

國立交通大學

生物科技學院

生化工程研究所

碩士論文

以血管內皮生長因子受器競爭物抑制血管新生與

B16/F10 腫瘤生長之活體研究

Inhibition of Angiogenesis and Tumor Growth of B16/F10 with A Novel

VEGF Receptor Antagonist *in vivo*

研究生：陳昱丞

指導教授：廖光文

中華民國九十七年七月

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研 究 生：陳昱丞

Student : Yu-Cheng Chen

指 導 教 授：廖光文

Advisor: Kuang-Wen Liao

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本授權書所授權之論文為本人在 國立交通大學 生物科技學院 生化工程 研究所
九十七 學年度第 二 學期取得 碩 士學位之論文。

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(親筆正楷)

學號： 9529503
(務必填寫)

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研究生簽名：
(親筆正楷)

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(英文) Inhibition of angiogenesis and tumor growth of B16/F10 with a novel VEGF receptor antagonist in vivo

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以血管內皮生長因子受器競爭物抑制血管新生與 B16/F10 腫瘤生長之活體研究

學生:陳昱丞

指導教授: 廖光文 博士

國立交通大學生物科技系生化工程所

中文摘要

血管新生成是腫瘤發育時的一個重要階段，此外血管新生也參與腫瘤由良性轉為惡性的過程。血管內皮細胞生長因子 (Vascular endothelial growth factor, VEGF) 是促進內皮細胞生長與血管新生的必要因子，在先前的實驗中，本實驗室建構了一嵌合蛋白 RBDV-Ig (Receptor binding domain of VEGF-Immuno-globin): 是以人類血管內皮細胞生長因子(VEGF)中第 1 至 108 個胺基酸序列做為指標區域 (targeting domain)，其具有與受器，血管內皮細胞生長因子受器 (VEGF Receptor)，結合的能力；加上人類免疫球蛋白 G1 (Immunoglobulin G1) 的 Fc 片段做為作用區域，以延長嵌合蛋白的半衰期。RBDV-Ig 已被證明可於細胞培養模式中藉由專一性地與人類臍帶靜脈內皮細胞 (human umbilical vein endothelial cells, HUVECs) 表面的血管內皮細胞生長因子受器結合而阻斷血管內皮生長因子的訊息傳導途徑，達到抑制人類臍帶靜脈內皮細胞增生與形成管狀結構。本研究欲探討於小鼠模式中，嵌合蛋白 RBDV-Ig 是否可藉由抑制血管新生達到抑制腫瘤發展的目的。人類血管內皮細胞生長因子之受器結合區域 (Human VEGF receptor binding domain, hRBDV) 與小鼠血管內皮細胞生長因子之受器結合區域 (Mouse VEGF receptor binding domain, mRBDV) 有 92% 相似度，顯示 RBDV-Ig 有可能可與小鼠血管內皮細胞生長因子結合，且細胞實驗證明 RBDV-Ig 可與已證實有表現小鼠血管內皮細胞生長因子受器 2 (mVEGFR2) 的 MS1 細胞結合。此外，RBDV-Ig 可抑制 SVEC4-10 內皮細胞的增生。在 C57/B6 小黑鼠活體實驗方面，皮下注射 RBDV-Ig 與靜脈注射 RBDV-Ig 皆可顯著抑制 B16/F10 腫瘤生長。以上結果顯示，RBDV-Ig 可能可藉由抑制血管新生而抑制腫瘤生長，是為一種有潛力的癌症治療用藥。

Inhibition of Angiogenesis and Tumor Growth of B16/F10 with A Novel VEGF Receptor Antagonist *in vivo*

Student: Yu-Cheng Chen

Advisor: Kuang-Wen Liao

Institute of Biochemical Engineering of National Chiao Tung University

ABSTRACT

Angiogenesis is not just an important stage in tumor growth but also involved in transforming a tumor from a benignancy to a malignant stage. Vascular endothelial growth factor (VEGF) is an essential factor in promoting endothelial cell growth and angiogenesis. In our previous experiments, we created a novel fusion protein, RBDV-Ig, which has a targeting domain, containing the amino acid sequences of VEGF from 1 to 108 with the binding activity to the human VEGF receptor, and an effector domain with Fc region of a human IgG1, used to increase a half-life of the fusion protein. RBDV-Ig has proven to inhibit tube-formation and proliferation through blocking the VEGF signal pathway by targeting the VEGF receptor on human umbilical vein endothelial cells (HUVECs) *in vitro*. Whether the RBDV-Ig chimeric protein can suppress tumor progression *in vivo* by anti-angiogenesis effect in the mouse model was verified in this study. The human VEGF receptor binding domain, RBDV is 92.7% similar to the mouse VEGF binding domain suggesting that the RBDV-Ig may bind to mouse VEGF receptors and later it was exactly proven to bind mouse VEGF receptor 2 (VEGFR2). It was also shown to bind to surface of mouse cells such as MS1 which have been proven with the VEGFR2 expression. Moreover, RBDV-Ig inhibits the proliferation of mouse SVEC4-10 endothelial cells in a dose-dependent way, but not of B16/F10. The results revealed that the tumor growths of B16/F10 tumors in C57/B6 mice were dramatically suppressed by subcutaneously injected RBDV-Ig. Tumor therapy with *i.v.* injection of RBDV-Ig also showed a similar result. Based on the results above, RBDV-Ig was shown to have the potent ability to inhibit tumor progression through suppression of angiogenesis and can be as a potential therapeutic drug in cancer therapy.

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CONTENTS

Abstract in Chinese	i
Abstract	ii
Acknowledgements	iii
Contents	iv
List of figures	vii
Abbreviations	viii

Chapter 1 Introduction

1.1 Tumor associated angiogenesis	1
1.2 Angiogenic factor	1
1.2.1 TGF-β	1
1.2.2 Interleukin-8	2
1.2.3 Basic Fibroblast Growth Factor	3
1.2.4 Platelet-Derived Endothelial Cell Growth Factor	4
1.2.5 Vascular Endothelial Growth Factor	4
1.2.5.1 Activities of VEGF	5
1.2.5.2 Tumor associated VEGF	5
1.2.5.3 VEGF gene and isoforms	6
1.2.5.4 Regulation of VEGF gene expression	8
1.3 VEGF Receptors	9
1.3.1 VEGFR-1	10
1.3.2 VEGFR-2	12
1.3.3 VEGFR-3 and Neuropilin	13
1.4 Negative regulators of angiogenesis and angiogenesis inhibitor	13
1.5 Anti-angiogenesis therapy through inhibiting VEGF signaling	15
1.5.1 Small molecules	15
1.5.2 Anti-VEGF antibody	16
1.5.3 Anti-VEGF receptors antibody	17
1.5.4 Soluble VEGF receptors	18

1.5.5 Peptides.....	18
1.5.6 Others.....	19
1.6 Research rationale and strategy.....	20

Chapter 2 Material and Methods

2.1 Material.....	22
2.1.1 Plasmid.....	22
2.1.2 Cell lines.....	22
2.1.3 Mice.....	22
2.2 Method.....	24
2.2.1 Amplification of pAAV-MCS/IgG1 Fc and pAAV-MCS /RBDV-IgG1 Fc.....	24
2.2.1.1 Transformation.....	24
2.2.1.2 Midi-preparation.....	24
2.2.1.3 Restriction enzyme digestion.....	26
2.2.2 Expression of chimeric proteins.....	26
2.2.2.1 Transfection of HEK-293T cell.....	26
2.2.2.2 Expression and purification of chimeric proteins.....	27
2.2.2.3 SDS-PAGE and Western blot.....	28
2.2.2.4 Stripping of Nitrocellulose membrane.....	29
2.2.3 Receptor binding assay <i>in vitro</i>	29
2.2.3.1 Biotin label of proteins.....	29
2.2.3.2 ELISA.....	29
2.2.4 Cell surface binding assay.....	30
2.2.5 SVEC 4-10 and B16/F10 cells proliferation assay.....	31
2.2.6 Tube formation assay.....	32
2.2.7 Cell migration assay.....	32
2.2.8 <i>In situ</i> tumor therapy.....	33
2.2.8.1 H&E staining.....	33
2.2.8.2 IHC staining.....	34
2.2.9 Tumor therapy with i.v. injection.....	35
2.2.10 Statistic analysis.....	35

Chapter 3 Results

3.1 Compare of receptor binding domain of VEGF between human and mouse.....	36
3.2 Expression and purification of chimeric proteins, IgG1 Fc and RBDV-IgG Fc....	36
3.3 The activity of RBDV-IgG1 Fc binding to mouse VEGF receptor.....	38
3.4 The activity of RBDV-IgG1 Fc binding to cells surface.....	39
3.5 The suppressive potency and efficacy of RBDV-IgG1 Fc to SVEC4-10 cells proliferation <i>in vitro</i>	39
3.6 Effect of blockade of VEGF receptors on tube formation <i>in vitro</i>	41
3.7 RBDV-Ig Fc inhibited the endothelial cell migration in a Transwell system.....	42
3.8 <i>In vivo</i> suppression of tumor growth with <i>in situ</i> RBDV-Ig Fc treatment.....	42
3.9 RBDV-Ig Fc-mediated <i>in vivo</i> therapy with i.v. injection of mice bearing B16/F10 tumors.....	43

Chapter 4 Discussion	45
-----------------------------------	----

References	80
-------------------------	----

Appendices	92
-------------------------	----

List of figures

Figure 1. Role of the VEGF receptors.....	52
Figure 2. Sequence alignment of human and mouse receptor binding domain of VEGF.....	53
Figure 3. Gene construction of the chimeric proteins.....	54
Figure 4. Restriction enzyme digestion of the pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc.....	55
Figure 5. The fluorescence expression in HEK-293 cell.....	56
Figure 6. Flow chart of procedure for the purification of chimeric proteins.....	57
Figure 7. SDS-PAGE analysis of recombinant proteins.....	58
Figure 8. Characterization of purified chimeric proteins.....	59
Figure 9. Mouse VEGF Receptor 1 binding activities of purified RBDV IgG1 Fc....	60
Figure 10. Mouse VEGF Receptor 2 binding activities of purified RBDV IgG1 Fc..	61
Figure 11. Cell surface binding ability.....	62
Figure 12. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of SVEC4-10 cells in a dose-dependent manner.....	64
Figure 13. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of SVEC4-10 cells, but not of B16/F10 cells.....	65
Figure 14. Effect of RBDV-IgG1 Fc on <i>in vitro</i> tube formation, 100X magnification.....	66
Figure 15. Effect of RBDV-IgG1 Fc on <i>in vitro</i> cell migration, 100X magnification.....	67
Figure 16. <i>In vivo</i> suppression of tumor growth with <i>in situ</i> RBDV-Ig Fc treatment.....	68
Figure 17. Subcutaneous vascularizing of mouse dorsum.....	69
Figure 18. H&E staining of tumor sections, 200X magnification.....	70
Figure 19. <i>In vivo</i> tumor therapy with <i>iv.</i> injection of RBDV-Ig Fc.....	72
Figure 20. Survival rate of tumor therapy with <i>iv.</i> injection of RBDV-Ig Fc <i>in vivo</i>	73
Figure 21. H&E staining of kidney sections, 200X magnification.....	74
Figure 22. H&E staining of spleen sections, 400X magnification.....	76
Figure 23. H&E staining of liver sections, 200X magnification.....	78

List of abbreviations

ADCC	Antibody dependent cell-mediated cytotoxicity
AMD	Age-related macular degeneration
aFGF	Acidic fibroblast growth factor
bFGF	Basic fibroblast growth factor
CDC	Cell dependent cytotoxicity
Cys	Cysteine
ECM	Extracellular matrix
EGF	Endothelial cell growth factor
FGFR	Fibroblast growth factor receptor
Flk	Fetal liver kinase
Flt	Fms-like tyrosine kinase
HIF-1	Hypoxia inducible factor-1
IFN- α	Interferon alpha
Ig	Immunoglobulin
IL-8	Interleukin 8
kDa	Kilo dalton
KDR	Kinase domain receptor
KO	Knockout
NK	Natural killer cell
NRP	Neuropilin
NSCLC	Non-small-cell lung cancer
PDGF	Platelet-Derived Endothelial Cell Growth Factor
PDGFR- β	Platelet-derived growth factor receptor-beta
PIGF	Placenta growth factors
PI3 kinase	Phosphatidylinositol kinase 3 kinase
RTK	Receptor tyrosine kinase
RBDV	Receptor binding domain of VEGF
RM	Room temperature
sFlt-1	Soluble fms-like tyrosine kinase-1
TGF- α	Transforming growth factor-alpha

TGF- β	Transforming growth factor-beta
TNF- α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor



Chapter 1 Introduction

1.1 Tumor associated angiogenesis

The history of tumor angiogenesis research can be traced back to over two centuries ago, pathologists in Germany observed that tumors are highly vascularized [1]. The development of tumor growth requires angiogenesis, the formation of new blood vessels [2]. In normal tissue, the blood vessels do not increase in size or number, because the endothelial cells that line these narrow tubes do not divide [3]. In the tumor region, endothelial cells are abnormal and including multi-layer morphology, extension of bridging and splitting vessels, uncontrolled permeability, in addition, the endothelial cells undergo constant remodeling [4]. The angiogenic process is stimulated in part by the tumor cells and the complex microenvironment consisting of many different cell types [1, 5, 6].

1.2 Angiogenic factor

Some evidence shows that tumor cells are triggered to secrete several pro-angiogenic molecules under a hypoxia environment, like TGF- β , VEGF, PDGF, bFGF, and IL-8 [1, 7, 8]. Moreover, these pro-angiogenic factors increase angiogenesis of endothelial cells has been associated with tumor growth in several human cancers [9-11].

1.2.1 TGF- β

Transforming growth factor (TGF)- β is a multifunctional protein that initiates its diverse cellular responses by binding to and activating its receptor, type I and type II serine/threonine kinase receptors. Although TGF- β is well known as an immunosuppressive growth factor [12], the importance of the signaling pathway in angiogenesis and vascular remodeling has been also highlighted during numerous recent studies [13].

In the studies of knockout (KO) mice and embryos, TGF- β and T β RII are critical for both formation of the primary vascular plexus (vasculogenesis) and the subsequent extension and remodeling into a complex network (angiogenesis) [14]. Furthermore, TGF- β was observed to be able to enhance the expression of VEGF, which could cause the excessive growth of endothelial cells. [15]

1.2.2 Interleukin-8

The chemokine interleukin-8 (IL-8), originally discovered as a chemotactic factor for leukocytes, has been shown to contribute to human cancer progression through its potential function as a mitogenic, angiogenic and motogenic factor [16]. IL-8 expression is regulated by the tumor microenvironment; tumor hypoxia and acid environment increase expression of IL-8. IL-8 may support tumor growth by direct or

indirect induction of angiogenesis. In a study of colon cancer, there was a strong correlation between constitutive expression of IL-8 and its receptors, CXCR1 and CXCR2, and increasing angiogenesis and metastasis [17]. In clinical studies, serum levels of IL-8 were significantly higher in human cancers, which was highly angiogenic and metastatic type [18, 19]. Thus, IL-8 may contribute to tumor progression and angiogenesis in several tumor types.

1.2.3 Basic Fibroblast Growth Factor

Basic fibroblast growth factor (bFGF) was a member of fibroblast growth factor and also named as FGF-2. FGFs are expressed in almost all tissues and play important roles in a variety of normal and pathological processes, including development, wound healing, and neoplastic transformation [20]. Moreover, bFGF was reported as a mitogen of endothelial cells, and promoter of tumor angiogenesis [21].

bFGF preferentially associates with the FGF receptor-3 (FGFR-3), which was also implicated in the development of a number of malignant tumors such as melanoma [22] or glioma [23]. FGFR-3 was expressed not only in endothelial cells but also tumor cells. bFGF and its receptor were reported to enhance the proliferation, migration, and angiogenesis of these cells [20]. Furthermore, antisense targeting of bFGF or its receptor in human melanoma inhibits tumor growth [22].

1.2.4 Platelet-Derived Endothelial Cell Growth Factor

Platelet-derived endothelial cell growth factor (PDGF), also known as thymidine phosphorylase, is another tumor angiogenic factor. In several cancer systems, PDGF has chemotactic activity for endothelial cells *in vitro* and angiogenic activity *in vivo* [24]. PDGF strongly induces neovascularization in the rat sponge model, and PDGF-transfected breast carcinoma cells exhibit accelerated growth in xenografts in mice [25]. In one study, Maeda and colleagues immunostained gastric cancer specimens for PDGF and microvessels and found a significantly higher microvessel density in tumors that expressed PDGF [26].

1.2.5 Vascular Endothelial Growth Factor

Vascular endothelial growth factor was first reported in 1989, and isolated an endothelial cell specific mitogen from medium conditioned by bovine pituitary follicular cells [27]. VEGF is potent, diffusible, and specific for vascular endothelial cells led to the hypothesis that this factor might play a role in the regulation of physiological and pathological growth of blood vessels. The role of VEGF in the regulation of angiogenesis has been investigated in two decades ago [21]. According to published literature, it has been indicated that new vessel growth are highly complex and

coordinated processes, requiring a serial of ligands binding to numerous receptors, which led to the proliferation, migration, and angiogenesis of endothelial cells [28].

1.2.5.1 Activities of VEGF

A well known *in vitro* activity of VEGF is a survival factor for endothelial cells derived from arteries, veins, and lymphatic. VEGF prevents endothelial apoptosis induced by serum starvation, which subsequently is mediated by the phosphatidylinositol 3-kinase (PI3 kinase)/Akt pathway [29]. Moreover, VEGF could induce expression of the anti-apoptotic proteins Bcl-2 and A1 in endothelial cells [30].

VEGF induces a potent angiogenic response in a variety of *in vivo* models and angiogenesis in tridimensional *in vitro* models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures [31, 32]. Moreover, VEGF elicits a pronounced angiogenesis response in a variety of *in vitro* models including the matrigel plug in mice [33].

1.2.5.2 Tumor associated VEGF

In contrast to normal vessels, vessels in solid tumors are often abnormally enlarged, and blood flow in tumor vessels is often chaotic, slow and not efficient in meeting metabolic demands [34]. Furthermore, tumor cells usually represent the main source

of VEGF, though several studies have shown that tumor-associated stroma is also a site of VEGF production [35, 36]. Many tumor cell lines particularly secrete VEGF *in vitro*, suggesting the possibility that this diffusible molecule may be a mediator of tumor angiogenesis [37]. *In situ* hybridization studies have demonstrated that VEGF mRNA is expressed in many tumors, including lung, breast, gastrointestinal tract, renal, and ovarian carcinomas [32].

1.2.5.3 VEGF gene and isoforms

The VEGF-related gene family of angiogenic and lymphangiogenic growth factors comprises five secreted glycol- proteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factors (PlGF) [38]. VEGF-A is the best studied, has been most strongly associated with angiogenesis, and is the target of most current anti-VEGF treatments. VEGF-A signals through two receptor tyrosine kinases, VEGFR1 and VEGFR2, and is the only member of the VEGF gene family found to be induced by hypoxia [39]. VEGF-B selectively binds to VEGFR1 and has a role in the regulation of extracellular matrix degradation, cell adhesion and migration [40]. Both VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 and regulate lymph-angiogenesis, and VEGF-C may also be involved in wound healing [41, 42]. PlGF selectively binds to VEGFR-1 and is the most abundantly expressed VEGF family

member in endothelial cells. PlGF may potentiate VEGF-A-induced endothelial cell proliferation, but on its own PlGF exerts only weak mitogenicity [39].

VEGF-A is a major angiogenic factor of the VEGF family, and is an important survival factor for endothelial cells, both *in vitro* and *in vivo*. VEGF-A was purified to be sequenced and cloned by Ferrara and collaborators. Alternative exon splicing of human VEGFs gene shows that it is comprised eight exons, denoted as: VEGFA121, VEGFA145, VEGFA165, VEGF165b, VEGFA189 and VEGFA206, gives rise to isoforms with different length of amino acids, and biological activities [43]. The properties of native VEGF closely correspond to those of VEGF165. VEGF121 is an acidic polypeptide that does not bind to heparin. VEGF189 and VEGF206 are highly basic and bind to heparin with high affinity. Whereas VEGF121 is a freely diffusible protein, VEGF189 and VEGF206 are almost completely sequestered in the extracellular matrix (ECM) [38].

VEGFA165 is a heparin-binding homodimeric glycoprotein of 46 kDa. Structurally, VEGFAs with intra-domain and inter-domain disulfide bonds between eight cysteine residues conserved positions. Anti-parallel homodimer covalently linked by two disulfide bridges between Cys-51 and Cys-60 [44]. VEGFA165 is secreted, but significant fraction remains bound to the cell surface and extracellular matrix (ECM), by virtue of its heparin-binding properties. In the present studies,

VEGFA and its receptors is the best characterized signaling pathway in developmental angiogenesis. Furthermore, much research has also established the VEGFA in tumor angiogenesis, and VEGFA action constitutes a rate-limiting step in normal and pathological blood vessel growth [38].

1.2.5.4 Regulation of VEGF gene expression

VEGF gene expression is up-regulated by hypoxia. Hypoxia allows the stabilization of hypoxia-inducible factors 1 (HIF-1) that binds to specific promoter elements that are present in the promoter region of VEGFA. This region is a 28-base sequence in the 5'- promoter of human VEGF gene, which mediates hypoxia-induced transcription [45]. Importantly, another study has implicated the PI3 kinase/Akt pathway in the regulation of HIF-mediated responses in a hypoxia-independent manner. Mutations in Akt also results in increased activation of HIF-1 and increased VEGF transcription [46]. Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of Ras leads to VEGF up-regulation, which indicates that mutant Ras-dependent VEGF expression is necessary for progressive tumor growth *in vivo* [47]. several major growth factors, including epidermal growth factor (EGF), TGF- α , TGF- β , FGF and PDGF, also up-regulate VEGF mRNA expression [48], suggesting that paracrine or autocrine release of such factors

cooperates with local hypoxia in regulating VEGF release in the microenvironment. Furthermore, inflammatory cytokines such as IL-8 also induce expression of VEGF in several cell types in agreement with the hypothesis that VEGF may be a mediator of angiogenesis in inflammatory disorders[49].

1.3 VEGF Receptors

VEGF binding sites were identified on the cell surface of vascular endothelial cells *in vitro* and *in vivo*. Subsequently, it became apparent that receptors for VEGF also occur on bone marrow-derived cells [50]. VEGF binds two highly related receptor tyrosine kinases (RTK), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin-like (Ig-like) domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence [51, 52]. VEGFR-3 (fms-like-tyrosine kinase (Flt)-4) is a member of the same family of RTKs, but is not a receptor for VEGF, binding instead to VEGF-C and VEGF-D [53]. In addition to these RTKs, VEGF interacts with a family of coreceptors, the neuropilins (NRP).

VEGFRs share similar regulatory mechanisms with well-characterized receptor tyrosine kinases, by which include receptor dimerization and activation of the tyrosine kinase. Moreover, VEGFRs perform cellular processes that are common to many

growth factor receptors such as cell survival and proliferation. The summary of VEGFRs shows in **Figure 1**, VEGF-A binds to VEGFR-1 and VEGFR-2, which are expressed in the cell surface of most blood ECs. In contrast, PLGF and VEGFB interact only with VEGFR-1. VEGFC and VEGFD bind VEGFR-2 and VEGFR-3, which is largely restricted to lymphatic EC. There is much evidence that VEGFR-2 is the major mediator of EC mitogenesis and survival, as well as angiogenesis and micro-vascular permeability. VEGFR-1 has an established signaling role in mediating monocyte chemotaxis. Also, in hematopoietic stem cells (HSC) or leukemic cells, both VEGFR-1 and VEGFR-2 may mediate a chemotactic and a survival signal [43].

1.3.1 VEGFR-1

VEGFR-1 or Flt-1 (fms-like-tyrosine kinase-1) was the first discovery of VEGF receptor [54]. The VEGFR-1 tyrosine kinase exhibits all the conserved motifs that are required for kinase activity. The crystal structure of part of the VEGFR-1 extracellular domain shows that the Ig domain-2 is the major ligand binding site on the receptor in physiology and pathology. VEGFR-1 expression is unregulated by hypoxia by a HIF-1 dependent mechanism [55]. VEGFR-1 binds not only VEGF-A but also PLGF and VEGF-B [40, 56]. The crucial role of VEGFR-1, as mentioned above, was determined by Fong, 1995, which revealed that when disruption of VEGFR-1 gene in

mice resulted in embryonic lethality.

In addition to the full length of VEGFR-1, there is an alternatively spliced soluble form of VEGFR-1 (sFlt-1), which has been shown to be an inhibitor of VEGF activity [57]. Hence, not only the full length membrane bound form of VEGFR-1 but sFlt-1 as well could perform a decoy function, which sequesters VEGF and prevent its interaction with VEGFR-2 [58].

In some cases, VEGFR-1 is expressed by tumor cells and probably mediates a chemotatic signal, thus potentially extending the role of this receptor in cancer growth. For instance, Wu and collaborates indicated that VEGF-A autocrine growth activity is acquired by certain human breast tumor cell lines defined by expression of VEGFR-1 [59].

Anchoring of the extracellular domain of VEGFR-1 to the cell membrane is important, as 50% of the mice that lack both of the tyrosine kinase domains and the transmembrane domain died at embryonic stage, owing to vascular malformation [60].

This study indicates that endothelial cells develop but fails to organize in vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and lethality, indicating that, at least during early development, VEGFR-1 is a negative regulator of VEGF action [61]. By contrarily, many evidence also indicated that VEGFR-1 is a positive regulator of monocyte and

macrophage migration [62, 63].

1.3.2 VEGFR-2

At the same time VEGFR-1 was discovered, Shalaby *et al.* found VEGFR-2, another receptor for VEGF, and proposed that VEGFR-2 had similar characteristics and continuously shows today. Biochemical analyses showed that the second and third Ig-like domains in VEGFR-2, also known as kinase domain region (KDR), is important for the determination of ligand binding specificity for VEGF [64]. VEGFR-2 undergoes RTK dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells.

There are many studies indicating that VEGFR-2 is the major mediator of the mitogenesis, survival, and permeability enhancing effects of VEGF-A in endothelial cells [32]. VEGF binds to VEGFR-2 and stimulates activation of Ras in HUVECs. Besides, Ras activation has been coupled to an angiogenic phenotype of endothelial cells [65]. The early finding that the binding of VEGF to VEGFR-2 is enhanced by heparin has been confirmed by recent studies, which shows that heparin amplifies signalling by VEGF. Byzova *et al.* have reported that VEGFR-2 activation by VEGF results in PI3 kinase/Akt-dependent activation of several integrins, which indicates that VEGF enhanced cell adhesion, migration, soluble ligand binding [66]. Moreover,

VEGFR-2 activation has been shown to be required for the antiapoptotic effects of VEGF for human umbilical vein endothelial cells (HUVECs) [29].

1.3.3 VEGFR-3 and Neuropilin

VEGFR-1 and VEGFR-2 are expressed on the surface of blood endothelial cells. By contrast, VEGFR-3 (Flt-4) is largely restricted to lymphatic endothelial cells. In recent studies, VEGFR-3 is important for lymphatic endothelial cell development and function [53].

Neuropilin-1 (NRP1), as its name suggests, is a molecular that is implicated in neuronal guidance and had been previously shown to bind the collapsin/semaphorin family. NRP1 is a cell surface glycoprotein that lacks intrinsic catalytic activity, a receptor for the heparin-binding isoforms of VEGF, and seems to present VEGF165 to VEGFR-2 in a manner that potentiates VEGFR-2 signaling [67]. This result shows that neuropilin stabilizes the VEGF/VEGFR-2 signaling complex when expressed on adjacent cells.

1.4 Negative regulators of angiogenesis and angiogenesis inhibitor

In a normal physiological condition, angiogenesis is a regulated process and seems to be under the control of both positive and negative regulatory factors. Several

fragments of large proteins have been discovered as endogenous inhibitor of angiogenesis; i.e, thrombospondin, endostatin, tumstatin and vasostatin. [68-71], but relatively little is known about their role in the physiological regulation of angiogenesis. The precise mechanism of action of these proteins remains to be more clearly defined, although several hypotheses have been proposed [72].

The similar concept was also used in cancer therapy. In 1971, Folkman proposed that inhibition of angiogenesis was a strategy to treat cancer, and ever since then it initiates the inhibitors that associated with angiogenesis. In 1989, interferon alpha (IFN- α), the first clinical trial of an antiangiogenic agent which decreases production of the angiogenic protein FGF made by tumors, was began for the treatment of life-threatening hemangioma. Furthermore, The United States Food and Drug Administration (FDA) approved TNP-470, a low molecular weight agent which selectively inhibits proliferation and migration of endothelial cells, to be used in clinical trials for a wide variety of cancers in clinics. From then on, there are many strategies toward this field, for example, inhibition of the influx of calcium into cells to suppress proliferation of endothelial cells [73], or inducement of inflammation in tumors, which destroying growing capillaries [74].

Antiangiogenesis therapy does not directly target tumors. Instead, it interferes with the expanding network of blood vessels and attacks blood supply in tumors to

suppress proliferation, and angiogenesis. So far, many angiogenesis inhibitors are proved successfully in vivo and in clinical trials, inhibitors based on anti-VEGF were major.

1.5 Anti-angiogenesis therapy through inhibiting VEGF signaling

VEGF is now general considered a central in the process of angiogenesis. Apart from the molecular interactions that are discussed above, several different strategies have been designed to target VEGF/VEGFR signal transduction, including small molecules inhibiting VEGFR signal transduction, humanized anti-VEGF monoclonal antibody, anti-VEGFR-1 antibody, anti-VEGFR-2 antibody, and a VEGFR chimeric protein [75].

1.5.1 Small molecules

Lots of small-molecule receptor tyrosine kinase inhibitors targeting the VEGF receptors have been developed. Such as SU5416 [76], ZD4190 [77], and PTK787/ZK2284 [78] are all small molecules as ATP mimics that target VEGFR-2 tyrosine kinase and have been shown to inhibit angiogenesis and tumorigenesis in animal models. The most advanced are BAY43-9006 and SU11248 [79]. BAY43-9006, also named Sorafenib, is a multi-targeted kinase inhibitor, including

VEGFR-2, PDGFR- β , Flt-3 and c-kit, which have been tested in large phase III clinical trials for treatment of metastatic kidney cancer. The FDA-approved SU11248, which named as Sunitinib, also targets multiple kinases, which inhibits VEGFRs, PDGFR, c-kit and Flt-3, for treatment of gastro-intestinal stoma tumor.

1.5.2 Anti-VEGF antibody

Tumor growth inhibition has been demonstrated by numerous laboratories using many anti-VEGF approaches. One of these is inhibiting angiogenesis by treatment with antibodies against VEGF. This reported anti-VEGF monoclonal antibodies exert a potent inhibitory effect on the growth of several tumor cells lines in nude mice, whereas the antibody had no effect on the tumor cells *in vitro* [80]. Subsequently, many other tumor cells lines were found to be inhibited *in vivo* by anti-VEGF monoclonal antibodies [81, 82] .

One of the most well-known monoclonal antibody to VEGF is bevacizumab (Avastin) which was a humanized antibody and approved for the treatment of metastatic colorectal cancer by US Food and Drug Administration (FDA) [83]. Furthermore, bevacizumab has applicability to other tumor types, such as non-small-cell lung cancer (NSCLC), prostate cancer, renal cell cancer [84]. Willett and his colleagues had shown that VEGF blockade with bevacizumab decreases

tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of viable circulating endothelial and progenitor cells in colorectal cancer patients [85]. The strategy of anti-VEGF acts by blocking tumor associated angiogenesis, which appears to be widely required by many different types of tumors. Therefore, this strategy may be usefully against different types of cancer.

1.5.3 Anti-VEGF receptors antibody

As anti-VEGF antibody, anti-VEGF receptors antibody was produced to inhibit angiogenesis through VEGF signaling transduction. Several anti-VEGFR-2 inhibitors are being clinically pursued. In 1999, Prewett and colleagues reported that anti-Flk (fetal liver kinase 1, VEGFR-2 in mouse) monoclonal antibody, and results showed that blockade of the Flk-1 receptor by systemic administration of the monoclonal antibody inhibits angiogenesis in animal models and the growth of several mouse and human tumors [86]. Skobe et al. have shown previously in a malignant keratinocyte invasion model that anti-Flk-1 monoclonal antibody treatment inhibits endothelial cell proliferation and induces endothelial cell apoptosis that leads to vessel regression. Furthermore, the use of anti-VEGFR-2 therapy in combination with conventional chemotherapy, radiotherapy or immunotherapy may improve the efficacy of anti-VEGFR-2 antibody [87].

1.5.4 Soluble VEGF receptors

Initial attempts to block VEGF by using a bevacizumab are beginning to show promise in human cancer patients, underscoring the importance of targeting VEGF pathway. Therefore, this clinical promise of humanized monoclonal antibody highlights the need to optimize blockade of this pathway. In 2002, Holash and collaborators engineered a soluble VEGF receptor, VEGF-Trap. The parental VEGF-Trap was created by fusing the first three Ig domains of VEGFR-1 to the Fc region of human IgG. Also, their variant, a chimeric soluble receptor consisting of domain 2 fused with domain 3 of VEGFR-2, was also created to determine the requirements to maintain high affinity while extending *in vivo* half-life [88]. Furthermore, it has been proposed that this fusion between the Fc region of human IgG1 and the Ig domain of VEGFRs represent an advantage over antibodies because they can result in higher binding affinity [89]. Luckily, VEGF-trap is also undergoing clinical development as an anti-cancer agent and is in Phase II/III trials for ovarian cancer.

1.5.5 Peptides

In recent researchers, some peptides selected with phage display were used as

antagonist of VEGF to its receptor [90-92]. Besides, a cyclic peptide corresponding to amino acids 79-93 of the VEGF sequence was reported to inhibit angiogenesis [93]. Moreover, the chemotherapeutic drugs combined with some small peptides were used currently cancer therapy. Peptide-mediated delivery of the drugs selectively to tumor and tumor associated neo-vessels [94].

1.5.6 Others

Another strategy to antagonist VEGF signaling transduction is aptamers. Aptamers were nucleic acids that can be highly potent antagonists of enzyme catalysis or of specific protein-protein interactions. In 1998, Ruckman proposed that an aptamer that interacts with the heparin-binding domain of VEGFA165. They have already isolated and modified 2'F-pyrimidine RNA nucleotide ligands by using the SELEX (systematic evolution of ligands by exponential enrichment) process. The results show that the binding ability of these aptamers to human VEGFA165 as well as murine VEGF164. Also, these aptamers potently inhibit the binding of VEGF to the human VEGF receptors, VEGFR-1 and VEGFR-2, expressed by transfected porcine aortic endothelial cells. Successfully, this VEGF-neutralizing aptamer, pegaptanib or Mucugen, became the anti-VEGF inhibitor to be approved by the FDA for the treatment of age-related macular degeneration (AMD), which is an effective at

slowing vision loss.

1.6 Research rationale and strategy

Here we report a novel antibody-like molecule, RBDV-IgG1 Fc chimeric proteins, constructed by fusing the receptor binding domain of VEGF (RBDV) with the constant region of human IgG1.

VEGF is an important growth factor of promoting specific mitogen and formation of blood vessels in pathological angiogenesis of tumor growth. The biological properties make VEGF as an important therapeutic target, and it has been shown that anti-VEGF signal pathway can inhibit tumor growth *in vivo*. At molecular level, VEGF activity is mediated by its interaction with two distinct receptors, VEGFR-1 and VEGFR-2. Charge reversal and alanine scanning mutagenesis have allowed identification of the receptor-binding domain of VEGF for VEGFR-2, which amino acids lined between 8 and 109 of full VEGFA165 [44]. Most importantly, the loss of the heparin-binding domain, the amino acids 111-165, results in a reduction in the mitogenic activity of VEGF [95].

The IgG1 subclass was selected because it is arguably the most active in the immune systems, being able to engage receptors on cytotoxic effective cells, called Fc γ R, and to activate complement and destroy target cells, effectively [96]. The Fc

component contains the hinge region, CH2 domain, and CH3 domain, but not the CH1 domain of IgG. The hinge and Fc regions form a conventional scaffold and flexibility for presenting functional RBDV domain. Furthermore, Fc provides a ready means for detection of the expression of chimeric receptors.

We expected RBDV as an antagonist to suppress VEGF signaling, and addition of constant region of human IgG1 could promote structure and stability of the fusion protein [97]. In previous data, RBDV-IgG1 Fc can bind to human VEGF receptors, VEGFR-1 and VEGFR-2, and be as an antagonist that inhibits the physiological interactions between VEGF to its receptors *in vitro*. Moreover, we determined that RBDV-IgG1 Fc could also be effective at inhibiting tumorigenesis and angiogenesis of B16/F10 melanoma cells inoculated into immunocompetent mice *in vivo*.

Chapter 2 Material and Methods

2.1 Material

2.1.1 Plasmid

PAAV-MCS/IgG1 Fc and pAAV-MCS /RBDV-IgG1 Fc were constructed by Yo-shong Chung and the map and full sequence of two plasmids are in the **Appendix1**.

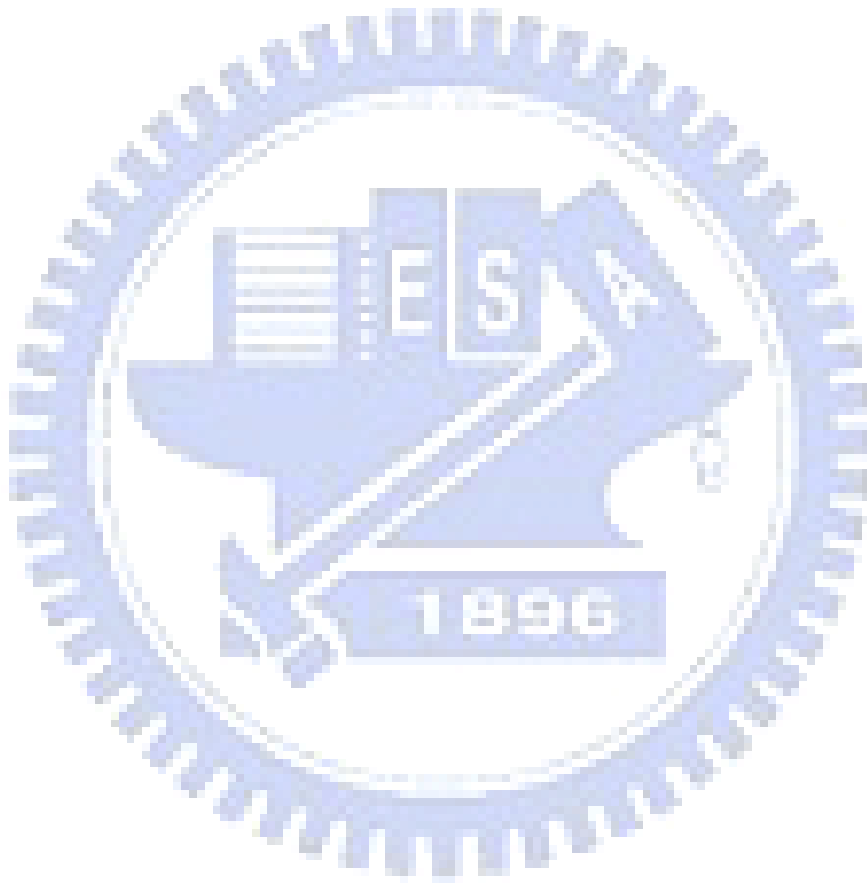
2.1.2 Cell lines

Human epithelial kidney (HEK) 293T cells, mouse melanoma cells (B16/F10), and mouse vascular epithelium cells (SVEC4-10) were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Gaithersburg, MD) containing 10% FBS (Fetal bovine serum qualified; Invitrogen, Gaithersburg, MD) and 1% penicillin-streptomycin amphotericin B (PSA; Biological industries, USA). Mouse endothelial cells (MS1) were cultured in DMEM supplemented with 5% FBS and 1% PSA. All the cells were incubated in tissue culture incubator with 5% CO₂ at 37°C. Except for SVEC4-10 was obtained from Dr. Tsai Nu-man, other cells were obtained from BCRC (Food Industry and Development Institute, Taiwan).

2.1.3 Mice

C57BL/6JNarl six- to eight-week female mice were purchased from the National

Taiwan University Laboratory Animal Center (Taipei, Taiwan, R.O.C.) and housed in the Animal Maintenance Facility of National Chiao Tung University (Hsin-chu, Taiwan, R.O.C.).



2.2 Method

2.2.1 Amplification of pAAV-MCS/IgG1 Fc and pAAV-MCS /RBDV-IgG1 Fc

2.2.1.1 Transformation

The competent cell, E. coli Top 10 strain (Invitrogen, USA), was used for transformation. Each plasmid was diluted with sterile water to 1µg/ml. One vial of stored competent cells (100 µl) was thawed and kept on ice. One µl of the diluted plasmid was first mixed into each vial containing the competent cells by stirring and then incubated for 30 minutes on ice. Then, the vials were heat shocked for exactly 90 seconds in a 42°C water-bath and placed on ice for additional 2 min. 250 µl of LB broth medium (1% tryptone, 0.5% yeast extract, 1% NaCl) were added to each tube and the vials were incubated at 37°C for 1 hour at 230 rpm shaking. The vials with the transformed cells were placed on ice after shaking. 100 µl of each transformation were spread out evenly on the agar plates with ampicillin (50µg/ml). Plates were then inverted and incubated at 37°C for 16 hours.

2.2.1.2 Midi-preparation

For further midi-preparation experiments, single colony was picked from each transformation, placed into 100 ml of LB broth medium containing ampicillin (50µg/ml) and incubated at 37°C for 20 hours at 230 rpm shaking incubator. Then, LB

broth medium with bacteria were centrifuged at 8,000 rpm for 15 min, the supernatant was discarded, and the bacterial pellet was resuspended in 4 ml of Buffer S1 (50 mM Tris-HCl, 10mM EDTA, 100µg/ml RNase A, pH8.0). Four ml of Buffer S2 lysis buffer (200 mM NaOH, 1% SDS) was added, the sample was gently mixed by inverting 6-8 times, and the bacteria were allowed to lyse for 3 min at room temperature. Four ml of ice-cold Buffer S3 neutralization solution (2.8 M KAc, pH 5.1) was added. The sample was gently mixed by inverting 6-8 times, before being incubated on ice for 5 min. Then, the mixture was centrifuged at 10,000 rpm for 30 min at 4°C. The clear solution was applied onto NucleoBond ion-exchange resin, which was pre-equilibrated with 5 ml Buffer N2 (100 mM Tris base, 15% ethanol, 900 mM KCl, 0.15% Triton X100, adjusted to pH 6.3 with H₃PO₄). The column was washed 2 times with 10 ml Buffer N3 (100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH6.3 with H₃PO₄). Plasmid DNA was eluted from the column with 5 ml high-salt Buffer N5 (100 mM Tris base, 15% ethanol, 1M KCl, adjusted to pH 8.5 with H₃PO₄). The plasmid DNA was precipitated with 0.7 volumes isopropanol, kept on ice for 10 min and centrifuged at 13,000 rpm for 30 min at 4°C in 1.5 ml tube. The DNA pellets were washed with ice-cold 70% ethanol, air-dried and dissolved in 20 µL sterile water. The concentration of the DNA was determined by measuring the absorbance at 260/280 nm.

2.2.1.3 Restriction enzyme digestion

Restriction enzyme digestion was used for checking plasmids. Restriction enzyme digestion of IgG1 Fc and RBDV-IgG1 Fc fragment were carried out in 20 µl volumes using the appropriate restriction enzyme buffer, as recommended by the manufacturer (Promega, WI, USA), supplied with the enzyme and incubated at 37°C. Digestion of plasmid DNA was fractionated by electrophoresis through a 1.5% agarose gel and was analyzed with UV photography system (EZlab, USA) after ethidium bromide (EtBr) staining.

2.2.2 Expression of chimeric proteins

2.2.2.1 Transfection of HEK-293T cell

HEK 293T cells were plated and distributed evenly at 4×10^6 cells per 100 mm tissue culture plate in 10 ml of DMEM growth medium. After 12 hours of incubation, 70-80% confluent of the plates. Pipette 10 µl of desired plasmid solution (1 µg/ml) into a 10 ml glass tube containing 1 ml of 0.25M CaCl₂ solution and mix gently. At the same time, pipette 1ml of 2×HBS buffer (280 mM NaCl, 1.5 mM Na₂HPO₄ and 50 mM HEPES, pH7.1) into a second 10 ml glass tube. Then, the 1.01 ml plasmids/CaCl₂ mixture was pipetted into the second tube containing 2×HBS solution.

All the reagents were mixed by repeated pipetting, immediately adding the mixture of plasmids/CaCl₂/HBS to the cell on the plate, and swirling gently to distribute the plasmid suspension evenly in the growth medium. After 6 hours of incubation, the medium was removed from the plate and replaced with 25 ml of fresh DMEM growth medium.

2.2.2.2 Expression and purification of chimeric proteins

The generated vectors were then transfected into HEK-293 cells using the calcium-phosphate method as described in the instruction manual of pAAV helper-free system (Stratagene, La Jolla, CA, USA). Supernatants from plasmid transfected HEK-293 cells were harvested (0.45 filtered) after a 48-hour incubation upon adding the fresh DMEM growth medium added. All supernatants were passed over a 2 ml protein G agarose affinity chromatography column (Upstate, Lake Placid, NY, USA). The column was next washed with TBS buffer (50 mM Tris-HCl, and 150 mM NaCl, pH 7.4). Bound proteins were eluted with TBS buffer, pH 2.7, and neutralized immediately with the neutralization buffer, pH 8.0 (1 M Tris-HCl, 1.5 M NaCl, and 1 mM EDTA). Following this neutralization, the eluted fractions from protein G column were loaded onto 1 ml nickel-charged HisTrap HP affinity column (Amersham Biosciences, Piscataway, NJ, USA), washed with binding buffer (20 mM

NaH₂PO₄, 0.5 M NaCl, 30 mM imidazole, pH7.4), and eluted with elution buffer (20 mM NaH₂PO₄, 0.5 M NaCl, 500 mM imidazole, pH7.4). The buffer was desalted by using sephadex G-25 (Amersham Biosciences). The recombinant proteins in PBS were concentrated by the Centricon centrifugal filter unit (Millipore, MA, USA). Protein concentration was determined by comparing it to a BSA standard curve.

2.2.2.3 SDS-PAGE and Western blot

The high-purity proteins, IgG1 Fc and RBDV-IgG1 Fc, were separated with 10% SDS-polyacrylamide gel electrophoresis, before being transferred to Nitrocellulose membrane (PALL, FL, USA). Membranes were blocked with 5% of skim milk in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH7.4). Protein bands were all recognized by using horseradish peroxidase (HRP)-conjugated antibodies, which are anti-human IgG antibody (AbD Serotec, Raleigh, NC, USA) and anti-his tagged antibody (Novus Biologicals, Littleton, CO, USA). The membrane was developed with enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to X ray film (Midsci, St. Louis, USA). PBST (0.05% Tween-20 in PBS) were used for all washing steps.

2.2.2.4 Stripping of Nitrocellulose membrane

The membrane was stripped with stripping buffer (20mM glycine, 3.5mM SDS, 1% Tween20 in PBS, pH2.2) twice, and washed with PBST twice. Finally, the membrane was washed with PBS, and ready to be blocked with skim milk.

2.2.3 Receptor binding assay *in vitro*

2.2.3.1 Biotin label of proteins

Five hundred μ l of proteins were reacted with 2 μ l of No-Weight™ Sulfo-SBED (Sulfosuccinimidyl [2-6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]-ethyl-1,3'-dithiopropionate; PIERCE, USA) in room temperature for 30mins. Nonreacted Sulfo-SBED was removed by using sephadex G-25 column, and the proteins were monitored in the fraction by mixing 10 μ l of each fraction with 200 μ l of BCA™ Protein Assay Reagent (PIERCE, USA) in 96-well microtiter plate (Nunc, Denmark).

2.2.3.2 ELISA

Enzyme link immuno-sorbent assays (ELISA) were carried out to examine the binding activities of the RBDV-IgG1 Fc. 0.5 μ g/well of the extracellular domain of mouse VEGFR-1 (R&D systems, MN, USA) or the extracellular domain of mouse

VEGFR-2 (R&D systems, MN, USA) in PBS were first coated onto the 96-well microtiter plate (Nunc, Denmark) at room temperature overnight. Wells were blocked by incubating with blocking buffer (2% skim milk, and 0.05% Tween-20 in PBS) for 1 hour. The plates were washed with PBST three times, and then the biotin-labeled proteins were added into the coated plates. After washing the plates three times with PBST, the plates were then incubated with HRP-conjugate streptavidin (R&D systems, MN, USA) at room temperature for an additional 20 minutes. The reactions were developed by the addition of TMB substrate (KPL, Gaithersburg, MD, USA), and the colorimetric reactions were stopped with 1N HCl after 10 min. The absorbance was measured by ELISA reader (TECAN, Austria) at 450nm.

2.2.4 Cell surface binding assay

Flow cytometry was used to analyze the cell surface binding ability. MS1, B16/F10 and SVEC4-10 cells were seeded at a density of 5×10^6 in a T75 flask. Viable cell counts were determined by trypan blue dye exclusion. MS1, B16/F10 and SVEC4-10 cells were washed with PBS, detached by Versine (0.2% EDTA in PBS, pH=7.4), washed, and resuspended in flow cytometry buffer (1% bovine serum albumin in PBS, pH 7.4). Aliquots of 100 μ l containing 5×10^5 cells were distributed in 15 ml centrifuge tube (Corning Inc., Corning, NY, USA). Cells were pre-incubated with 10

$\mu\text{g/ml}$ of purified recombinant proteins for 1 hour at 4°C , followed by 1 hour of incubation with goat anti-human IgG fluorescein isothiocyanate (FITC) conjugate (Acris Antibodies GmbH, Hiddenhausen, Germany). The cells were washed three times with ice-cold flow cytometer buffer after incubation. Cell pellets were suspended in 1 ml flow cytometer buffer and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Negative controls were the cells incubated with anti-goat FITC, with no prior exposure to recombinant proteins.

2.2.5 SVEC 4-10 and B16/F10 cells proliferation assay

Five thousand SVEC 4-10 and B16/F10 cells were seeded onto the flat-bottomed well of a 96-well plate (Corning Inc., Corning, NY, USA) in DMEM growth medium, allowed to settle for 16 hours, incubated with recombinant proteins for 2 hours, and then challenged for 70 hours with human VEGF165 (Upstate Inc., Lake Placid, NY, USA) at a final concentration of 10 ng/ml . The proliferative response was measured by adding 1.9 mg/ml of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, WI, USA) to each well followed by a further 2 hour incubation and spectrophotometric analysis at 492nm . The % of survival was calculated as the ratio of average OD values in wells containing recombinant proteins-treated cells to average OD of wells containing only

cells with MTS containing medium.

2.2.6 Tube formation assay

Fifty microliter of growth factor reduced matrigel (BD Biosciences, San Jose, CA, USA) was added to each well of a 96-well plate (Corning Inc., Corning, NY, USA), and allowed to be polymerized for 2 hours at 37°C. A suspension of 3×10^4 SVEC 4-10 cells were incubated with recombinant proteins for 1 hour and passed into each well containing 16 ng/ml of VEGF. As the positive control, SVEC 4-10 cells were incubated with 16 ng/ml of VEGF (Upstate Inc., Lake Placid, NY, USA) alone. Cells were incubated for a further 16 hours at 37°C and photographed using a Olympus microscope (40× magnification). The total number of network formations was counted.

2.2.7 Cell migration assay.

8-µm pore Transwell (Costar, Corning, NY) was used. SVEC4-10 cells were pre-cultured with 10 µg/ml protein for 1 h (37 °C, 5% CO₂), then washed with sterile PBS. These cells were next resuspended in DMEM growth medium, and plated (30,000 cells/well) in the upper well. The SVEC4-10 cells were allowed to migrate toward DMEM growth medium with 10ng/ml VEGF for 6 hrs (37 °C, 5% CO₂), cells

on the top of the filter were removed with a cotton swab, and migrated cells on the underside were fixed with methanol, stained with Propidium Iodide (PI, 50 μ g/ml) and counted with fluorescence microscope.

2.2.8 *In situ* tumor therapy.

Female C57/BL6 (6-8 week age) was inoculated with 1×10^6 B16/F10 cells subcutaneously in 200 μ l PBS. When the tumor average volume was up to 50 mm³, mice were injected with 150 μ g protein *in situ*. Tumor volume was measured every 2 days after injecting with proteins. Mice were sacrificed on the final day of the experiment, and tumors were removed, fixed by paraformaldehyde (4% paraformaldehyde in PBS, pH=7.4), and examined by microscopic H&E staining and IHC (immune- histochemistry) staining.

2.2.8.1 HE staining

For the histological HE staining of the B16/F10 melanoma tumor tissues (s.c. melanoma tumors with or without RBDV treatments), the tumors were harvested and fixed with 10% neutral formalin. The tissues were dehydrated using graded alcohols, cleared with xylene, and infiltrated with paraffin wax. The tissue was subsequently embedded with paraffin wax in molds, which facilitate tissue sectioning. Collect

sectioned tissues (4 μm / section) from paraffin-embedded blocks on clean glass slides and dehydrated in an oven for 30 minutes at 60°C. Prior to staining, tissue slides were deparaffinized and rehydrated, then stained with Mayer's hemotoxylin and Eosin Y solution for 3 minutes, respectively. After air-dry, tissue slides were mounted with mounting media and visualized under a Nikon light microscope camera system.

2.2.8.2 Immunohistochemical staining (IHC staining)

The Paraffin-embedded sections (7 μm / section) were obtained from the tumors and were processed for immune-histochemical staining. A rat anti-mouse CD31 monoclonal antibody (1/150 dilution; Novus Biologicals, Littleton, CO, USA) were used in immune-histochemical studies for 4°C overnight incubation. Briefly, the slides were treated with 3% hydrogen peroxide in 1×PBS for 10 min to block endogenous peroxidase activity after dewaxed and rehydrated processes. Next, the sections were washed three times with PBS-T (1×PBS containing 0.05% tween-20) for 5 min each time and non-specific reactions were blocked by 10% FBS in PBS for 10 min at room temperature. The sections were incubated with the anti-CD31 first antibody for 4°C overnight, biotin-conjugated anti-rat IgG secondary antibodies (1/1000 dilution) for 1 hour at RT, and the immune complexes were visualized using the horseradish peroxidase-conjugated straptavidin. LSAB2 system (DAKO, Carpinteria, CA) was

used to visualize the immune complexes, and incubated with 0.5mg/ml diaminobenzidine and 0.03% (v/v) H₂O₂ in PBS for 10 min. Finally, sections were counterstained with hematoxylin, mounted and observed under a light microscope at magnifications of × 400 and photographed.

2.2.9 Tumor therapy with i.v. injection.

The female C57/BL6 (6-8 week age) was inoculated with 1×10^6 B16/F10 cells subcutaneously in 200µl PBS. When the tumor average volume was up to 30 mm³, the mice were intravenously (i.v.) injected with 150µg/kg proteins. The tumor volume was measured every 2 days after injecting with proteins. Mice were sacrificed when tumor grew up to 2,500mm³. Tumors and organs were obtained, fixed by paraformaldehyde, and examined by microscopic H&E staining and IHC staining.

2.2.10 Statistic analysis

Statistical analyses were done using SPSS statistics software (SPSS Inc., Chicago, IL, USA). The t-test was used when comparing two independent samples and ANOVA test was used when comparing multiple samples. A value of $p < 0.05$ was considered significant.

Chapter 3 Results

3.1 Compare of receptor binding domain of VEGF between human and mouse.

The mature VEGF₁₆₅ of human has an 88.5% similarity to the VEGF₁₆₄ of murine. Besides, many studies have already shown that the signal transduction of VEGF and its receptors can cross over between human and murine [98, 99]. It could not be confirmed whether the same part of VEGF had the same function between human and murine. Because of this doubt, the amino sequence of the receptor binding domain of VEGF (RBDV) between human and mouse was necessary to be determined.

The amino acid sequence of mouse VEGF was obtained from the NCBI protein database (NCBI No: NP_001020421). Two sequences was compared to find out the mouse receptor binding domain of VEGF (mRBDV), and the highest similarity was obtained when RBDV was compared with 179-288 amino acids of mouse VEGF. There was a 92.7% similarity between RBDV and mRBDV (**Figure 2**). According to the result *in silico*, it was thought that RBDV-Ig Fc might bind to mouse VEGF receptors *in vitro*.

3.2 Expression and purification of chimeric proteins, IgG1 Fc and RBDV-IgG Fc.

The IgG1 Fc and RBDV IgG1 plasmids were amplified by transforming into the *E. coli* DH5 α strain, and extracted by Midi-preparation as described in 2.2.1.2. The

recombinant plasmid was confirmed by restriction enzyme digestion (**Figure 4**). The resulting constructs, pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc, were transiently transfected into HEK-293 cells using calcium phosphate based methods as described in section 2.2.2.1. The efficiency of transfection of this method had been confirmed by pAAV-hrGFP vector (Stratagene, USA; **Figure 5**). Expression of the secreted IgG1 Fc and RBDV-IgG1 Fc fusion proteins were confirmed by directly harvesting the culture supernatant followed by SDS-PAGE under reducing conditions, and transferred and blotted as described in 2.2.2.3. IgG1 Fc and RBDV-IgG1 Fc had immunoreactivity against both anti-his tag antibody; or an anti-human IgG antibody was detected (data none shown), where no signal could be detected in the vehicle group.

To purify the IgG1 Fc and RBDV-IgG1 Fc fusion proteins, the cultured supernatants were harvested after the initial transfection. The resulting supernatants were first loaded into a protein G affinity column. Elutes of the protein G affinity chromatography were subsequently applied onto a nickel-charged HisTrap affinity chromatography and were desalted by using sephadex G-25. **Figure 6** is the flow chart of the purification. To determine the purity, coomassie-stained SDS-PAGE was performed (**Figure 7**). The protein was a single band at ~38 kDa for IgG1 Fc and at ~48 kDa for RBDV-IgG1 Fc with >95% of purity. The purified protein was also

confirmed by using the western blot with anti- human IgG antibody (**Figure 8A**) or an anti- his tag antibody (**Figure 8B**), the stripping buffer was used between recognition of antibodies.

3.3 The activity of RBDV-IgG1 Fc binding to mouse VEGF receptor

To directly confirm that RBDV-IgG1 Fc can also bind to mouse VEGF receptors as well as to human VEGF receptors, the sandwich enzyme-linked immune sorbent assay (ELISA) was used here. Because the recombinant extracellular domain of mouse VEGFR-1 and VEGFR-2 were fused with human Ig Fc domain and poly His, it was difficult to assay the binding activity with the ELISA. The sandwich ELISA was modified by coupling the biotin to the chimeric proteins, and detecting the binding activity of RBDV-IgG1 Fc with HRP-conjugate streptavidin. The immobilized mouse VEGFR-1 and VEGFR-2 extra- cellular domains were incubated with the RBDV-IgG1 Fc biotin-labeled protein, respectively. The results showed that the binding activity of the RBDV-IgG1 Fc to the immobilized VEGFR-1 (**Figure 9**) and VEGFR-2 (**Figure 10**) were strong and in a dose dependent manner. In contrast, the human IgG1 Fc could not bind to the mouse VEGF receptors, either in the VEGFR-1 or VEGFR-2.

3.4 The activity of RBDV-IgG1 Fc binding to cells surface

RBDV-IgG1 Fc was proven and confirmed to have a binding activity to the mouse VEGF receptors with ELISA. To further characterize the *in vitro* properties of the chimeric proteins, its ability to target the VEGF receptors expressed on the cell surface of MS1, B16/F10 and SVEC4-10 cells was tested for. MS1 cells were strongly probed with RBDV-Ig Fc (**Figure 11A**), because of the high expression of VEGFR-2 [100]. The B16/F10 and SVEC4-10 cells were less bound with RBDV-Ig Fc compared with MS1 cells (**Figure 11B, C**). The B16/F10 and SVEC cells had also been demonstrated to express VEGFR-1 and VEGFR-2. [100, 101]. According the results, we demonstrated that RBDV-Ig Fc can bind to the mouse VEGFR-1 and VEGFR-2 expressed on mouse cells.

3.5 The suppressive potency and efficacy of RBDV-IgG1 Fc to SVEC4-10 cells proliferation *in vitro*.

The VEGF signal transduction through the VEGFR-2 does indeed stimulate the proliferation of endothelial cells (Ferrara, 2003). After confirming the binding ability of RBDV-Ig Fc to mouse VEGFRs, *in vitro* proliferation assay was used as described in section 2.2.5 to determine the bio-function of RBDV-Ig Fc. The effect of RBDV-IgG1 Fc on SVEC4-10 cells was examined first to confirm whether the specific

binding activity could suppress post-receptor signal through the VEGF, which enhances proliferation of endothelial cells. The SVEC4-10 cells (5×10^3 cells/well in a 96-well plate) were incubated in the presence of IgG1 Fc, RBDV-IgG1 Fc or PBS, as a negative control, for 72 hours and the proliferation assay was determined by MTS. Previous data showed that the presence of RBDV-IgG1 Fc (2.5 $\mu\text{g/ml}$), HUVECs did not show any significance in proliferation, as well as IgG1 Fc (2.5 $\mu\text{g/ml}$) (**Appendix 2, Figure 1A**), but it was different on mouse endothelial cells. The results show that RBDV-Ig Fc could inhibit proliferation of SVEC4-10 cells with dose-dependence (**Figure 12A**), but there was no significant variation in efficiency by treating it with IgG1 Fc (**Figure 12B**). According to the previous results, we observed that RBDV-Ig Fc could inhibit the proliferation of HUVECs with VEGF stimulating *in vitro* (**Appendix 2, Figure 1B**). The SVEC4-10 cells were also treated with RBDV-Ig Fc in the condition of adding of the VEGF (10 ng/ml) to determine the proliferation of SVEC4-10 cells. As expected, the proliferative response of endothelial cells was significantly enhanced by the addition of 10 ng/ml of VEGF (**Figure 12A**). Besides, when the VEGF was co-incubated with different concentrations of RBDV-IgG1 Fc, cellular proliferation was reduced dose-dependently, whereas an equivalent concentration of the control, IgG1 Fc, had no significant effect on the cell growth, indicating this response was specific to the VEGF and an antagonistic action of

RBDV-IgG1 Fc.

Because of the binding ability of RBDV-Ig Fc to B16/F10 (**Figure 11B**), we also wondered whether RBDV-Ig Fc had the ability to inhibit proliferation of B6/F10. We treated B16/F10 cells with 2.5 μ g/ml RBDV-Ig Fc. No matter whether presenting VEGF stimulation or not, the proliferation of B16/F10 showed no significant difference from the negative group (**Figure 13B**). At the same time, the SVEC4-10 cells were also treated in the same condition (**Figure 13A**).

3.6 Effect of blockade of VEGF receptors on tube formation *in vitro*

To investigate the angiogenesis mechanism *in vitro*, by which VEGFR-1 promotes capillary morphogenesis, the effect of RBDV-IgG1 Fc on capillary-like structures in SVEC4-10 cells was examined. SVEC4-10 cells were cultured with the presence of IgG1 Fc (10 μ g/ml), RBDV-IgG1 Fc (10 μ g/ml) or PBS for 1 hour before being given stimulation with VEGF (10 ng/ml), and allowed to incubate at 37 $^{\circ}$ C for 6 hours. The RBDV-IgG1 Fc strongly inhibited the VEGF-induced tube formation and capillary connections compared with VEGF alone or IgG1 Fc within VEGF stimulation. (**Figure14 A, B, and C**). In the presence of RBDV-IgG1 Fc, SVEC4-10 cells could not extend morphology to form a tube-like structure, and accumulated in aggregates. In contrast, IgG1 Fc alone did not cause a significant decrease in the VEGF-induced

tube and network formation (**Figure 14B**). These data showed that capillary connections were apparently blocked by the RBDV-IgG1 Fc. The number of capillary network connections in each group was also calculated (**Figure 14D**).

3.7 RBDV-Ig Fc inhibited the endothelial cell migration in a Transwell system

RBDV-Ig Fc (10 $\mu\text{g/ml}$) induced a significant inhibition of VEGF-stimulated endothelial cell migration from the upper chamber to the lower one through the membrane (**Figure 15C**) whereas IgG1 Fc had no any suppressive effect on migration ability of SVEC4-10 cells (**Figure 15B**). This result indicates that RBDV-Ig Fc could suppress the angiogenic factors-stimulated cell locomotion in responding to chemo-attractive surrounding.

3.8 *In vivo* suppression of tumor growth with *in situ* RBDV-Ig Fc treatment.

After confirming the function of RBDV-Ig Fc to inhibit proliferation, tube formation, and migration of endothelial cells, the efficiency of RBDV-Ig Fc by *in situ* injection was first determined. Female C57/BL6 (6-8 week age) was inoculated with 1×10^6 B16/F10 cells to right dorsum in 200 μl PBS subcutaneously. When the tumor average volume was up to 50 mm^3 , mice were injected with 150 μg proteins *in situ*. Tumor volume was measured every 2 days after being injected with proteins. After being

treated with 150 μg of RBDV Ig-Fc, the tumor growth was significantly inhibited in 2 days (**Figure 16**). Moreover, RBDV-Ig Fc could suppress the B16/F10 tumor growth under 500 mm^3 while tumor growth of other groups was up to 2500 mm^3 .

After scarifying mice, their dorsal skin was regularly cut from them. The vessels were obvious in the negative (**Figure 17A**) and the Ig-G1 Fc (**Figure 17B**) groups, but not in the RBDV-Ig group (**Figure 17C**), regardless of whether the fountainhead of new vessels formed were from axilla. The results showed that RBDV-Ig Fc could be an antagonist of VEGF and inhibit B16/F10 tumor growth through antiangiogenesis.

Based on tumor sections of the H&E staining, the tumor associated blood vessels and around tumor were disrupted (**Figure 18C**) with the RBDV-Ig Fc treatment. Moreover, the tumor cells were also damaged randomly with the IgG1 Fc group (**Figure 18B**), which did not show any destruct effects in the negative group (**Figure 18A**).

3.9 RBDV-Ig Fc-mediated *in vivo* therapy with i.v. injection of mice bearing B16/F10 tumors.

After confirming the suppressive ability of RBDV-Ig Fc to tumor growth with *in situ* treatment, the mice bearing B16/F10 were treated with an i.v. injection of RBDV-Ig

Fc twice every five days. As expected, the tumor growth after treatment with RBDV-Ig Fc was significantly suppressed (**Figure 19**) when compared with the negative group or IgG1 Fc group after the injection 4 days later. Besides, the damage of the tumor region was similar to the RBDV-Ig Fc treatment *in situ* (data not shown), the blood vessels and tumor around blood vessels were disrupted, whereas there was a random damage of tumor section with the IgG1 Fc treatment. Moreover, the survival rate of the mice was extended with RBDV-Ig Fc treatment (**Figure20**).

The mice's organs were separated, and stained with H&E to confirm the physiological toxicity of RBDV-Ig Fc. The kidney tissue with RBDV-Ig Fc treatment was damaged and lots of hollows were present in the junctions between cells (**Figure 21C**). On the other hand, the kidney sections with IgG1 Fc treatment (**Figure 21B**) or negative group (**Figure 21A**) did not have any hollows or serious damage. In the spleen sections, there were some clots in IgG1 Fc (**Figure 22B**) and RBDV-Ig Fc (**Figure 22C**), and the connections within splenocyte were broken in the RBDV-Ig Fc groups. RBDV-Ig Fc caused detrimental breaks in the liver (**Figure 23C**), in the meantime, the liver cells had little damage with the IgG1 Fc treatment (**Figure 23B**). According to the tumor and organs section, RBDV-Ig Fc has efficient ability to suppress angiogenesis and tumor growth, meanwhile, 150 μ g of chimeric protein caused physiological toxicity in C57/BL6 mouse model.

Chapter 4 Discussion

In cancer therapy, inhibition of the VEGF/VEGF receptors signal pathway has been shown to suppress angiogenesis in lots of models, including genetic models of cancer, leading to clinical development of a lot of VEGF inhibitors. Consequently, a novel chimeric protein is confirmed by fusing the receptor binding domain of human VEGFA165 to the Fc portion of the human IgG1 as an affibody, which is designed as antagonist that blocks the physiological interaction between VEGF ligands and its receptors, VEGFR-1 and VEGFR-2. It has been reported that Ig Fc portion could help maintain the tertiary structure of their CH-2 domain after glycosylation of IgG molecule [97]. Besides, glycosylation of the IgG molecule has been shown to significantly affect the resulting the ability of the antibody to participate in ADCC and complement system [102].

The stimulation of endothelial cells by growth factors, such as VEGF, is required for the process of angiogenesis [103]. In previous lab results, it has been shown that RBDV-Ig Fc could bind to human VEGFRs, VEGFR-1 and VEGFR-2, and inhibit angiogenesis through VEGF and its receptors interaction in HUVE cells *in vitro*. This can be strongly confirmed since the sequence alignment of receptor binding domain of VEGF (RBDV) of human and mouse share a 92.7% similarity. As expected, RBDV-Ig Fc fusion proteins can bind to immobilized mouse VEGF receptors, not

only VEGFR-1 but also VEGFR-2, in ELISA. We, therefore, demonstrated this receptor binding domain containing fusion protein could also bind to mice cells which express VEGF receptors on cell surface. According to the results, RBDV-Ig Fc could bind both endothelial cells and the melanoma cell, B16/F10, of which was reported VEGFR expression [101]. We also needed to confirm whether RBDV-Ig Fc could inhibit signal transduction cascades in mouse endothelial cells, SVEC4-10. Clearly, in the course of this study, we discovered that RBDV-Ig Fc consistently and significantly inhibited the proliferation of SVEC4-10 cells, and has also suppressed the proliferation when compared with their natural interactions with VEGF alone. This can be supported by the research of the carboxyl terminal of VEGF. The heparin binding region (amino acid 110-165) has been strongly indicated and that the heparin, through simultaneous binding to VEGF and its receptors increases in signal amplitude and duration [93]. Here our results have shown that the RBDV-Ig Fc inhibits VEGF receptor activation and mitogenesis in mouse endothelial cell no matter whether VEGF has been presented or not. Our results suggest that the mechanism of endothelial cell growth inhibition mediated by the chimeric proteins may due to the direct blocking of VEGF/VEGFR-2 interaction, resulting in inhibition of VEGF-induced VEGFR-2 activation.

It was proposed that the disturbance of VEGFR-1 activity results in vascular

malformation [60], though the role of VEGFR-1 in angiogenesis is still the object of debate. As we expected, VEGF-driven, capillary-like tube formation in SVEC4-10 cells was strikingly decreased by RBDV-Ig Fc. Morphological changes of SVEC4-10 cells, induced by VEGF, were inhibited by RBDV-Ig Fc. Moreover, the migration ability of SVEC4-10 cells, which was induced by VEGF, was partially suppressed by RBDV-Ig Fc. Base on the above results, we confirm that RBDV-Ig Fc could inhibit the signal transduction through VEGF and VEGFR-1 *in vitro*. According to the results above, RBDV-Ig Fc could block the angiogenesis signal transduction through competing against VEGF.

Several strategies have previously been reported to function as VEGF binding antagonists, including anti-VEGF antibody [81, 82], anti-VEGFR-2 receptor antibody [86], RNA-based aptamers [104], and various peptides [90-92]. Compared with the above molecules, RBDV-IgG1 Fc possesses some theoretical advantages, because it can target both VEGF main receptors, VEGFR-1 and VEGFR-2. Furthermore, coupling of the receptor binding domain of VEGF sequence to human IgG Fc region sufficiently blocks VEGF receptors and antagonizes VEGF activity. There are several theoretical advantages for targeting VEGFR receptors on endothelial cells as cancer therapeutics. First, VEGFR-2 is expressed exclusively on proliferating endothelial cells at tumor sites [105]; therefore, antagonists against the receptor may offer higher

specificity compared to other agents. Moreover, tumor vessel endothelial cells are in direct contact with the blood, and they have greater accessibility compared to antibodies against markers expressed on individual tumor cells.

With *in situ* injection of RBDV-Ig Fc, B16/F10 tumor growth was suppressed and was significantly smaller than control groups, PBS and IgG1 Fc; however, the proliferation of B16/F10 was not inhibited by RBDV-Ig Fc *in vitro*. We speculated that the proliferation of B16/F10 was not only driven by VEGF signal transduction, but also other growth factors in signal pathway; for example the hepatocyte growth factor (HGF), scatter factor (SF), and thrombin [106, 107]. Besides, blockade of angiogenesis which was activated by VEGFR-1 and VEGFR-2 signaling was necessary to efficiently inhibit B16/F10 melanoma growth and metastasis [108]. Hence, RBDV-Ig Fc could inhibit tumor growth of B16/F10 through the suppressing of the angiogenesis and proliferation of endothelial cells *in vivo*.

Tumor sections were obtained and stained with H&E, we discovered that tumor associated blood vessels and the area surrounding the tumor were disrupted (**Figure 18C**) with RBDV-Ig Fc treatment. Moreover, the tumor cells were also damaged randomly within the IgG1 Fc group (**Figure 18B**), which did not show any destructive effects in negative group (**Figure 18A**). IgG-like fusion proteins may prove to be better choices over smaller molecule substances or fragments such as peptides for

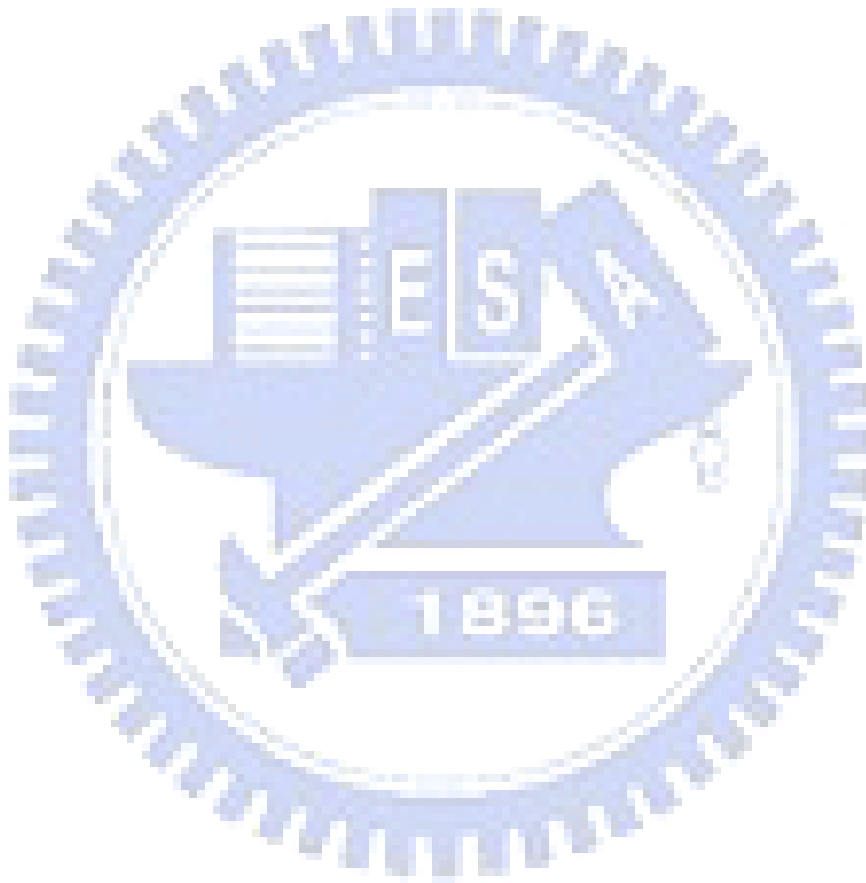
other *in vivo* applications by providing the Fc domain that not only confers long pharmacokinetic half-life [97] but also supports secondary immune functions, such as antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In a previous study, we have shown that RBDV-IgG1 Fc can induce cell-mediated cell cytotoxicity by NK cells (**Appendix2, Figure2**). Tumor cell killing by ADCC is triggered by the interaction between the Fc region of an antibody bound to a tumor cell, and the Fc γ receptors on immune effector cells, such as neutrophils, macrophages and natural killer (NK) cells. Among these receptors, Fc γ RIIIa is a predominant Fc γ R on NK cells [109, 110]. CDC is initiated by complement component C1q binding to the Fc region of IgG, which is bound to the surface of a tumor cell. Of these two isotypes, the IgG1 isotype is widely accepted as the most effective at recruiting the immune system. This is because the Fc regions of IgG1 interact strongly with all types of Fc γ R on lymphoid and myeloid effectors and are strong complement activators [111, 112]. Because the damage of tumor cells was in cytoplasm, and there were not a lot of immune cells in the tumor region, we believe that the Ig Fc region of chimeric protein can damage tumor cells and endothelial cells by CDC.

After confirming the suppressive ability of RBDV-Ig Fc to tumor growth with *in situ* treatment, we treated mice bearing B16/F10 with *i.v.* injection of RBDV-Ig Fc

twice every five days. Tumor growth was effectively suppressed with RBDV-Ig Fc treatment and showed significant difference from other groups in 4 days after injection. The damage of the tumor region was similar to the RBDV-Ig Fc treatment *in situ* (data not shown). It is strongly believed that RBDV-Ig Fc not only could bind VEGF receptors *in vitro*, but also serve a function of targeting VEGF receptors *in vivo*. Heart, lung, spleen, liver, and kidney were also separated from mice. We discovered that RBDV-Ig Fc caused physiological damage in kidney (**Figure 21C**), spleen (**Figure 22C**), and liver (**Figure 23C**). The organ sections of the mice with IgG1 Fc treatment also showed a bit of break (**Figure 21B, 22B, 23B**). Although RBDV-Ig Fc caused physiological damage to mice, it indeed extended the survival rate of the mice with treatment of RBDV-Ig Fc (**Figure 20**). According to the histological results, we believe that RBDV, as a targeting domain of RBDV-Ig Fc, could bind cells which expressed VEGF receptors *in vivo*; in addition, the systemic immune response was enhanced by the IgG1 Fc portion of the chimeric protein resulted in organ damage.

In summary, an effective strategy was demonstrated in the engineering of receptor binding domain of ligands against specific targeted cells which was fused with an appropriate Fc portion of IgG for their therapeutic capacity. Also, this chimeric protein, RBDV-Ig Fc, was effective in antiangiogenesis *in vitro* and suppressing B16/F10 tumor growth in C57/BL6 mouse model. It is believed that

RBDV-Ig Fc can be used in the treatment of the growth of human cancer cells, which was dependent on angiogenesis. The concept of using a receptor binding domain of growth factor conjugated with Fc portion of IgG might be a useful strategy for cancer therapy.



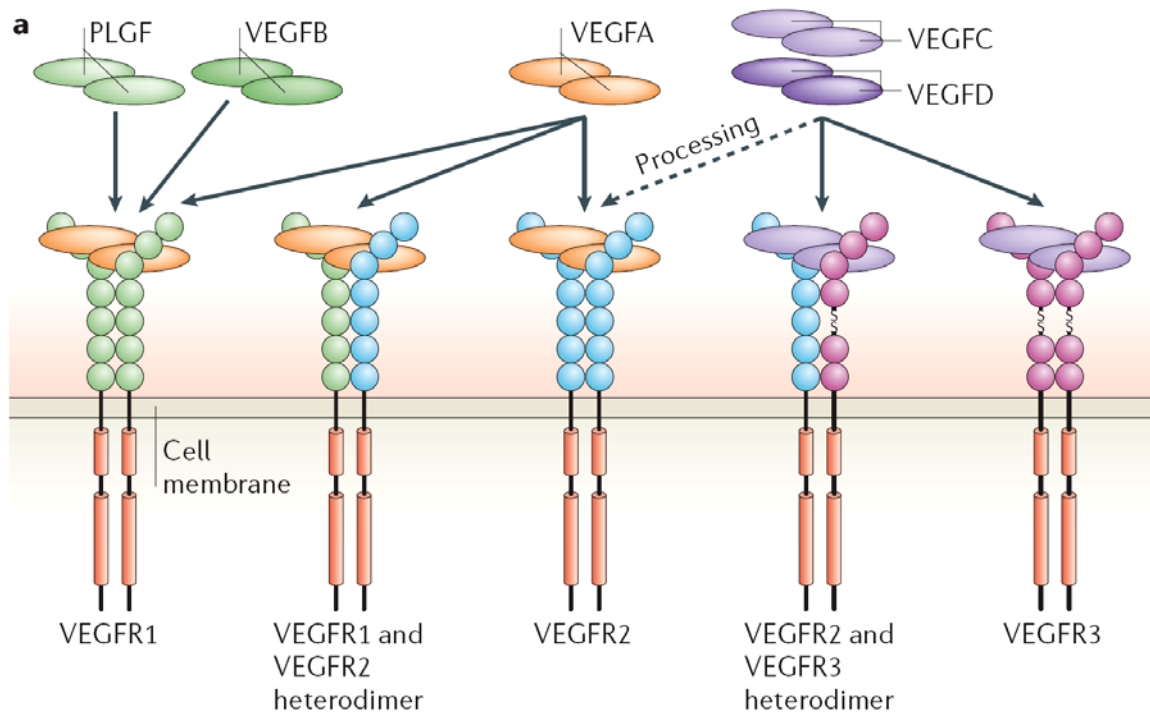
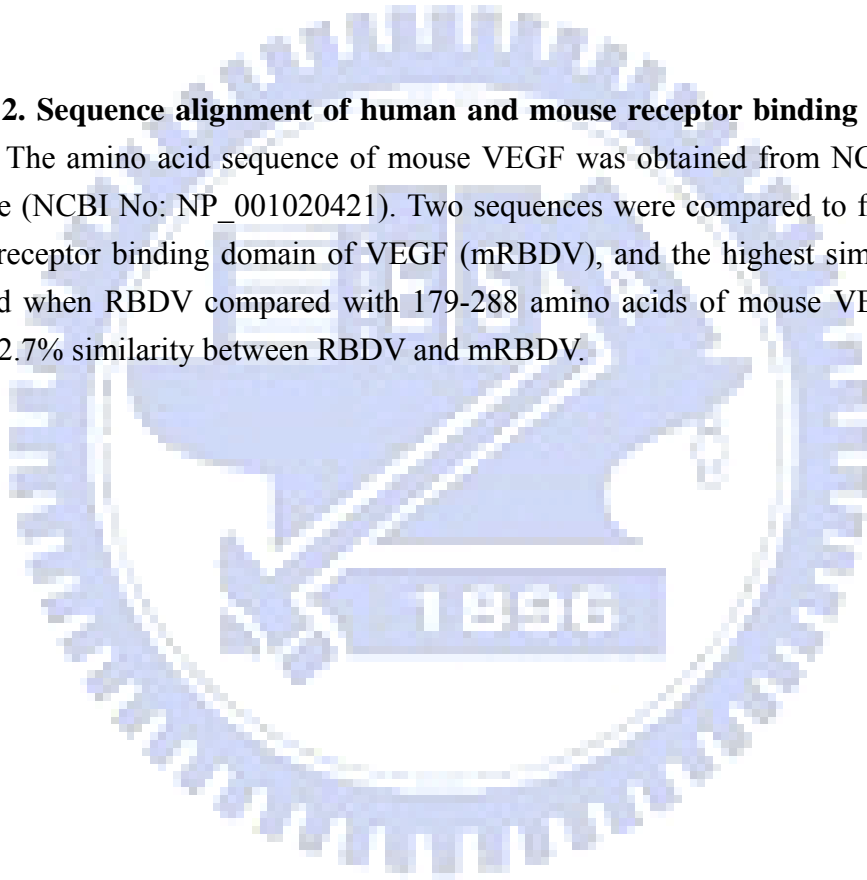


Figure 1. Role of the VEGF receptors. VEGFR-1 and VEGFR-2 are expressed in the cell surface of most blood ECs. Instead, VEGFR-3 is largely restricted to lymphatic EC. VEGF-A binds both VEGFR-1 and VEGFR-2. In contrast, PLGF and VEGFB interact only with VEGFR-1. VEGFC and VEGFD bind VEGFR-2 and VEGFR-3. There is much evidence that VEGFR-2 is the major mediator of EC mitogenesis and survival, as well as angiogenesis and microvascular permeability. VEGFR-1 has an established signaling role in mediating monocyte chemotaxis. Also, in hematopoietic stem cells (HSC) or leukemic cells, both VEGFR-1 and VEGFR-2 may mediate a chemotactic and a survival signal. R1, VEGFR-1, R2, VEGFR-2; R3, VEGFR-3. (Olsson, A.K. et al. *Nat. Rev. Molecular Cell Biology* 2006)

		1	10	20	30	44
mouse RBDV	(1)	MN	FLLSWVHW	T	LALLLLYLHHAKWSQAAP	TTEGE-QKSHEVTKFM
human RBDV	(1)	MN	FLLSWVHW	S	LALLLLYLHHAKWSQAAP	MAEGGGQNHHEVVKFM
Consensus	(1)	MN	FLLSWVHWS	L	LALLLLYLHHAKWSQAAP	EG Q HEVIKFM
		45	50	60	70	88
mouse RBDV	(44)	DVYQRSYC	RPIETLVDIFQEY	PDEIEYIFK	PSCVPLMRC	AGCCN
human RBDV	(45)	DVYQRSYC	HPIETLVDIFQEY	PDEIEYIFK	PSCVPLMRC	GCCN
Consensus	(45)	DVYQRSYC	PIETLVDIFQEY	PDEIEYIFK	PSCVPLMRC	AGCCN
		89	100	110		
mouse RBDV	(88)	DE	ALECVPTS	ESNITMQIMRIK		
human RBDV	(89)	DE	GLECVPTE	ESNITMQIMRIK		
Consensus	(89)	DE	ALECVPT	ESNITMQIMRIK		

Figure 2. Sequence alignment of human and mouse receptor binding domain of VEGF. The amino acid sequence of mouse VEGF was obtained from NCBI protein database (NCBI No: NP_001020421). Two sequences were compared to find out the mouse receptor binding domain of VEGF (mRBDV), and the highest similarity was obtained when RBDV compared with 179-288 amino acids of mouse VEGF. There was a 92.7% similarity between RBDV and mRBDV.



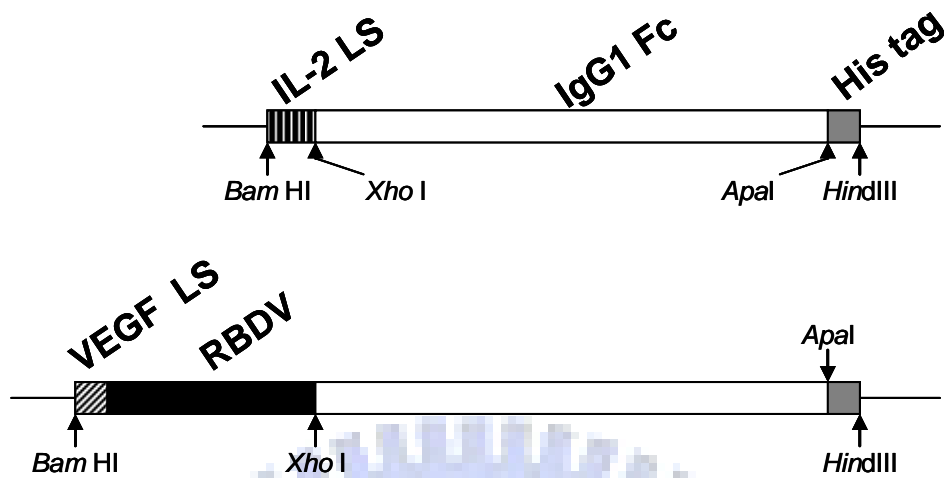


Figure 3. Gene construction of the chimeric proteins. The receptor binding domain of the human vascular endothelial growth factor (VEGF) was fused to the N terminal of the Fc fragment of IgG1, followed by the 6X his tag for protein purification. LS means the leader sequence; RBDV, the receptor binding domain of vascular endothelial growth factor; IgG1 Fc is the constant region of the Fc domain of human immunoglobulin G1. Human IgG1 Fc molecule was also created as a control.

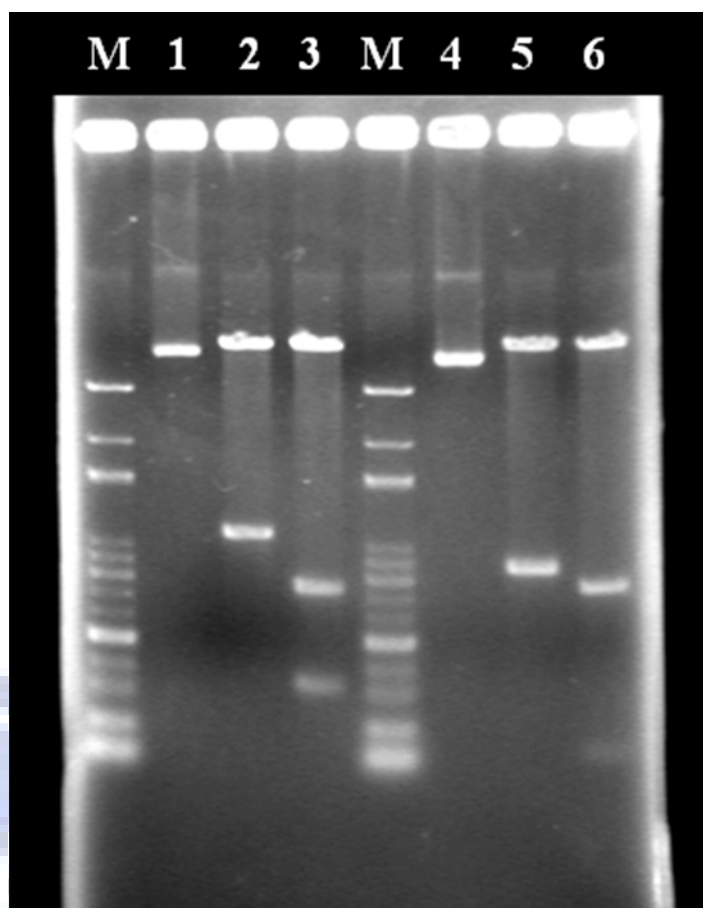


Figure 4. Restriction enzyme digestion of the pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc. In lanes 1 and 4: uncut plasmids, lane 1 is pAAV-MCS/RBDV-IgG1 Fc, and lane 4 is pAAV-MCS/IgG1 Fc. In lanes 2 and 5: *Bam*HI and *Hind*III digestion and the expected fragments of 1080 and 855 bp are obtained, respectively. In lanes 3 and 6: *Bam*HI and *Xho*I digestion and the expected fragments of 340 and 753 bp in pAAV-MCS/RBDV-IgG1 Fc plasmid, 120 and 753 bp in pAAV-MCS/IgG1 Fc plasmid (arrow indicated) can be observed. M means 100 bp ladder marker.

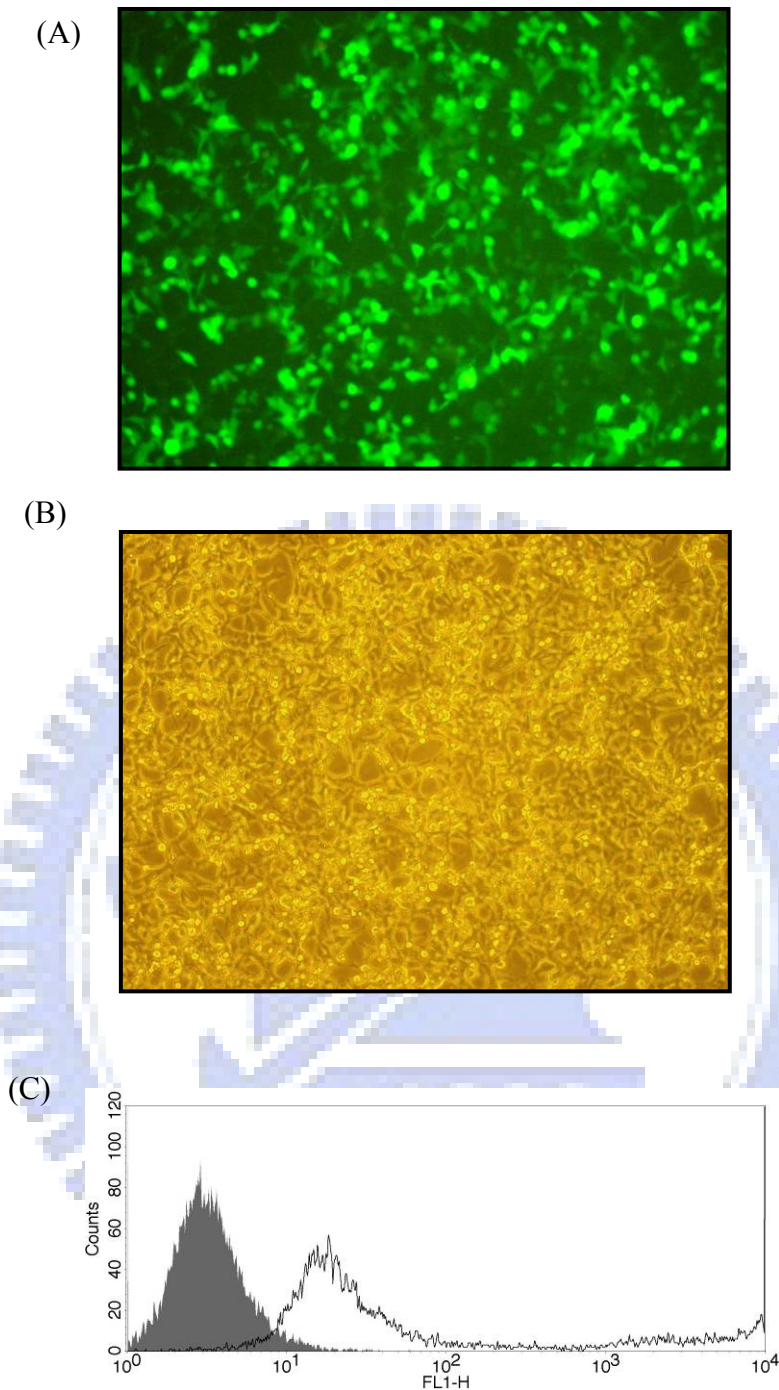


Figure 5. The fluorescence expression in HEK-293 cell. HEK-293 cells were transfected with pAAV-MCS-hrGFP vector using calcium phosphate based transfection methods. (A) Light microscope picture of hrGFP-transfected HEK 293 cells, 100X magnification. (B) Fluorescence microscope photograph of the hrGFP-transfected HEK-293 cell, 100X magnification. (C) Histograms of the hrGFP-transfected cells from 10,000 events measured with flow cytometer. The transfection efficiency is over 70%.

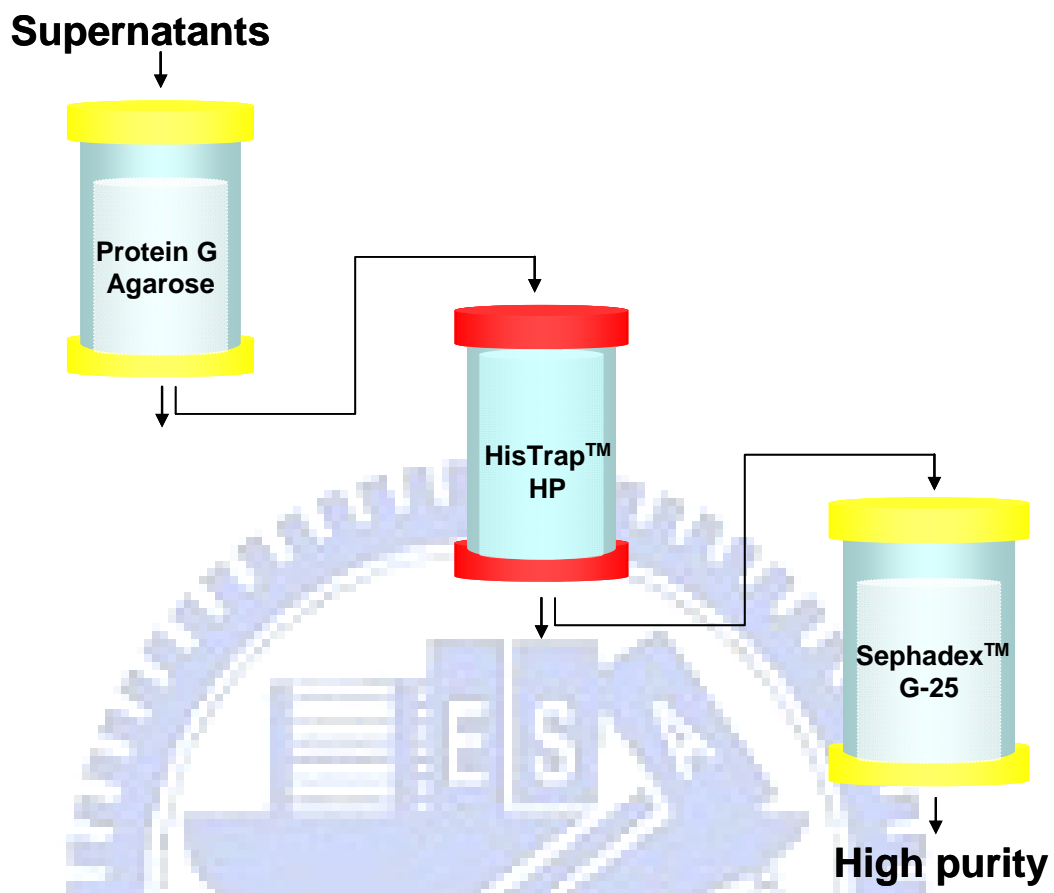


Figure 6. Flow chart of procedure for the purification of chimeric proteins. The culture supernatants of HEK-293T cells were harvested after transfection. The resulting supernatants were first applied into protein G affinity chromatography. Eluted fractions of Protein G were successively loaded into a nickel-charged HisTrap affinity chromatography and were desalted by using sephadex G-25.

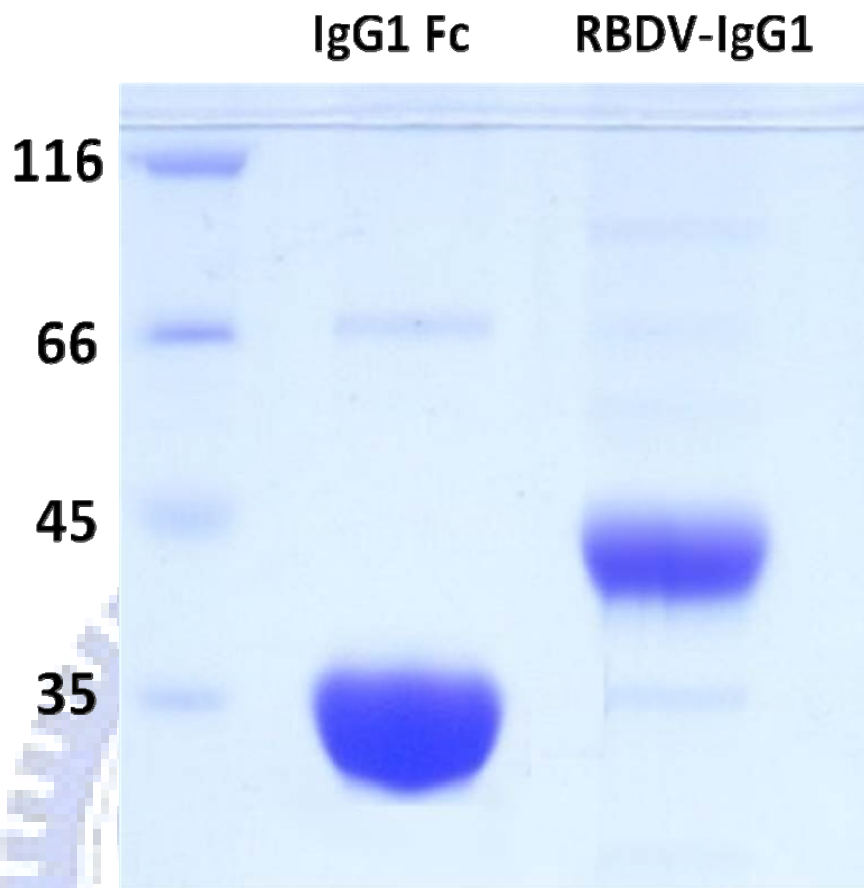


Figure 7. SDS-PAGE analysis of recombinant proteins. IgG1 Fc and RBDV-IgG1 Fc proteins were expressed and first purified by protein G affinity chromatography, followed by HisTrap affinity chromatography and desalted by using sephadex G-25. Purified proteins were resolved by 10% SDS-PAGE under reducing conditions and stained with Coomassie blue. The molecular markers in kilodaltons (kDa) are shown on the left.

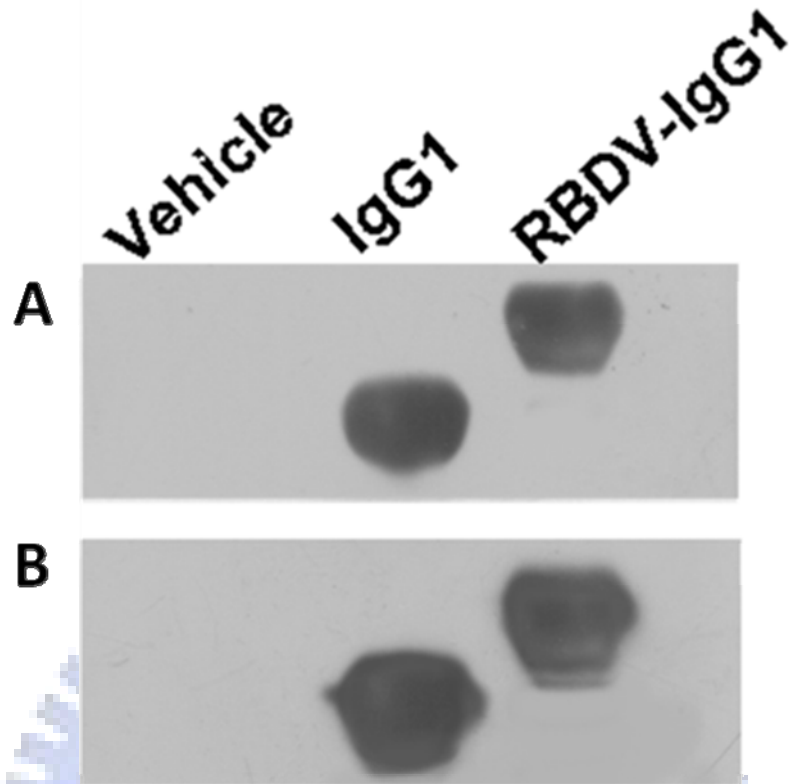


Figure 8. Characterization of purified chimeric proteins. Plasmid pAAV-MCS/IgG1 Fc or pAAV-MCS/RBDV-IgG1 Fc, driven by a human cytomegalovirus (CMV) immediate-early promoter, was transfected into human kidney epithelial (HEK) 293 cells. When the color of cultured medium changed to orange, cultured medium of transfectants were collected, run on reducing 10% SDS-polyacryamide gel electrophoresis, and then analyzed by Western blotting withAs for the purity of chimeric proteins, SDS-PAGE was further transferred onto NC membrane, analyzed by Wesern blotting with the antibody to human IgG (A) and to poly His (B).

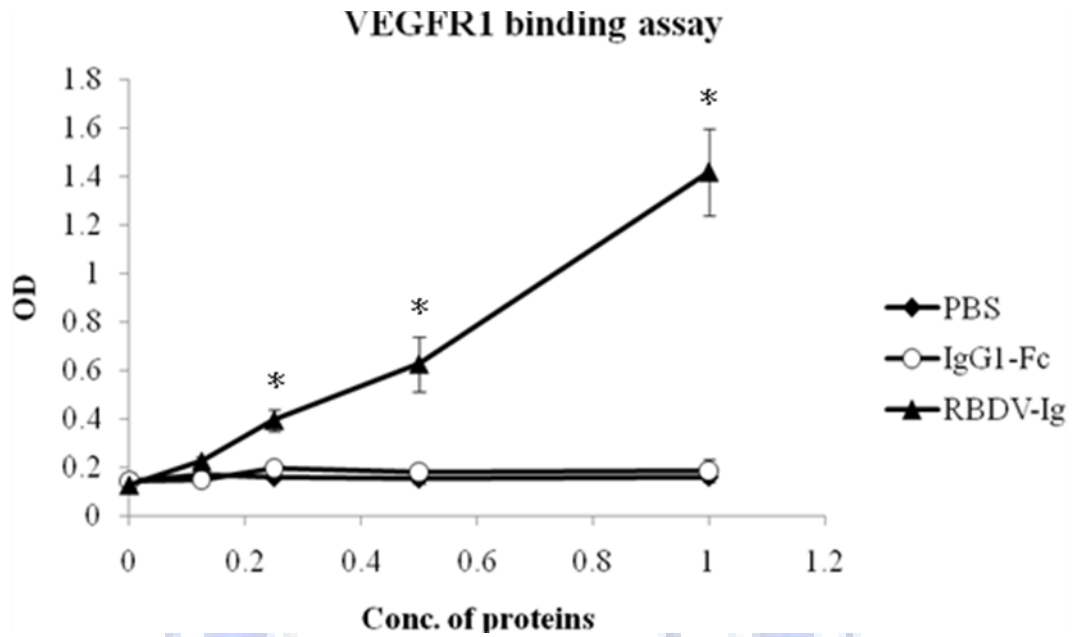


Figure 9. Mouse VEGF Receptor 1 binding activities of purified RBDV IgG1 Fc. The binding activity of the purified RBDV-IgG1 Fc to immobilized mouse VEGFR-1. 96-well plates was precoated with mouse VEGFR1- Fc (0.4 μ g/well in 100 μ l) at room temperature overnight. Different quantities of purified proteins with biotin labeled were added and incubated at room temperature for 1 hr, followed by incubation with HRP conjugate streptavidin for an additional 20 mins. The plates were then incubated with a peroxidase TMB substrate, followed by reading of the absorbance at 450 nm. (* P <0.05). The data performed here are the mean \pm SD of three independent experiments (n=6) corresponding to PBS or IgG1 Fc control.

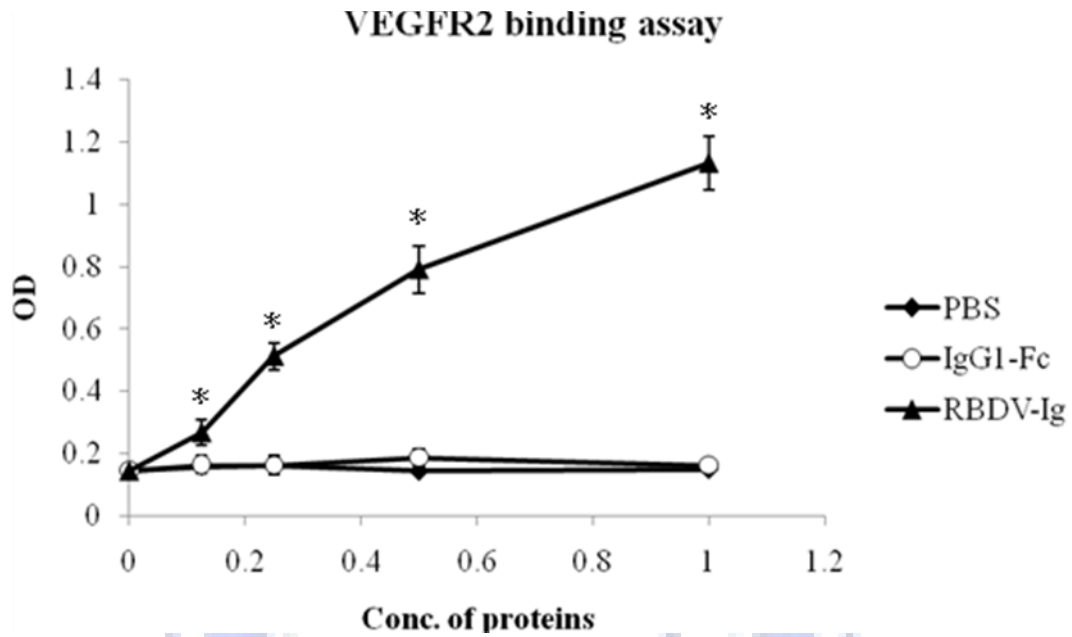
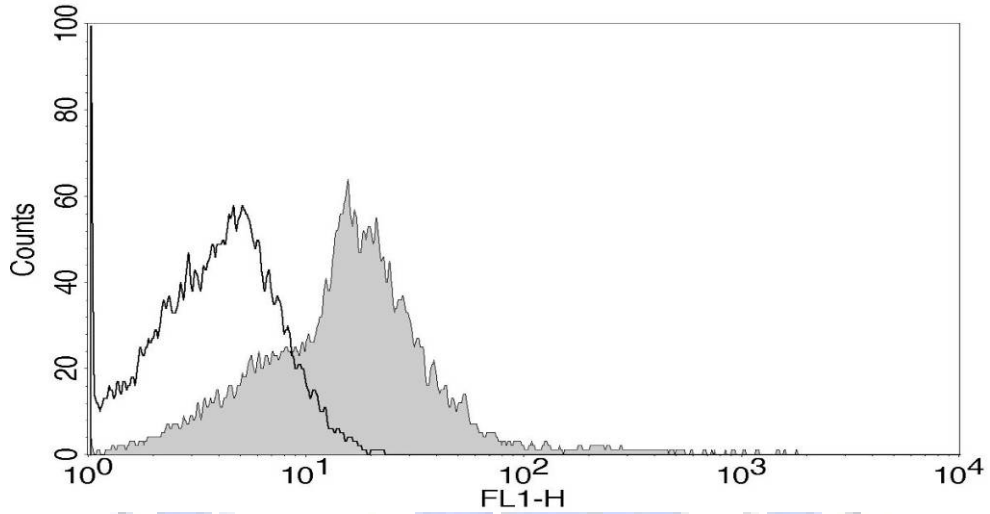


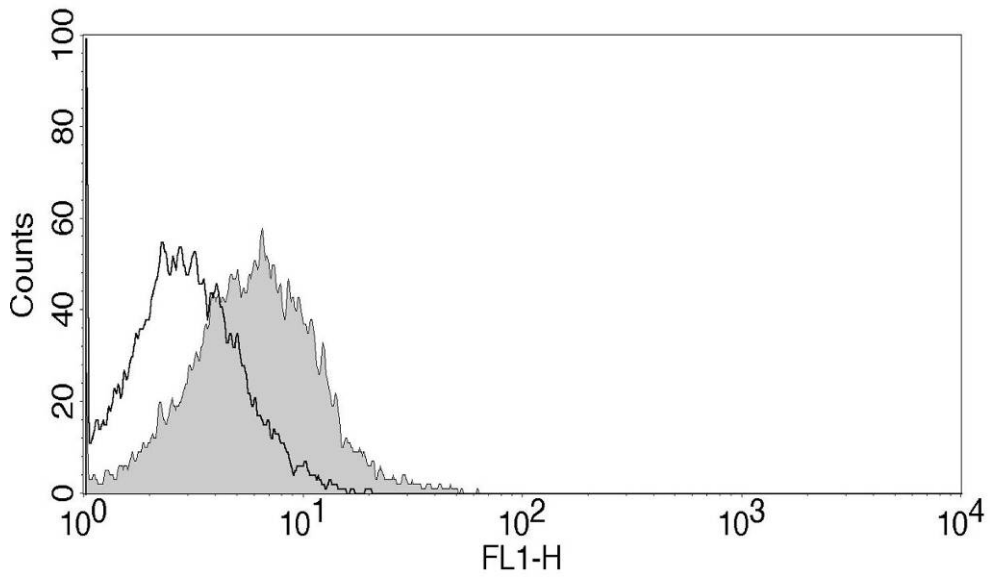
Figure 10. Mouse VEGF Receptor 2 binding activities of purified RBDV IgG1 Fc. The binding activity of the purified RBDV-IgG1 Fc to immobilized mouse VEGFR-2. 96-well plates was precoated with mouse VEGFR2- Fc (0.4 μ g/well in 100 μ l) at room temperature overnight. Different quantities of purified proteins with biotin labeled were added and incubated at room temperature for 1 hr, followed by incubation with HRP conjugate streptavidin for an additional 20 mins. The plates were then incubated with a peroxidase TMB substrate, followed by reading of the absorbance at 450 nm. (* P <0.05). The data performed here are the mean \pm SD of three independent experiments (n=6) corresponding to PBS or IgG1 Fc control.

Figure 11.

(A) MS1 cells



(B) B16/F10 cells



(C)SVEC4-10 cells

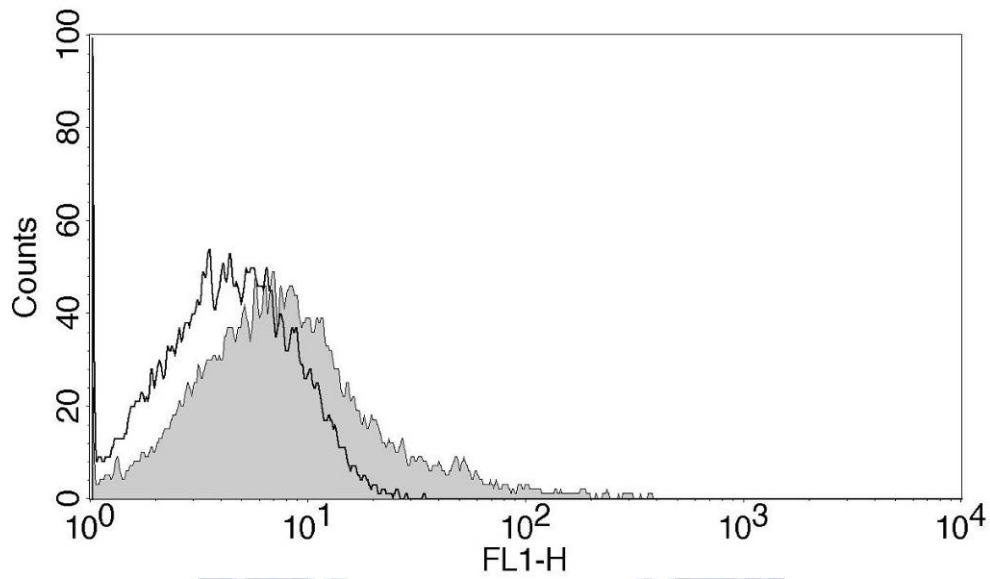
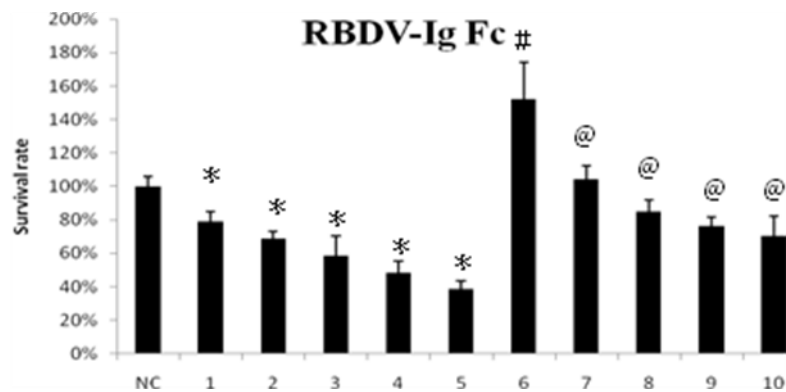


Figure 11. Cell surface binding ability. RBDV-IgG1 Fc chimeric protein was detected by flow cytometric analysis using MS1(A), B16/F10(B), and SVEC4-10(C). The cells were incubated with chimeric proteins at 4 °C for 1 hour, followed by incubation with FITC-labeled goat anti-human IgG antibody for an additional hour (gray area). Cells stained with IgG1-Fc and followed staining with FITC-labeled goat anti-human IgG antibody were used as the negative control (solid line).

(A)



(B)

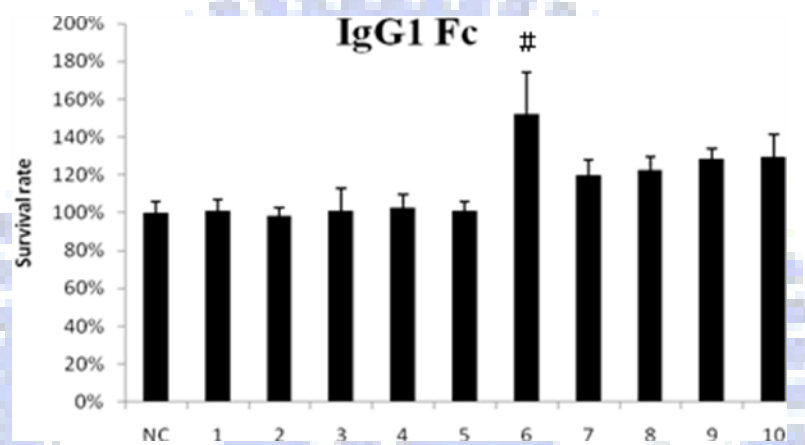
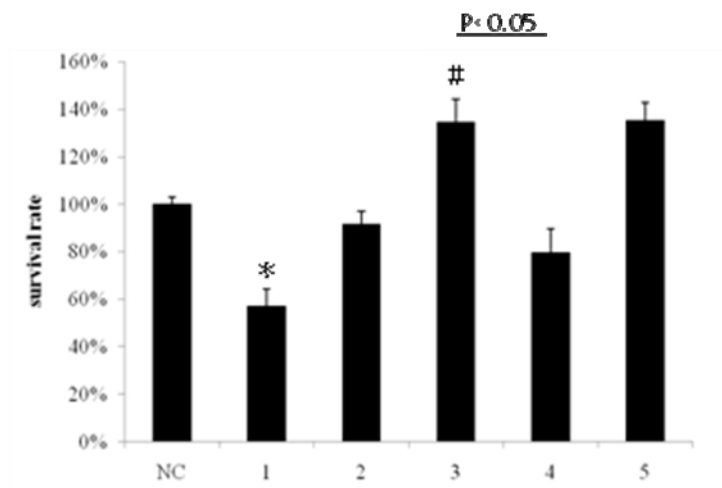


Figure 12. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of SVEC4-10 cells in a dose-dependent manner. SVEC4-10 cells were pre-cultured in the presence of 1, 2.5, 5, 10, and 20 μ g/ml of RBDV-IgG1 Fc(A) or IgG1 Fc(B) (In lanes 1, 2, 3, 4 and 5, respectively). In another condition, cells were pre-cultured in the presence of 2.5, 5, 10 and 20 μ g/ml of RBDV-IgG1 Fc(A) or IgG1 Fc(B) before stimulation with 10ng/ml VEGF (In lanes 7, 8, 9, and 10, respectively) for 1 hour at room temperature. Cell proliferation was then measured with MTS proliferation assay as described in the “Materials and Methods”. The data shown as mean of proliferation inhibition percentages \pm SD obtained from three independent experiments (n=9), * means $p < 0.05$ when compared with the negative group. The VEGF (In lane 6, 10 ng/ml) stimulated a significant increase in endothelial cell proliferation as compared to the control, PBS (Lane 1; # $P < 0.01$). compared with VEGF stimulated proliferation, the growth of SVEC4-10 cells were significantly inhibited by RBDV-IgG1 Fc in a dose-dependent way. (@, $p < 0.05$)

(A)SVEC4-10



(B)B16/F10

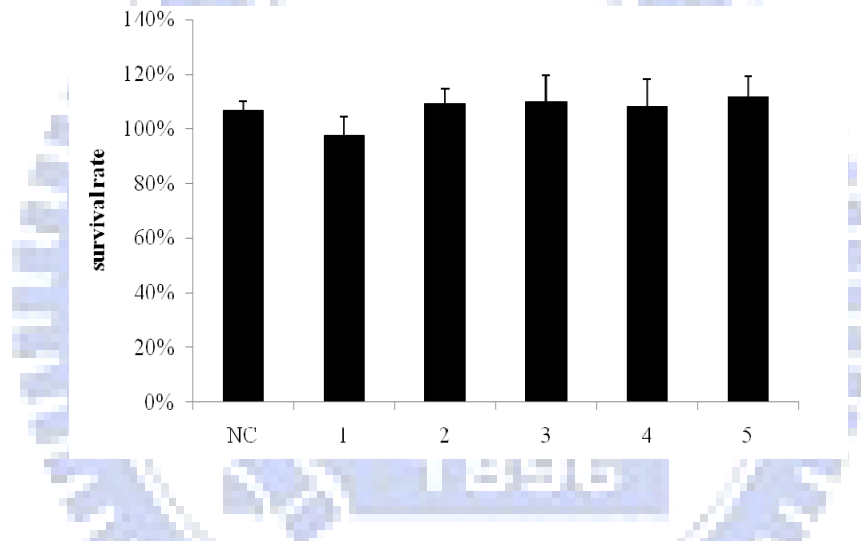


Figure 13. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of SVEC4-10 cells, but not of B16/F10 cells. (A) SVEC4-10 cells and (B) B16/F10 cells were incubated with chimeric proteins for 72 hours and proliferation profile was determined by 1.9 mg/ml MTS. NC, PBS control. In lane 1, 5 μ g/ml of RBDV-Ig was added. In lane 2, 5 μ g/ml of IgG1-Fc was added. In lane 3, 10ng/ml VEGF was added. In lane 4, RBDV-Ig and VEGF were added. In lane 5, IgG1 Fc and VEGF were added. The data shown here are the mean of proliferation inhibition percentages \pm SD obtained from three independent experiments (n=9). VEGF (In lane 3, 10ng/ml) stimulated a significant increase in endothelial cell proliferation as compared to the negative control, PBS (# $P < 0.001$). The proliferation of SVEC4-10 cells were significantly inhibited by RBDV-IgG1 Fc when compared with VEGF or IgG1 Fc group (* $P < 0.05$). There was no significant effect of VEGF or RBDV-Ig on B16/F10 cells.

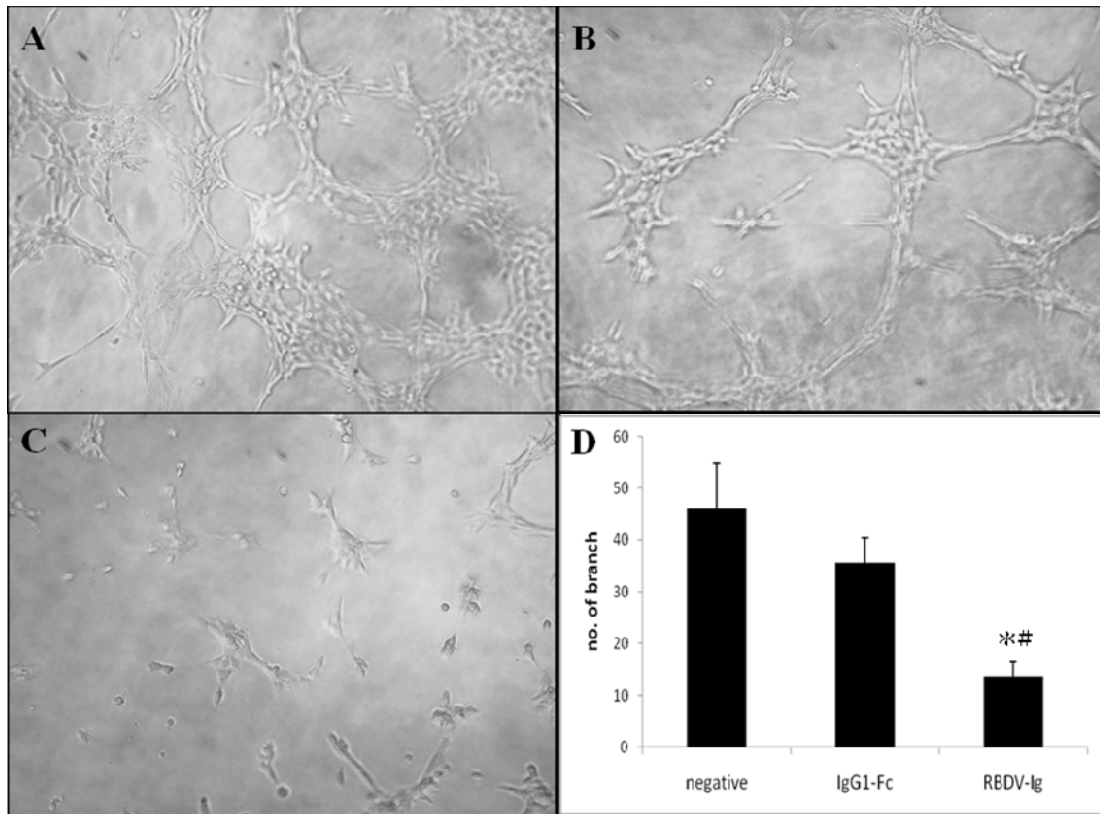


Figure 14. Effect of RBDV-IgG1 Fc on in vitro tube formation, 100X magnification. To determine the angiogenic activity, the SVEC4-10 cells (3×10^4 cells/well/100 μ l) were pre-incubated with IgG1 Fc (10 μ g/ml) or RBDV-IgG1 Fc (10 μ g/ml) for 1 hour, and were added onto growth factor-reduced Matrigel with VEGF (10 ng/ml) in DMEM growth medium for 6 hours at 37°C. Under normal conditions, SVEC4-10 cells formed a network of tubes (A) that was enhanced when stimulated with VEGF (10 ng/ml). (B) SVEC4-10 cells were treated with IgG1 Fc. (C) Tube formation was apparently inhibited with RBDV-IgG1 Fc. (D) Three random fields were counted per well, and the total branches of the tube-like structures per field were calculated. The data represents a mean of the branch of tube-like structures \pm SD obtained from three independent experiments (n=6). * $P < 0.05$ when compared with VEGF group. # $P < 0.05$ when compared with VEGF+IgG1 Fc group.

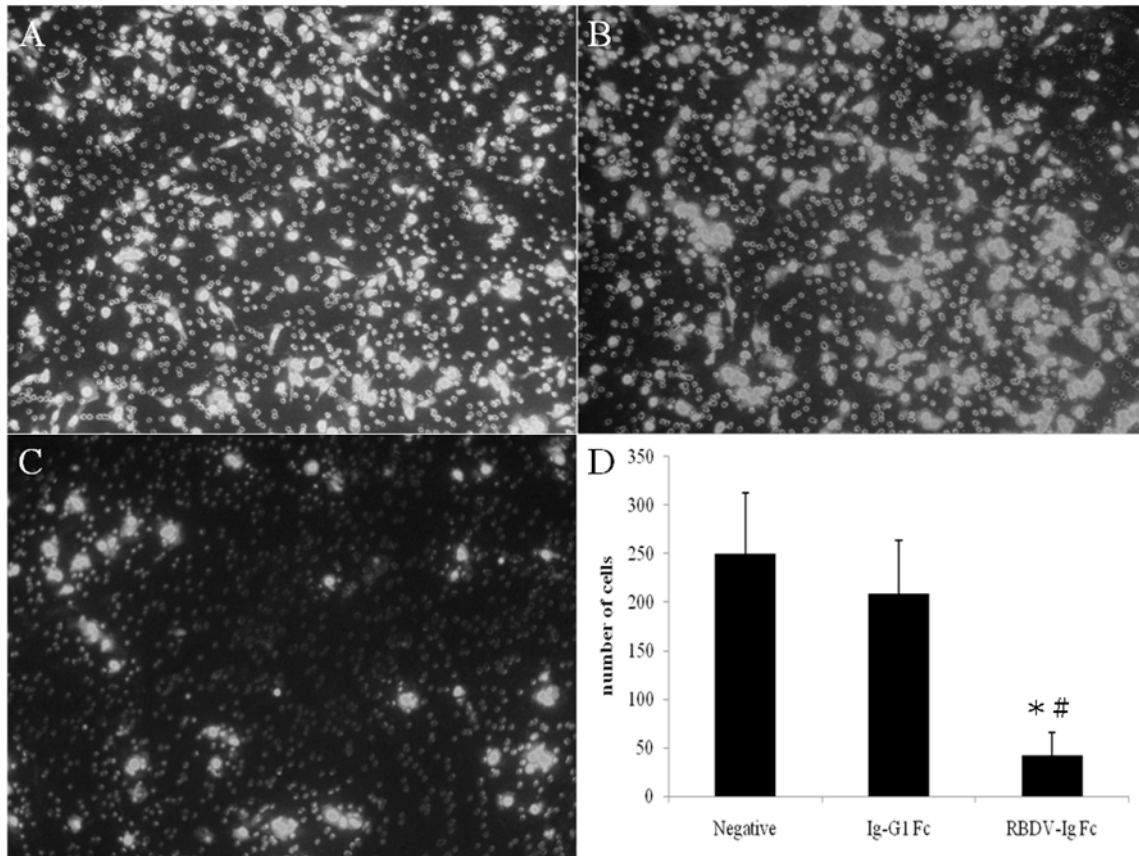


Figure 15. Effect of RBDV-IgG1 Fc on *in vitro* cell migration, 100X magnification.

To confirm the migration activity, the SVEC4-10 cells (3×10^4 cells/well/100 μ l) were pre-incubated with IgG1 Fc (10 μ g/ml) or RBDV-IgG1 Fc (10 μ g/ml) for 1 hour, and were added into the upper well of Transwell whose lower well contains 10 ng/ml of VEGF in DMEM growth medium for 6 hours at 37°C. After 6 hours, the cells in the upper well were removed by cotton buds, and the migrated cells on the other side of membrane were fixed and stained with PI. (A) Migration ability was enhanced when stimulated with VEGF (10 ng/ml). (B) SVEC4-10 cells were treated with IgG1 Fc. (C) migration ability of SVEC4-10 cells was apparently inhibited with RBDV-IgG1 Fc. The small circular pores were 8 μ m pore of Transwell. (D) Three random fields were count per well, and the total number of cells were calculated. The data represents a mean of the branch of tube-like structures \pm SD obtained from two independent experiments (n=4). * $P < 0.05$ when compared with VEGF group. # $P < 0.05$ when compared with IgG1 Fc group.

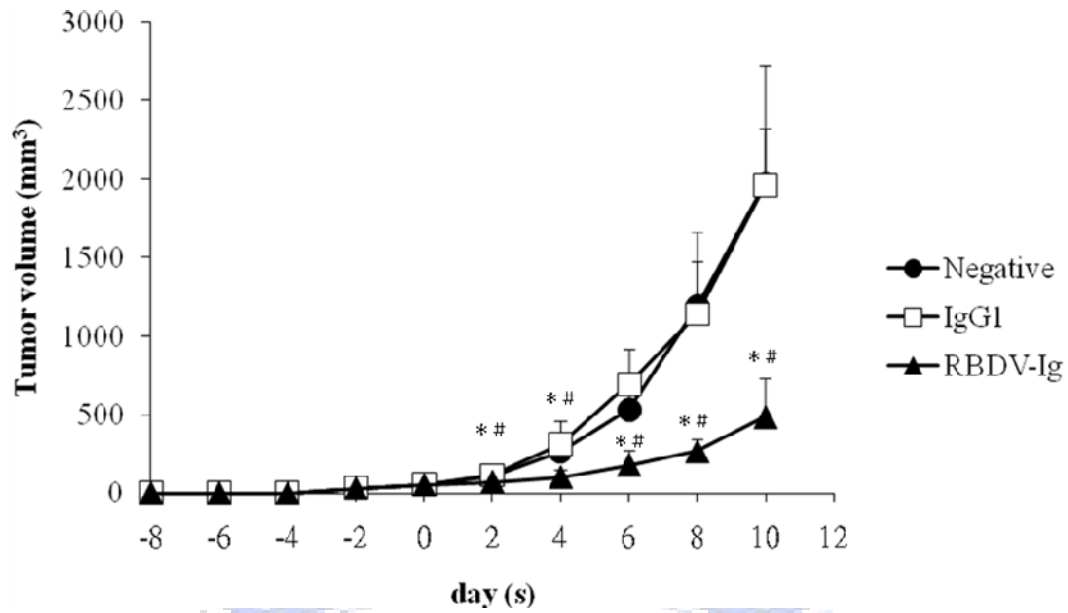
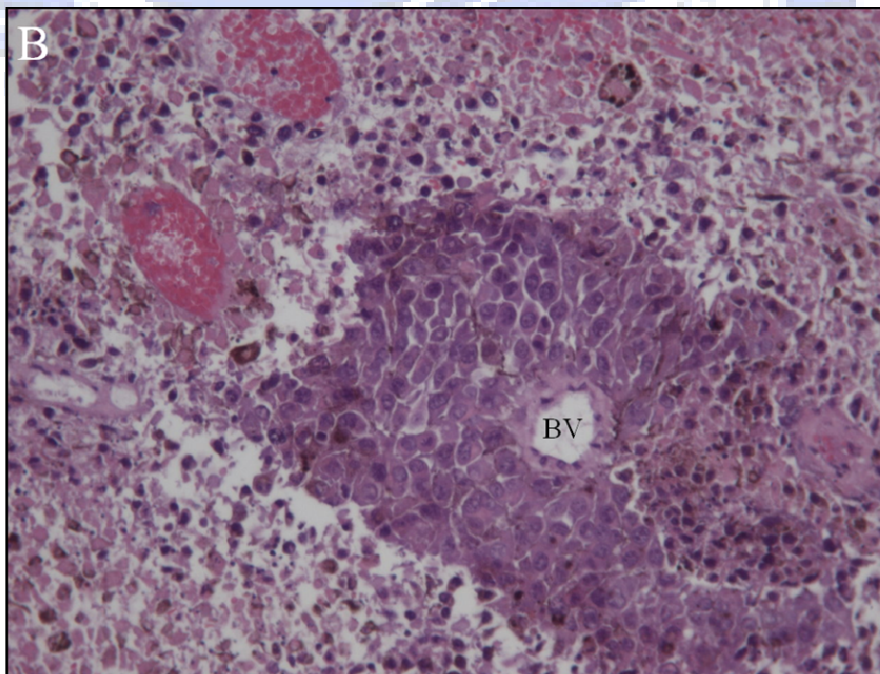
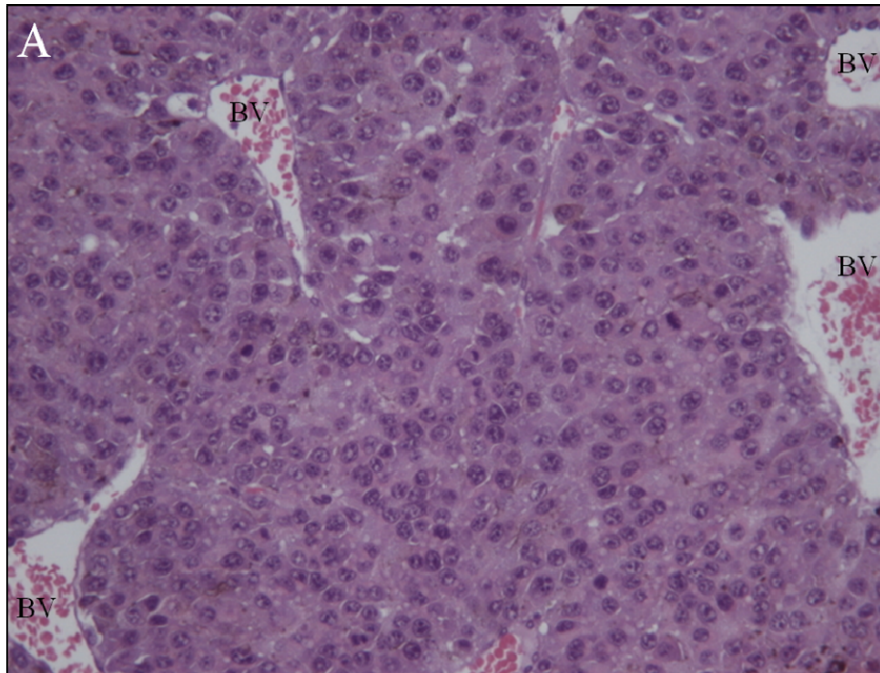


Figure 16. *In vivo* suppression of tumor growth with *in situ* RBDV-Ig Fc treatment. Female C57/BL6 (6-8 weeks of age) was inoculated with 1×10^6 B16/F10 cells subcutaneously in 200 μ l PBS. When the tumor average volume was up to 50 mm³, mice were injected with 150 μ g proteins *in situ*. Tumor volume was measured every 2 days after injecting with proteins. The data represent a mean of tumor volume \pm SD obtained from two independent experiments (n=6). For RBDV-Ig group, *means $P < 0.05$ when compared with the negative group, # means $P < 0.05$ when compared with IgG1 Fc group.



Figure 17. Subcutaneous vascularizing of mouse dorsum. Mice were sacrificed when the tumor volume of negative group was up to 2,500 mm³. Dorsal skin was regularly cut from the mice. The vessels were obvious in (A) negative and (B) Ig-G1 Fc groups, but not in RBDV-Ig group (C).

Figure 18.



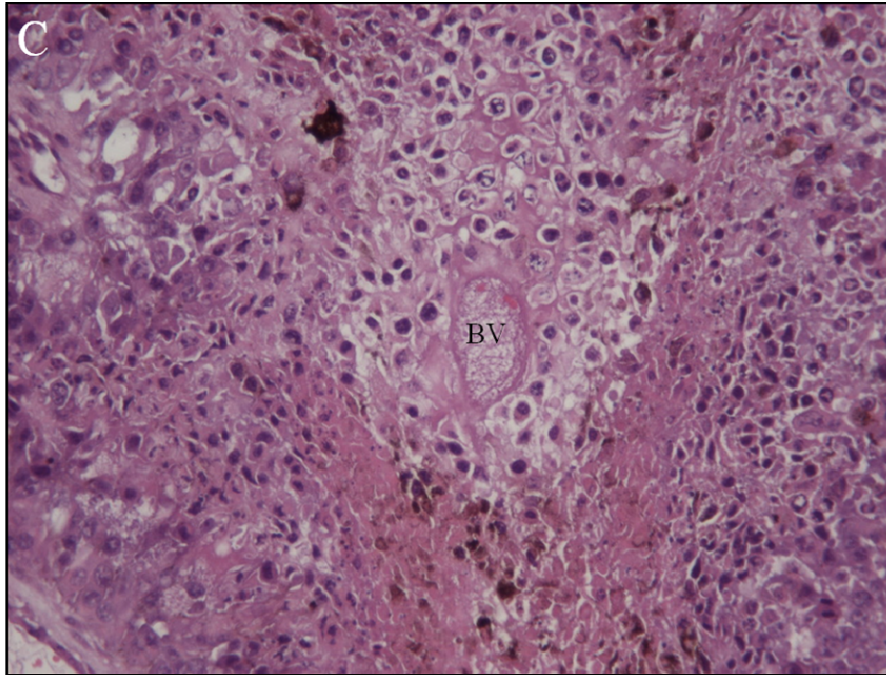


Figure 18. H&E staining of tumor sections, 200X magnification. Portions of the tumors were fixed in 4% paraformaldehyde overnight and subsequently paraffin embedded and cut in slides. Tumor sections were stained with H&E. (A) Negative group which was injected with 200 μ l PBS had lots of disorder blood vessels (BV) and the growth of the melanoma tumor B16/F10 was active. Tumors of the IgG1 Fc group (B) were randomly damaged, whereas tumors of the RBDV-Ig (C) group were disrupted around blood vessels.

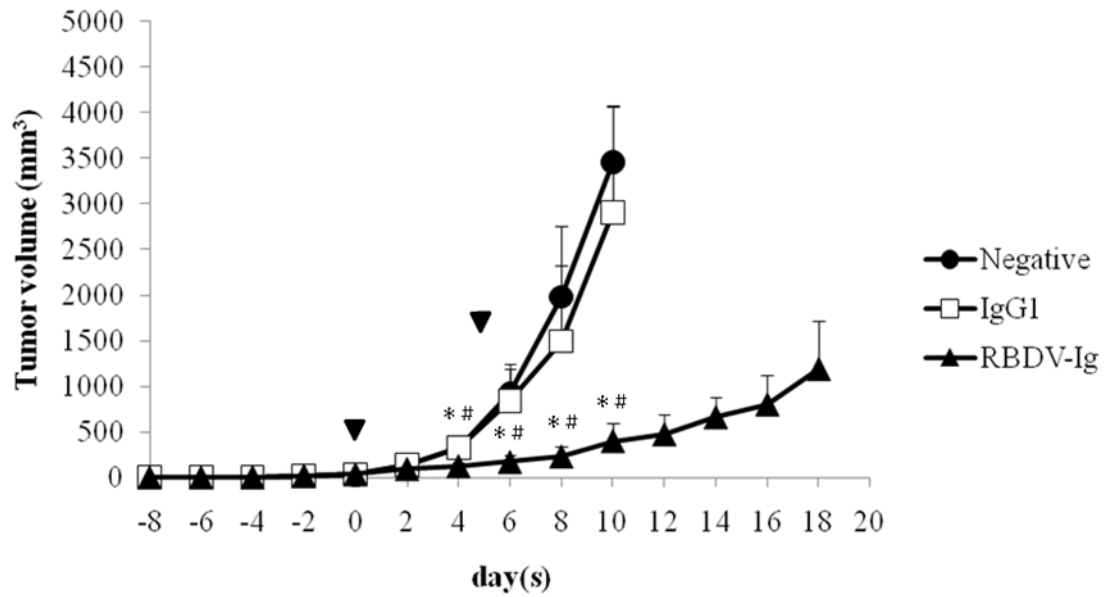


Figure 19. *In vivo* tumor therapy with *iv.* injection of RBDV-Ig Fc. Female C57/BL6 (6-8 weeks of age) was inoculated with 1×10^6 cells subcutaneously in 200 μ l PBS. When the tumor average volume was up to 30 mm^3 , mice were intravenously (i.v.) injected with 150 μ g protein and injected again after five days. ▼ means the day with protein injection. Tumor volume was measured every 2 days after injecting with proteins, and mice were sacrificed when tumor grew up to 2,500 mm^3 . The data represents a mean of tumor volume \pm SD obtained from two independent experiments (n=9). For RBDV-Ig group, *means $P < 0.05$ when compared with negative group, # means $P < 0.05$ when compared with IgG1 Fc group.

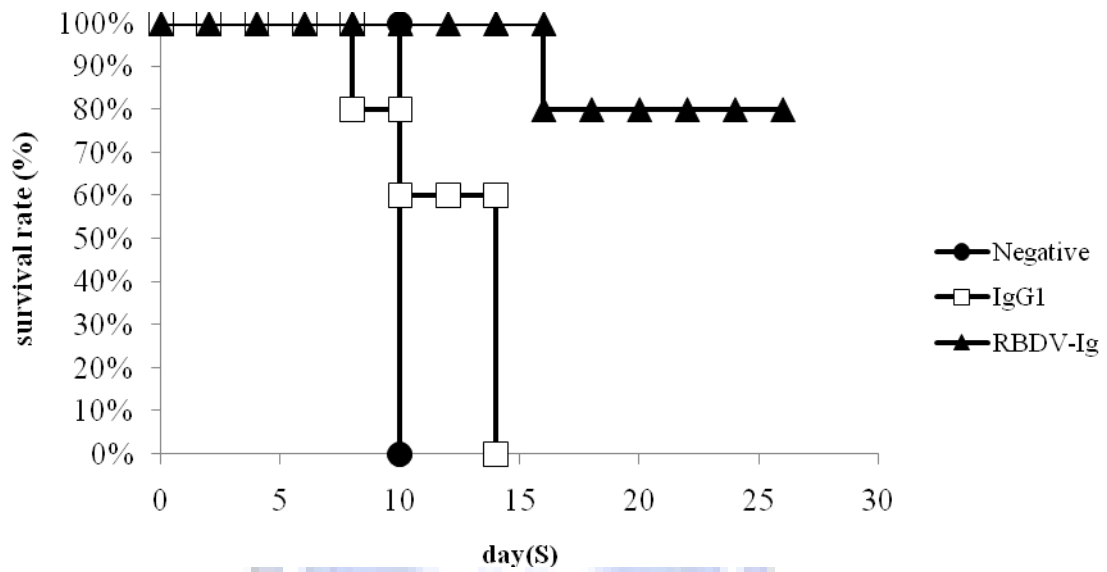
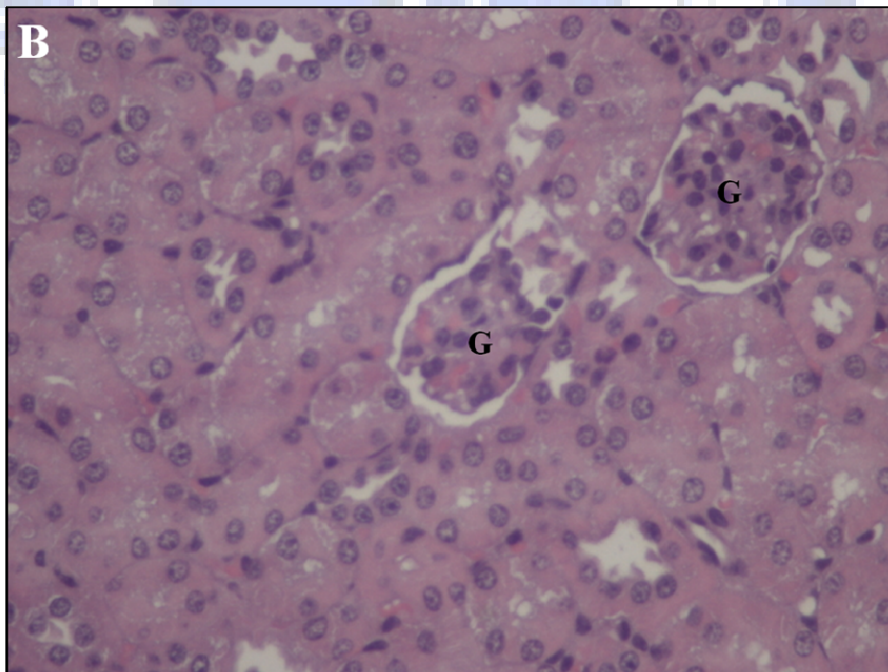
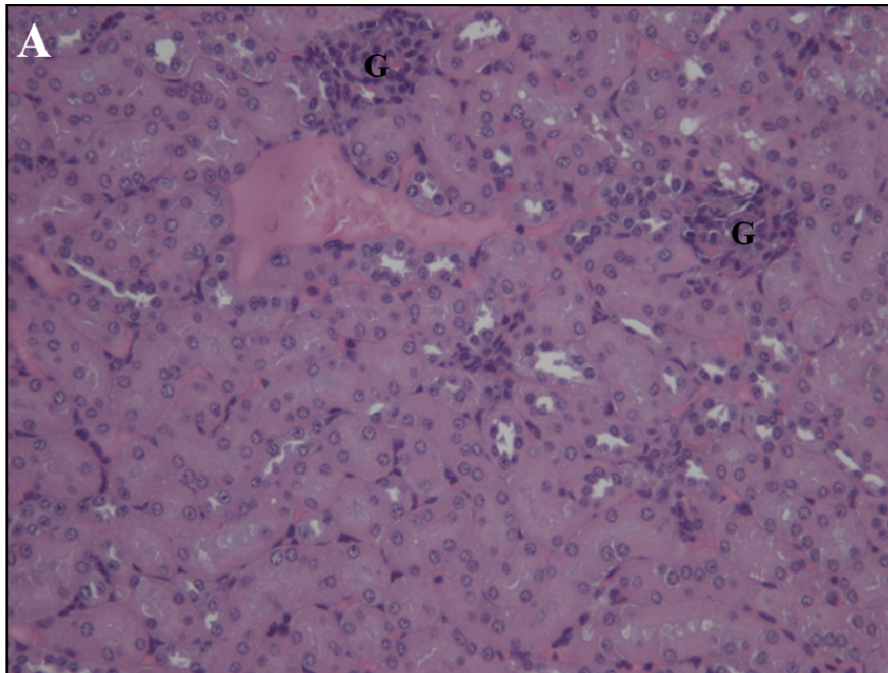


Figure 20. Survival rate of tumor therapy with *iv.* injection of RBDV-Ig Fc *in vivo.* Female C57/BL6 (6-8 weeks of age) was inoculated with 1×10^6 cells subcutaneously in $200 \mu\text{l}$ PBS. When the tumor average volume was up to 30 mm^3 , mice were intravenously (*i.v.*) injected with $150 \mu\text{g}$ protein and injected again after five days. Mice were looked on as dead when tumor volume up to 2500 mm^3 . RBDV Ig-Fc could efficiently extend survival of mice bearing B16/F10 tumor.

Figure 21.



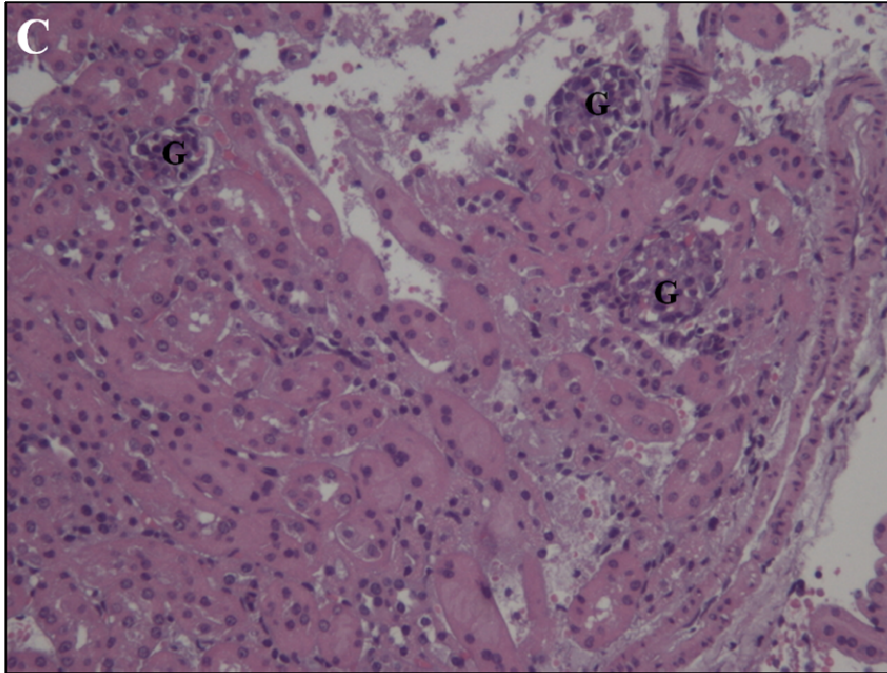
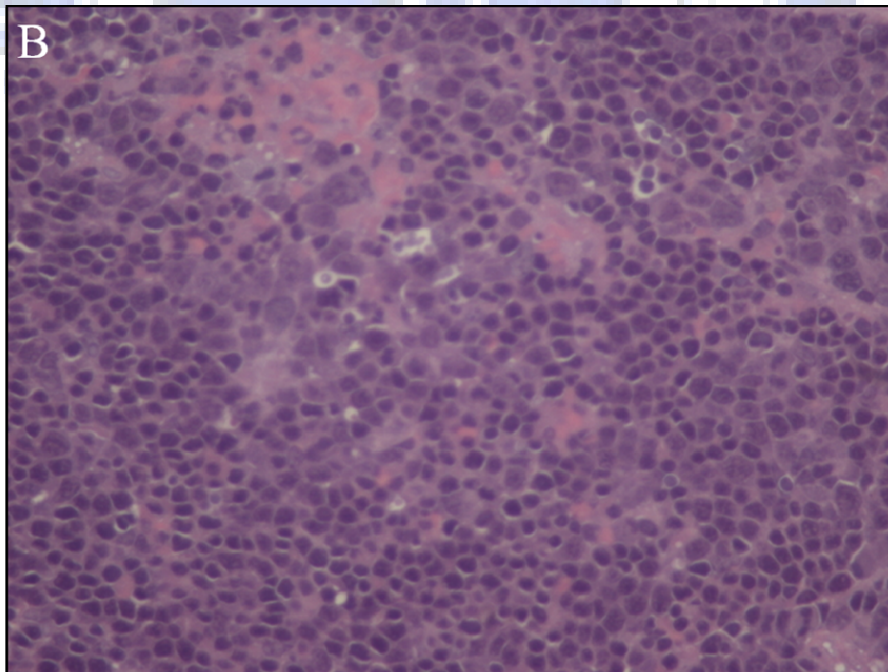
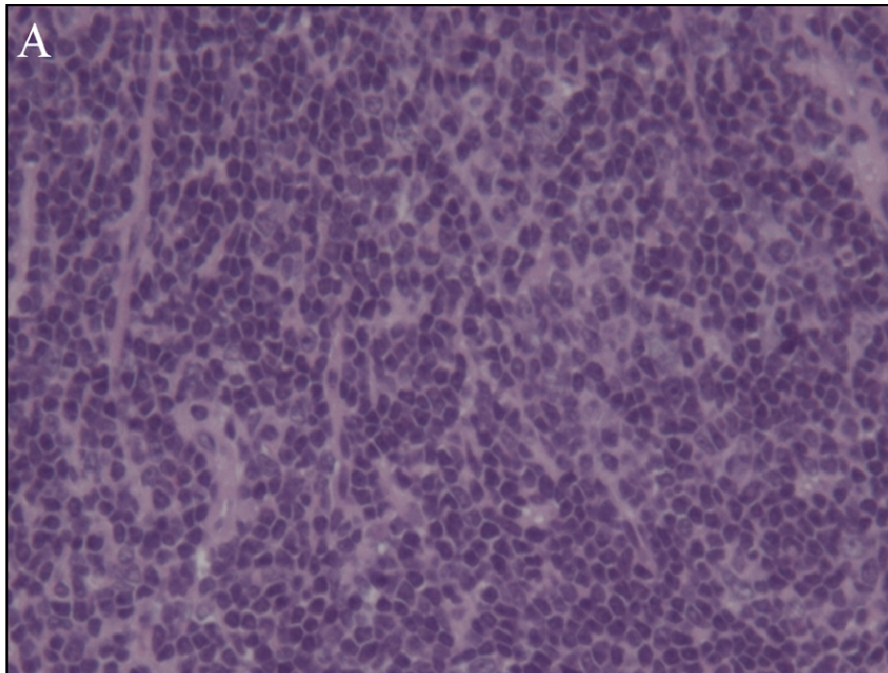


Figure 21. H&E staining of kidney sections, 200X magnification. Portions of the kidneys were fixed in 4% paraformaldehyde overnight and subsequently paraffin embedded and cut in slides. kidney sections were stain with H&E. (A) Negative group, (B) IgG1 Fc group, and (C) RBDV-Ig Fc group. G means glomerulus.

Figure 22.



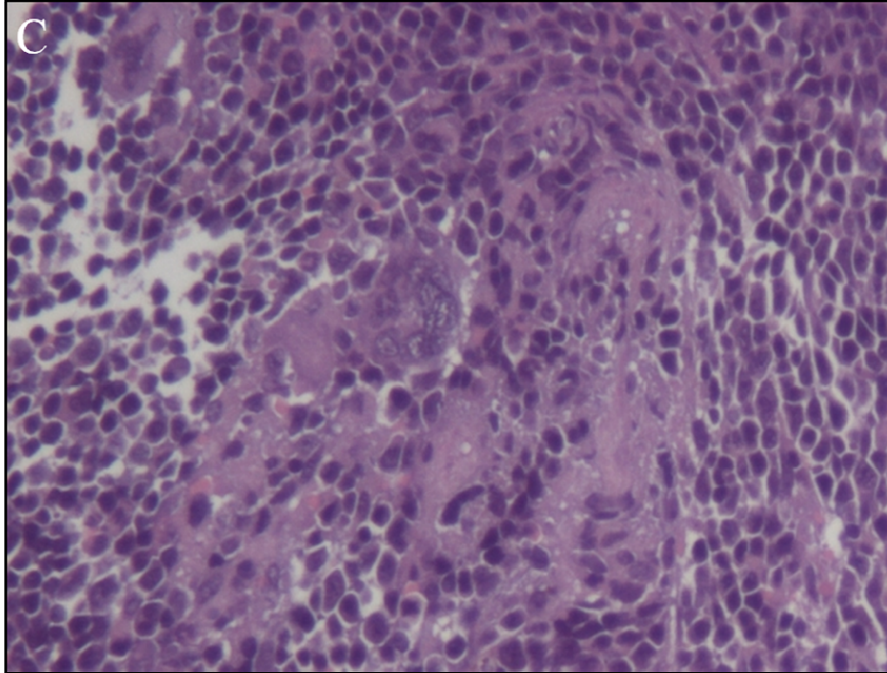
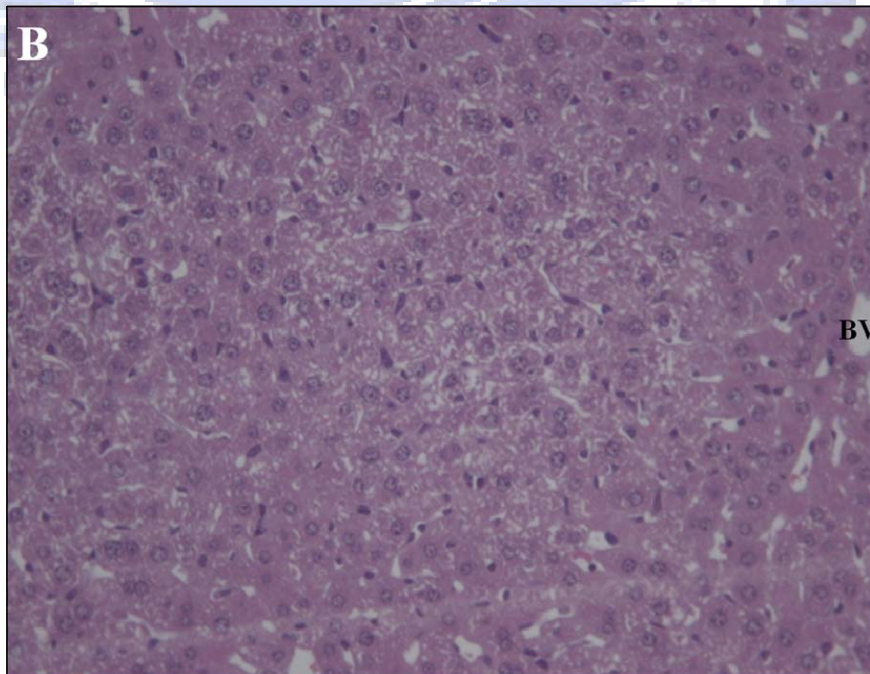
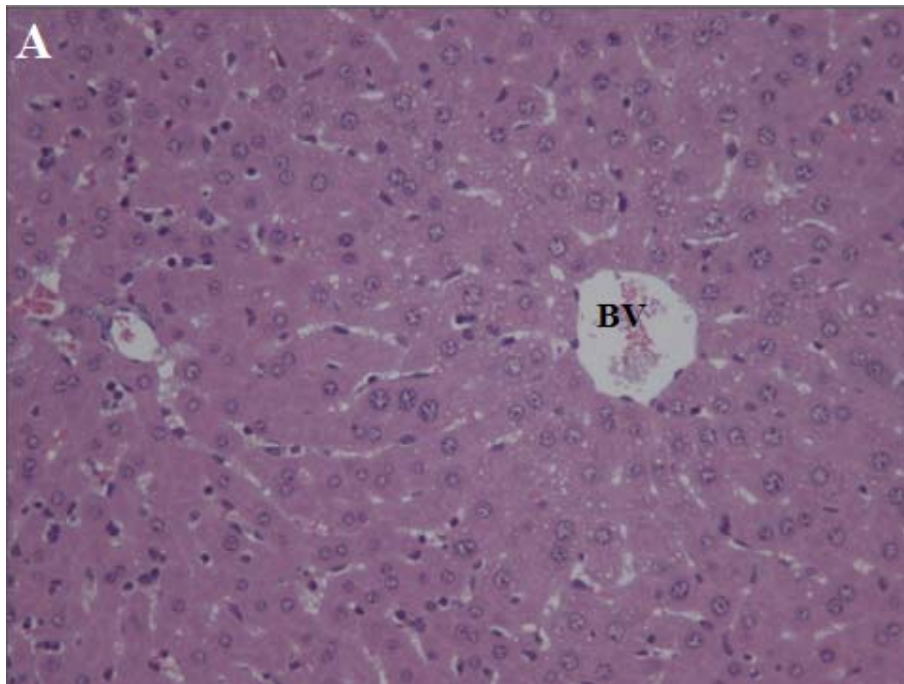


Figure 22. H&E staining of spleen sections, 400X magnification. Portions of the spleens were fixed in 4% paraformaldehyde overnight and subsequently paraffin embedded and cut in slides. Spleen sections were stained with H&E. (A) Negative group, (B) IgG1 Fc group, and (C) RBDV-Ig Fc group.

Figure 23.



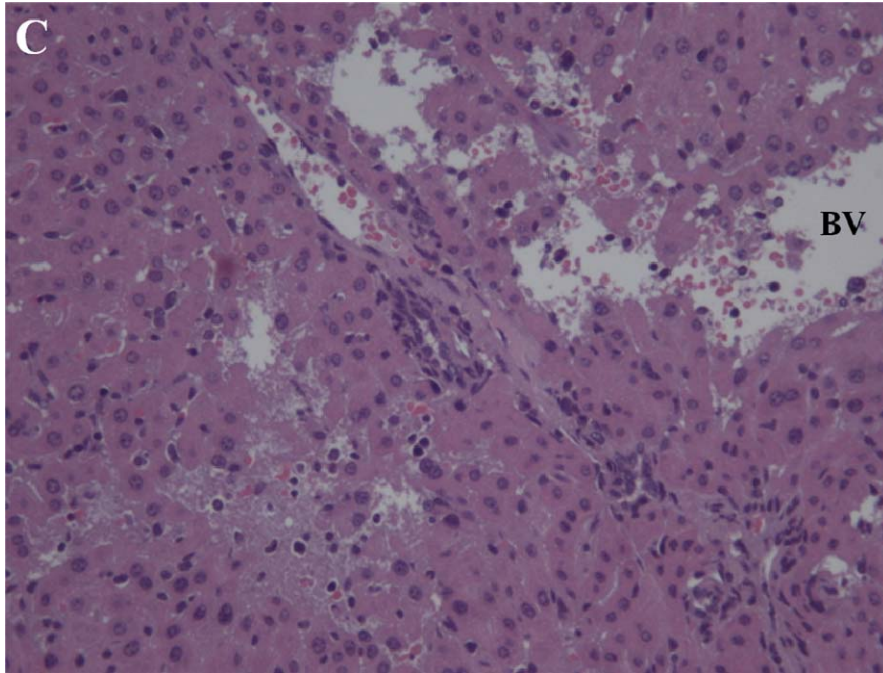


Figure 23. H&E staining of liver sections, 200X magnification. Portions of the livers were fixed in 4% paraformaldehyde overnight and subsequently paraffin embedded and cut in slides. Liver sections were stain with H&E. (A) Negative group, (B) IgG1 Fc group, and (C) RBDV-Ig Fc group. BV means blood vessels.

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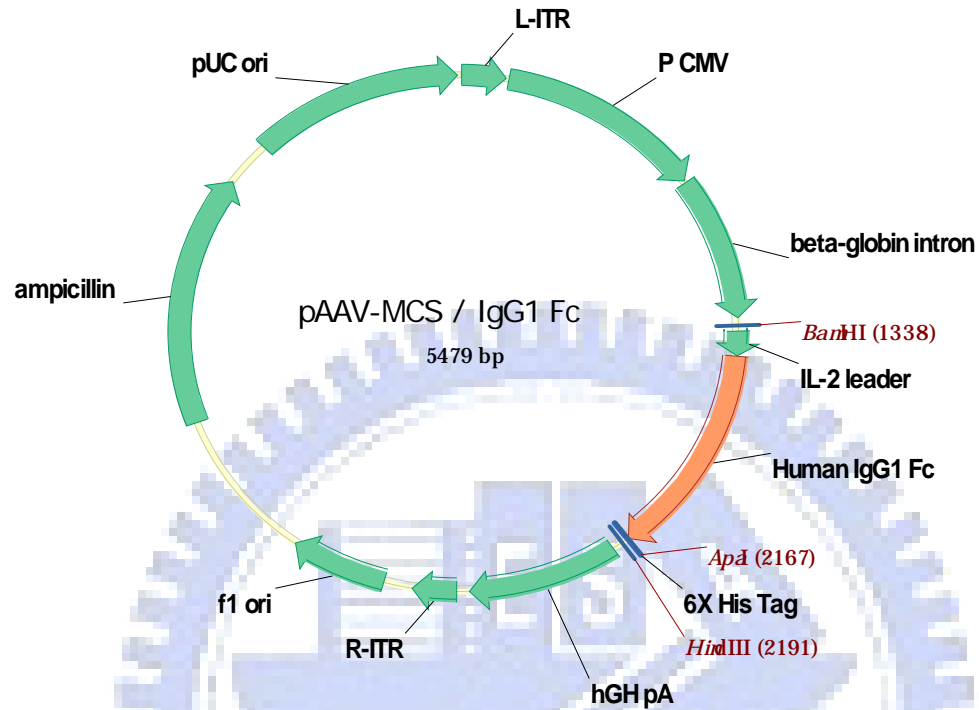
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Appendix 1. Map of using plasmids

The map of pAAV-MCS/IgG1-Fc



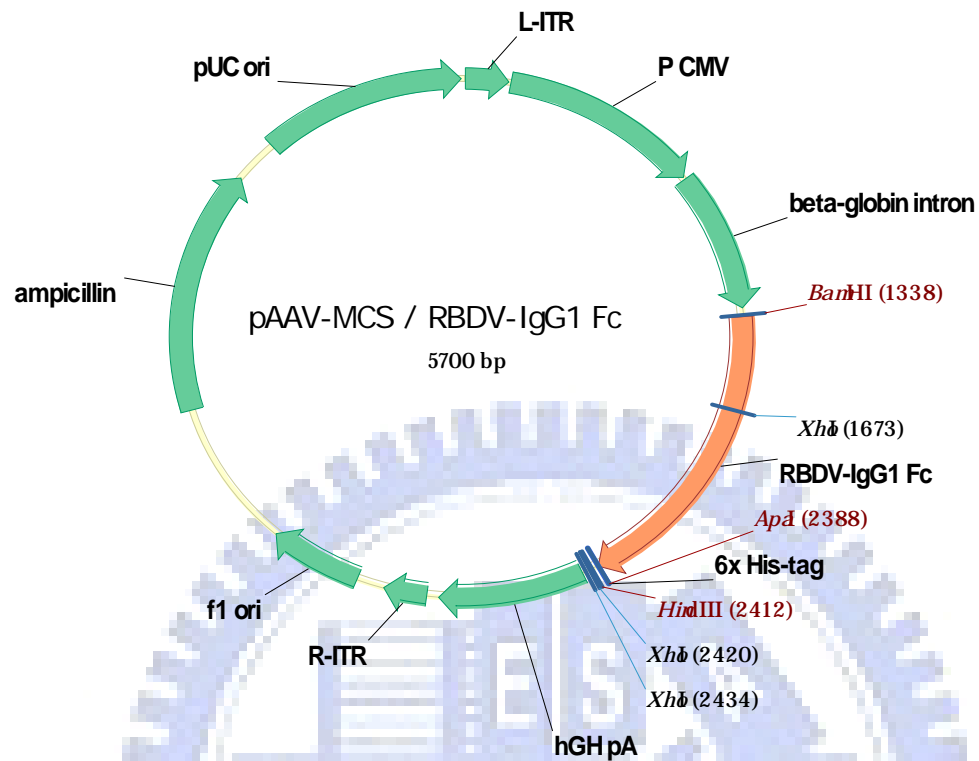
Description	start	end
IL-2 leader sequence	1357	1440
Human IgG1 Fc	1441	2166
L-ITR	1	141
CMV	150	812
Beta globin intron	820	1312
6X His Tag	2169	2189
Hgh Pa	2224	2702
R-ITR	2742	2882
F1 ori	2974	3280
Ampicillin	3799	4656
pUC ori	4807	5474
Apal	2167	
BamH1	1338	
HindIII	2191	
XhoI	1452	

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51 CCCGGGCGTC GGGCGACCTT TGGTCGCCCC GCCTCAGTGA GCGAGCGAGC
101 GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC TGGGCGCGCA
151 CGCGTGGAGC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT
201 AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC
251 TGGCTGACCG CCCAACGACC CCCGCCATT GACGTCAATA ATGACGTATG
301 TTCCCATAGT AACGTCAATA GGGACTTTC ATTGACGTCA ATGGGTGGAG
351 TATTTACGGT AAAC TGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
401 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
451 ATGCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC
501 GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA
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651 AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA
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1251 ACAGCTCCTG GGCAACGTGC TGGTCTGTGT GCTGGCCCAT CACTTTGGCA
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1401 GCACTAAGTC TTGCACTTGT CACAAACAGT ATCCAGCACA GTGGCGGCCG
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The map of pAAV-MCS/RBDV-IgG1 Fc



Description	Start	End
RBDV	1343	1671
Human IgG1 Fc	1671	2388
L-ITR	1	141
CMV	150	812
Beta globin intron	820	1312
6X His Tag	2390	2407
Hgh Pa	2445	2923
R-ITR	2963	3103
F1 ori	3195	3501
Ampicillin	4020	4877
pUC ori	5028	5695
ApaI	2388	
BamHI	1338	
HindIII	2412	
XhoI	1673	

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251 TGGCTGACCG CCCAACGACC CCCGCCATT GACGTCAATA ATGACGTATG
301 TTCCCATAGT AACGTCAATA GGGACTTTC ATTGACGTCA ATGGGTGGAG
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Appendix 2.

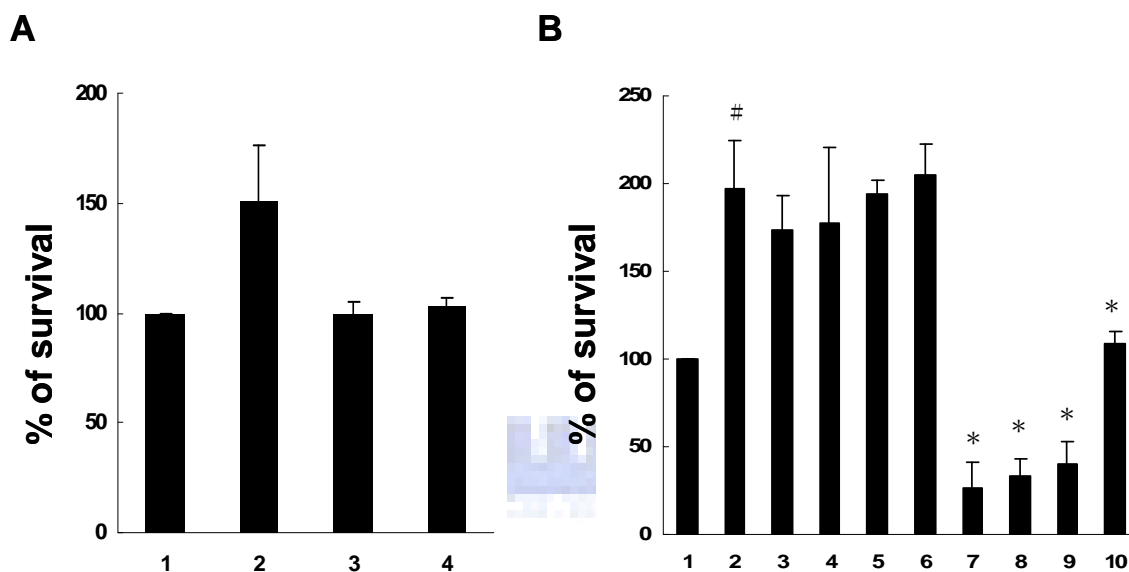


Figure 1. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of HUVECs in a dose-dependent manner. (A) HUVECs were incubated with chimeric proteins for 72 hours and proliferation profile was determined by 5 mg/ml MTT. Lane 1, PBS. Lane 2, VEGF (10ng/ml). Lane 3, IgG1 Fc (2.5 µg/ml). Lane 4, RBDV-IgG1 Fc (2.5 µg/ml). (B) Before stimulation with VEGF, HUVECs were pre-cultured in the presence of 15, 10, 5, and 1 µg/ml of IgG1 Fc (Lane 3, 4, 5, and 6, respectively) or 15, 10, 5, and 1 µg/ml of RBDV-IgG1 Fc (Lane 7, 8, 9, and 10, respectively) for 1 hour at room temperature in M199 medium containing 20% FCS and 25U/ml heparin, and then supplemented with VEGF (8 ng/ml). Cell proliferation was then measured on the basis of MTS as described under “Materials and Methods”. The data shown here are the mean of proliferation inhibition percentages \pm SD obtained from two independent experiments (n=4). The VEGF (Lane 2, 8 ng/ml) stimulated a significant increase in endothelial cell proliferation as compared to the control, PBS (Lane 1; # $P=0.021$). HUVECs growth were significantly inhibited by RBDV-IgG1 Fc when compared with VEGF or IgG1 Fc group (* $P<0.05$).

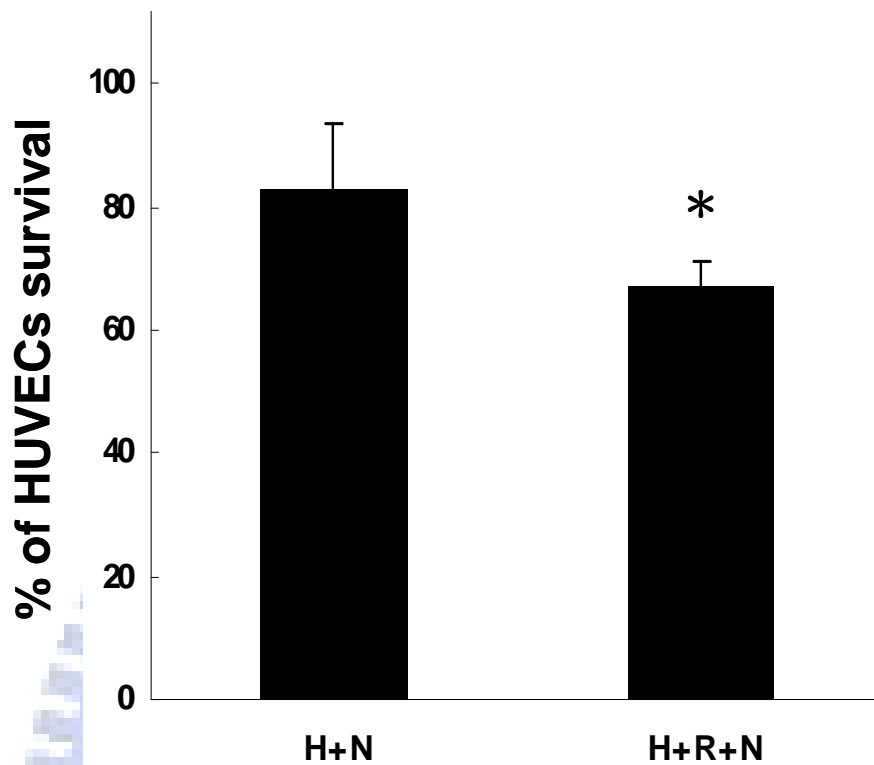


Figure 2. IL-2 activated human NK cytotoxicity. IL-2 activated human NK-92MI cells were co-incubated with HUVECs in the presence of RBDV-IgG1 Fc at an E/T ratio of 1 for 5 hours. Specific lysis was determined by MTS as described in materials and methods. H: HUVECs. N: NK-92MI cells. R: RBDV-IgG1 Fc. The data represent a mean \pm SD of two independent experiments (n=4). * P <0.05 when compare with H+N group.