a modified method described previously [21]. Tumor microvessel elements were scored five fields per tumor at 400× magnification by two blinded observers. The average vessel count in five areas was taken as the MVD.

2.10. 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 unpaired 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. Generation and biochemical characterization of anti-HGF RabMAbs neutralize HGF

Four rabbits were immunized with Fc-hHGF and the rabbit whose sera with the best neutralizing activities by the receptor-ligand binding assay were selected (data not shown). A total of 275 hybridomas with specific binding to human HGF were identified. 11 antibodies were selected from these 275 positive clones by blocking hHGF binding to c-Met. The antibodies were recombinated and purified for further evaluation.

A quantitative binding-neutralization assay was developed to assess whether RabMAbs blocked HGF/c-Met interaction. Fig. 1A illustrates the percentage inhibition of HGF binding to c-Met with eight representative RabMAbs. IC_{50} values are presented in Table 1. RabMAbs were able to completely block HGF/c-Met binding with IC_{50} values in the range of 0.1–5 nmol/l. We further evaluated the binding cross-reactivity of the anti-HGF RabMAbs with mouse HGF. Seven out of eleven antibodies bound to both human and mouse HGF (Fig. 1B and Table 1). We next evaluated the inhibition of HGF-mediated c-Met phosphorylation by anti-HGF RabMabs in PC-3 cells, which highly expressing both HGF and c-Met. To mimic a

Table 1
Summary of 11 Recombinant anti-HGF RabMAbs bioactivities.

Anti-HGF RabMAb#	Mouse HGF crossreactivity	Blocking HGF/c-Met interaction IC50(nM)	Blocking HGF phosphorylation[a]
R46-2	–	4.125 ± 1.133	+
R100-2	–	0.963 ± 0.756	++
R170-1	+	2.272 ± 1.652	–
R182-1	+	2.058 ± 0.921	–
R184-1	+	0.163 ± 0.123	–
R196-2	+	3.099 ± 0.712	++
R253-3	–	4.646 ± 0.494	+
R261-1	+	3.508 ± 0.445	++
R267-3	+	1.176 ± 0.669	++
R270-6	+	1.795 ± 1.23	++
R272-6	–	1.216 ± 0.559	+

[a] '–', inhibition <20% baseline signal; '+' inhibition between 20% and 50% baseline signal; '++', inhibition >50% baseline signal.

paracrine model of ligand-mediated receptor activation, anti-HGF antibody was introduced into HGF-containing medium before its addition to the cells. HGF stimulated phosphorylation of the receptor c-Met and eight RabMAbs showed essentially inhibition of the stimulation (Fig. 1C, Table 1). PC-3 cells also had a basal level of phosphorylated c-Met which can be blocked by anti-HGF RabMAbs. One anti-HGF RAbMAb, R267-3 with mouse cross-reactivity and good *in vitro* activities was chosen for further evaluation.

3.2. RabMAb R267-3 inhibits c-Met phosphorylation and downstream signaling pathways in c-Met highly expressed prostate cancer cell lines

First, we tested the gene expression of both HGF ligand and c-Met receptor in prostate cancer cell lines, PC-3, DU145, and LnCaP cells. HGF mRNA could be detected in PC-3 but not DU145 or LNCaP cells (Fig. 2A). C-Met gene was highly expressed in PC-3 cells and was higher than DU145 (6.5 fold) but not expressed in LNCaP cells.

To show the inhibition of RabMAb R267-3 on HGF/c-Met signaling pathways, cells were treated with RabMab R267-3 followed by HGF stimulation. In prostate cancer cell lines, 20 μg/ml RabMab R267-3 completely suppressed HGF-triggered c-Met phosphorylation both in PC-3 and DU145 cells (Fig. 2B). Phosphorylation of Akt (S473) and extracellular signal-regulated kinase (ERK; Y187/Y204) also was blocked by RabMAb R267-3. More obvious inhibition was observed in PC-3 than DU145 cells.

3.3. RabMAb R267-3 inhibit HGF-mediated cellular proliferation

We examined the inhibition of anti-HGF RabMAb R267-3 on cell growth by MTS assays. As shown in Fig. 2C, after 72 h, HGF increases PC-3 cells proliferation by ~33%. 30ug/ml of the anti-HGF RabMAb

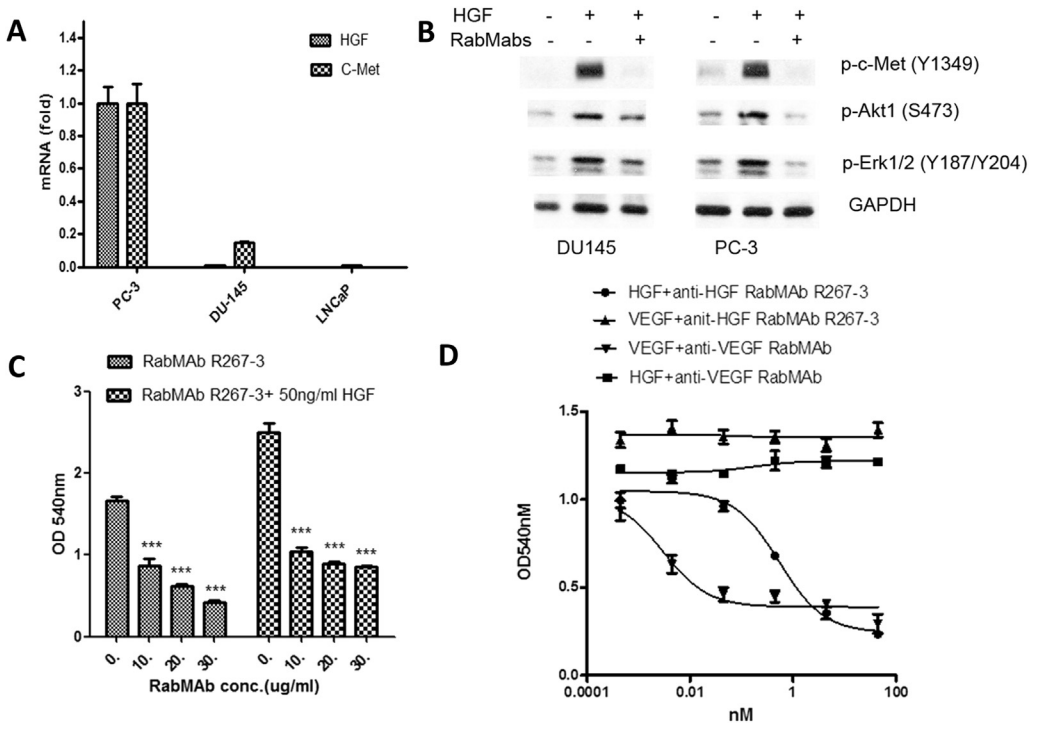

Fig. 2. Effects of RabMAb R267-3 *in vitro*. (A) Detection of HGF and c-Met in prostate cancer cells. qPCR analysis of HGF and c-Met RNA levels in PC-3, DU145 and LNCaP cells. Gene expression in PC-3 cells was defined as "1". Columns, mean; bars, SD (n = 3). (B) RabMAb R267-3 blocks the HGF-stimulated c-Met signaling pathways in androgen insensitive prostate cancer cell lines. Cells were serum starved and stimulated with HGF (50 ng/ml) for 10 min treated with RabMAb R267-3 (20 μg/ml) for 1 h. Whole-cell lysates were processed for Western blot analysis. Membranes were probed with respective primary antibodies. GAPDH is used as a loading control. (C) Anti-HGF RabMabs inhibit PC-3 cells proliferation. Points represent the means of six replicates and error bars represent standard deviations. ***, $P < 0.0001$, statistically significant compared with control IgG group. (D) Dose-dependent inhibition of HGF-induced HUVECs proliferation by RabMAb R267-3. An anti-VEGF RabMAb was included as control.

caused ~75% reduction in the HGF-untreated cells and ~66% reduction in the HGF-treated group. In HUVECs, only the HGF-mediated proliferation can be blocked by the anti-HGF antibody with an IC50 = 0.54 nM (Fig. 2D). An anti-VEGF RabMAb was included as control, which can only inhibited the VEGF-induced cell proliferation.

3.4. Anti-HGF RabMAbs inhibit tumor growth in PC-3 xenograft mouse models

We evaluated efficacy of the anti-HGF RabMAb R267-3 on PC-3 xenograft models. The RabMAb significantly inhibited tumor growth at a dose of 5 mg/kg ($P = 0.0087$ compared with control group, $P < 0.01$, Fig. 3A). As expected, tumor weights were also markedly reduced at the end of the experiment and correlated with tumor volumes and the tumor growth inhibition was 61.50% (data not shown). Tthe inhibition had dose dependence. At a lower dose of 1 mg/ml, the ant-HGF RabMAb also showed inhibition on tumor growth ($P = 0.0319$, $P < 0.05$), and the tumor growth inhibition was 36.90%. In all of the tumor xenograft experiments done, there was no evidence of overt toxicity based on body weight and overall appearance of the treated animals even in the high dose group.

Histological examination of PC-3 tumor xenograft was processed at the end of the study. H&E staining showed more necrosis areas in anti-HGF RabMAbs treated group compared to control group (Fig. 3B). To evaluate whether anti-HGF RabMab can inhibit tumor growth by reducing tumor vasculature, quantitative image analysis was done on tumor sections that were stained with the endothelial cell marker, CD34 (Fig. 3C). Administration of a dose of 5 mg/ml RabMAb R267-3 showed a weaker CD34 staining and a lower density of blood vessels compared to control group ($P = 0.0098$, $P < 0.01$) and 1 mg/ml/dose showed a slightly weaker but still significant decrease in the area of CD34-positive blood vessels ($P = 0.0168$, $P < 0.05$) (Fig. 3C, D).

4. Discussion

In this report, we have identified and characterized a panel of 11 RabMAbs that potently neutralized human HGF. Seven out of 11 antibodies had cross-reactivity with mouse HGF. These RabMAbs can block HGF binding to its receptor c-Met and inhibited HGF/c-Met pathways in c-Met expressed prostate cancer cell lines, including kinase phosphorylation and cell proliferation. In previous studies, some investigators did not observe that the expression of HGF in PC-3 cells [22,23], while others suggested that the existence

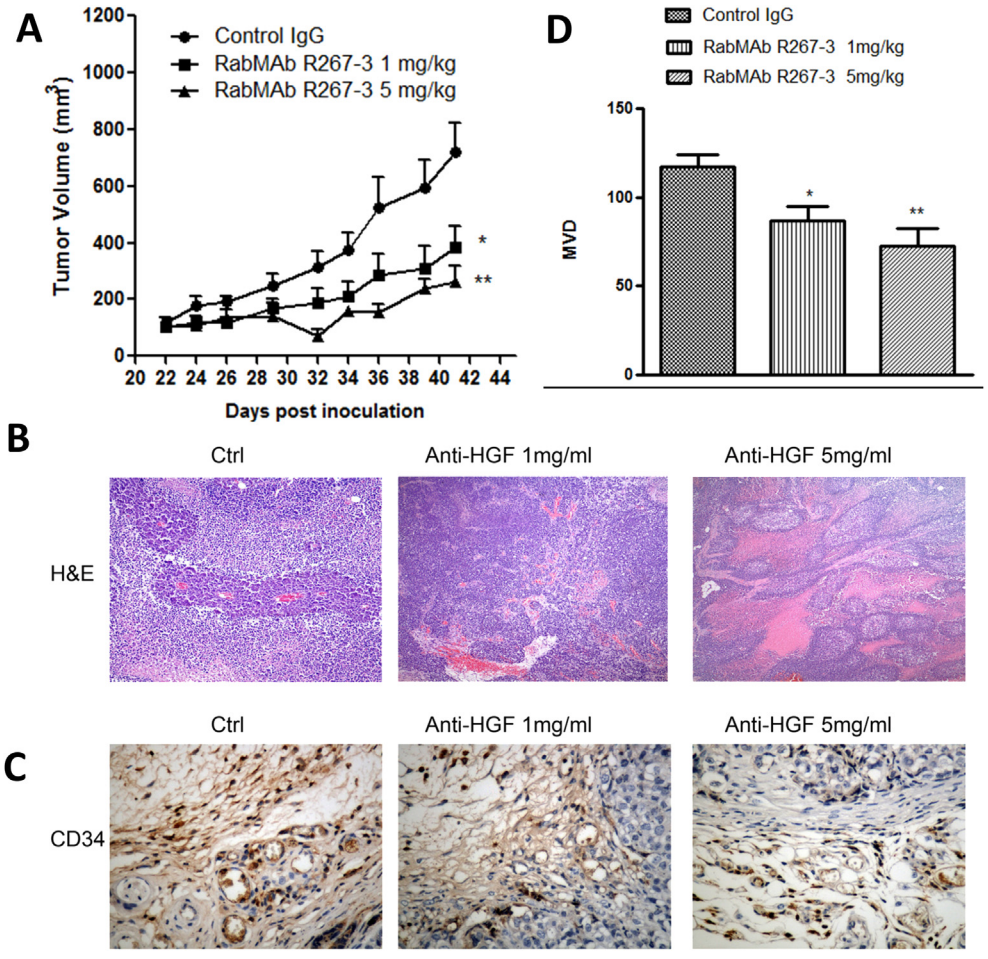

Fig. 3. In vivo efficacy of anti-HGF RabMAbs in inhibiting PC-3 cells xenografts. (A), Inhibition of tumor growth by different doses of RabMAb R267-3 (5 mg/kg/dose or 1 mg/kg/dose). PC-3 cells were injected *s.c.* into nude mice and tumors were allowed to reach 50–200 mm^3. On day 22, mice were randomized into individual groups and treatment with anti-HGF RabMAbs was initiated and continued 3 times per week at the doses indicated in the legends. Points, mean (n = 8); bars, SE. *, $P < 0.05$, **, $P < 0.01$. (B), Histology of tumor section from PC-3 mice models assessed by H&E staining. Anti-HGF RabMAbs treated tumors have more necrosis areas than that in control group (×100). (C), PC-3 tumor xenograft staining with anti-CD34 anitibody (×400). (D), MVD scoring of CD34 staining in PC-3 xenograft tumors. Significant difference when compared to the control group was denoted with *, $P < 0.05$, **, $P < 0.01$. Non-relevant rabbit IgG was included as a Control.

of a HGF/c-Met autocrine loop [24,25]. Nakashiro et al. [26] reported that in an androgen-independent human prostatic carcinoma cell line CWR22R, hepatocyte growth factor could switch from paracrine to autocrine phenotype. Our finding support that there was constitutive c-Met activation in PC-3 cells even in an environment that lacks HGF and provide important information about the potential therapeutic application of targeting HGF/c-Met pathway in cancer patients whose tumors possess cells with constitutively activated c-Met.

Moreover, we demonstrated that anti-HGF antibody R267-3 inhibited tumor growth in PC-3 xenograft models. Tumor microenvironment interactions are essential for prostate cancer growth, metastasis and drug resistance. The HGF/c-Met signaling pathway is an important contributor to epithelial–stromal interactions [15]. The lack of binding and functional activity of the conventional mouse-derived antibodies (e.g. rilotumumab) on mouse, rat, or rabbit HGF precluded the use of these species for preclinical studies, including efficacy and safety evaluation [17]. Rilotumumab (AMG102) did not show treatment advantage in patients with CRPC in a previous randomized phase II study [27]. In fact, as we discussed above, stromal effects from the host cannot be assessed because rilotumumab does not recognize or neutralize mouse HGF, in another words, rilotumumab has not be evaluated in prostate cancer models during preclinical stages. It was tested in an HGF/c-Met autocrine tumor, U87 glioma cells xenograft model, however, the antibodies did not affect the tumor vasculature [22], because the tumor angiogenesis mainly driven by the mouse-derived HGF which could not be neutralized by their antibodies. Different from rilotumumab, our RabMAbs showed significant inhibition on HGF/c-Met downstream tyrosine kinase activities in prostate cancer cells. Furthermore, we took advantage of our RabMAbs with mouse cross-reactivity, which neutralized not only the endogenous HGF but also the HGF secreted by mouse stromal cells, can be easily evaluated in mouse models. In the PC-3 mouse model, RabMAb R273 showed simultaneous inhibition of prostate cancer cell growth and angiogenesis.

In conclusion, we have successfully developed a panel of anti-HGF RabMAbs, which have inhibitory effects of tumor growth and angiogenesis. Anti-HGF RabMAb R267-3 would be a novel therapeutic agent against prostate cancer. Our studies provide preliminary proof for the potential therapeutic application of targeting HGF/c-Met pathway in advance prostate cancer patients. Further development is needed.

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Transparency document

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