CHAPTER ONE

INTRODUCTION

1.1 Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels, rather than through *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells. This involves three steps in angiogenic sprouting: vasodilation, endothelial permeability and periendothelial support (for review, see Carmeliet 2000). Angiogenesis first initiates with NO-induced vasodilation, followed by VEGF-stimulated vascular permeability. And then, they need to loosen interendothelial cells contacts and to relieve periendothelial cell support for migration. Once the path has been cleared, proliferation and migration of endothelial cells will occure.

History of tumor angiogenesis research can be traced back to over two centries ago, since pathologists in German observed that tumor are highly vascularized (for review, see Ferrara 2002). Under normal condictions, capilliaries, the tiny blood vessels, do not increase in size or number, because the endothelial cells that line theses narrow tubes do not divide (Folkman 1996). However, endothelial cells in tumors are abnormal in many ways: they are mutilayered, protrude extensions bridging and splitting vessels, show relatively uncontrolled permeability and undergo constant remodeling (Maniotis, Folberg et al. 1999). In fact, angiogenesis appears to be one of the crucial steps in solid tumors, because the process transforms a small (~2 mm in diameter), harmless cluster of abnormal cells into a large, maligant growth, and even has ability to spread to other organs.

Currently, cancer research has been focused on the ability of tumors to induce new blood

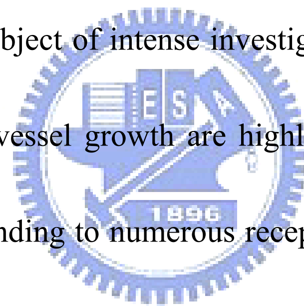
vessel formation since Folkman initiated angiogenesis hypothesis many decades ago. The molecular basis of blood vessel growth is now gradually understood, therefore angiogenesis has been significant in inspiring many investigators. Moreover, angiogenesis is also implicated in the pathogenesis of a variety of disorders; proliferative retinopathies, tumors, rheumatoid arthritis (Folkman 1995).

In 1971, Folkman proposed that anti-angiogenesis might be an effective anticancer strategy. In the following years, Folkman and collaborates initiated efforts aimed at the isolation of tumor angiogenesis factor from human and animal tumors (Folkman, Merler et al. 1971). And ever since then, the so called angiogenesis factors, including epidermal growth factor (EGF), TGF-alpha, TGF-beta, TNF-alpha, angiogenin, etc., were reported (Folkman and Klagsbrun 1987). Yet, much of the attention was directed toward two related potent angiogenic factors, acidic and basic fibroblast growth factors (aFGF and bFGF). However, an unexpectd finding was that the genes for both aFGF and bFGF do not encode for secretory signal peptides, which indicates that these factors are mostly cell associated (Gospodarowicz, Ferrara et al. 1987).

1.2 Vascular endothelial growth factor (VEGF)

In 1989, Ferrara and his colleagues reported the isolation of a diffusible endothelial cell specific mitogen from medium condiction by bovine pituitary follicular cells, which named

vascular endothelial growth factor (VEGF) to reflect the restricted taret cells specificity of this angiogenic factors. (Ferrara and Henzel 1989). VEGFA165 is a heparin binding glycoprotein with a single glycosylation site (at Asn75) and is secreted as a homodimer of ~45kDa. Also, the structure shows that VEGF forms an antiparallel homodimer covalently linked by two disulfide bridges between Cys51 and Cys60 (Muller, Li et al. 1997). Therefore, this finding that 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. For more than about two decades, the role of VEGF in the regulation of angiogenesis has been the object of intense investigation. Although literture that has been published indicades that new vessel growth are highly complex and coordinated processes, requiring a serial of ligands binding to numerous receptors (Yancopoulos, Davis et al. 2000); however, VEGF is still known to be central to angiogenesis.



1.2.1 Biological activities of 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 (Helmlinger, Yuan et al. 1997). Here, VEGF and its receptors play a vital role in increasing lumen formation, and vessel length. Furthermore, tumor cells usually respresent the main source of VEGF, though several studies have shown that tumor-associated stroma is also a site

of VEGF production. (Fukumura, Xavier et al. 1998; Gerber, Kowalski et al. 2000; Tsuzuki, Fukumura et al. 2000).

So far, ther are many studies indicating that VEGF is a survival factor for endothelial cells, both *in vitro* and *in vivo*. *In vitro*, VEGF prevents endothelial apoptosis induced by serum starvation, which is mediated by the phosphatidylinositol 3-kinase (PI3 kinase)/Akt pathway (Gerber, Dixit et al. 1998). Also, VEGF has the ability to promote growth of vascular endothelial cells derived from arteries, veins, and lymphatics. VEGF promotes angiogenesis in tridimensional *in vitro* models, inducing confluent microvascular endothelial cells to invade collagen gels and form capilliary-like structures (for review, see Ferrara and Davis-Smyth 1997). Moreover, VEGF elicts a pronounced angiogenesis response in a variety of *in vitro* models including the matrigel plug in mice (Mesri, Federoff et al. 1995). In particular, many tumor cell lines secrete VEGF *in vitro*, suggesting the possibility that this diffusible molecule may be a mediator of tumor angiogenesis (Ferrara, Houck et al. 1992). *In vivo*, the pro-survival effects of VEGF are developmentally regulated. VEGF inhibition results extensive apoptotic changes in the vasculature of neonatal mice (Gerber, Hillan et al. 1999). For more than a decade, *in situ* hybridization studies have demonstrated that VEGF mRNA is expressed in many tumors, including lung, breast, gastrointestinal tract, renal and ovarian carcinomas (for a review, see Ferrara, Hillan et al. 2004).

In the present studies, VEGFA and its receptors are the best characterized signalling

pathway indevelopmental 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 (Ferrara, Gerber et al. 2003).

1.2.2 Properties of VEGF gene and isoforms

In mammals, the VEGF family consists of five members, VEGFA, B, C, D and placenta growth factor (PLGF), whereas VEGFC and VEGFD were involved in lympagenesis. All of the five VEGFs are secreted as dimeric glycoprotein (Olsson, Dimberg et al. 2006). VEGFA is purified to sequenced and cloned by Ferrara and collaborates. Alternative exon splicing of human VEGFs gene shows that its comprises eight exons , denoted VEGFA121, VEGFA145, VEGFA165, VEGF165b, VEGFA189 and VEGFA206, gives rise to isoforms with different length of amino acids, and biological activities (Olsson, Dimberg et al. 2006). VEGFA189 retains coding full sequence with the secretion signal, whereas VEGFA165 lacks exon 6 and VEGFA121 lacks the residues encoded by exon 6 and 7 (see reviews, Ferrara, Gerber et al. 2003).

VEGFA165 is a haparin-binding homodimeric glycoprotein of 46 kDa. Structurally, VEGFAs with intrachain and interchain disulfide bonds between eight cysteine residues an conserved positions. Anti-parrallel homodimer covalently linked by two disulfide bridges between Cys-51 and Cys-60 (Muller, Li et al. 1997). VEGFA165 is secreted, but significant

fraction remains bound to the cell surface and extracellular matrix, by virtue of its heparin-binding properties. The main focus in the thesis is VEGFA165 (or generally denoted VEGF).

1.2.3 Regulation of VEGF gene expression

Oxygen tension has been indicated of a key role in regulating the expression of a variety of genes. This section, however, is focus on the path of VEGF.

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 (Semenza 2003). Importantly, other study has implicated the PI3 kinase/Akt pathway in the regulation of HIF-mediated reponses in a hypoxia-independent manner. Mutations in *Akt* also results in increased activation of HIF-1 and increased VEGF transcription (Li, Yen et al. 1997). 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* (Okada, Rak et al. 1998). Several major growth factors, including epidermal growth factor, TGF-alpha, TGF-beta, FGF and PDGF, also up-regulate VEGF mRNA expression (Pertovaara, Kaipainen

et al. 1994), 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-6 also induce expression of VEGF in several cell types in aggerment with the hypothesis that VEGF may be a mediator of angiogenesis in inflammatory disorders (Cohen, Nahari et al. 1996).

Taken together, VEGF upregulation in tumors is not only linked to hypoxia but a number of transforming events as well.

1.3 VEGF receptors

The key experiments towards a deeper understanding of the angiogenesis were the discoveries of two VEGF receptors. Fong and Shalaby with their collaborates reported that when the gene of VEGFR-1 (Fong, Rossant et al. 1995) or VEGFR-2 (Shalaby, Rossant et al. 1995) were knockout result in embryonic lethality.

VEGF binds two highly related receptor tyrosine kinases (RTK), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig)-like domains in the extracellualar domain, a single-transmembrane region, and a consensus tyrosine kinase sequence. A member of the same family of receptor tyrosine kinase is VEGFR-3, which, however, is not a receptor for VEGF, but instead binds VEGFC and VEGFD (Pajusola, Aprelikova et al. 1992). As a result, VEGFR-3 is important for lymphatic endothelial cell

development and function. In addition to these receptor tyrosine kinases, VEGF interacts with a family of coreceptors, the neuropilin (NP).

VEGFRs share similar regulatory mechanisms with well-characterized receptor tyrosine kinases, by which include receptor dimerization and activiation of the tyrosine kinase. Moreover, VEGFRs perform cellular processes that are commom to many growth factor receptors such as cell survival and proliferation.

1.3.1 VEGFR-1

VEGFR-1 or Flt-1 (fms-like-tyrosine kinase-1) was the first discovery of VEGF receptor (de Vries, Escobedo et al. 1992). The VEGFR-1 tyrosine kinase exhibits all the conserved motifs that are required for kinase activity. The crystal structure of part of the extracellular domain of VEGFR-1, alone and incomplex with lignad, shows that the Ig domain-2 is the major ligand binding site on the receptor in physiology and pathology. VEGFR-1 binds not only VEGFA but also PIGF and VEGFB. The crucial role of VEGFR-1, as mentioned above, was determined by Fong, 1995, which reveled 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 (Kendall and Thomas 1993). 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 (Carmeliet, Moons et al. 2001).

In some cases, VEGFR-1 is expressed by tumor cells and may mediated a chemotatic signal, thus potentially extending the role of this receptor in cancer growth. For instance, Wu and collaborates indicated that VEGFA autocrine growth activity is acquired by certain human breast tumor cell lines defined by expression of VEGFR-1 (Wu, Hooper et al. 2006).

Anchoring of the extracellular domain of VEGFR-1 to the cell membrane is importmant, as 50% of the mice that lack both of the tyrosine kinase domain and the transmembrane domain died at embryonic stage, owing to vascular malformation (Hiratsuka, Kataoka et al. 2005). This study indicates that endothelial cells develop but fail to organize in vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and leathality, indicating that, at least during early development, VEGFR-1 is a negative regulator of VEGF action (Fong, Zhang et al. 1999). On the contrary, many evidence also indicated that VEGFR-1 is a positive regulator of monocyte and macrophage migration.

The significance of VEGFR-1 in the regulation of angiogenesis is more complex and the precise functon of VEGFR-1, however, is still the object of debate.

1.3.2 VEGFR-2

When the discovery of VEGFR-1 was lethality with mice embryo, in the meantime, Shalaby poposed that VEGFR-2 has the same characteristics. 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 (Fuh, Li et al. 1998). VEGFR-2 undergoes RTK dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic, and prosurvival signal in endothelial cells.

Although the VEGFRs are primarily expressed in vascular system, and the demostration that the VEGF high-affinity binding sites were largely restricted to the vascular endothelium, however, many rescerch have shown that VEGFR also expressed in non-endothelial cells. For example, Dias *et al*. proposed that autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration (Dias, Hattori et al. 2000).

There are many studies indicated that VEGFR-2 is the major mediator of the mitogenesis, survival, and permeability enhancing effects of VEGF-A in endothelial cells (for review, see Ferrara 2004). VEGF binds to VEGFR-2 and stimulates activation of ras in HUVECs. Also, ras activation has been coupled to an angiogenic phenotype of endothelial cells. The early finding that binding 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 (Byzova, Goldman et al. 2000). Furhermore, VEGFR-2 activation has been shown to be required for the antiapoptotic effects of VEGF for human umbilical vein endothelial cells (HUVECs) (Gerber, McMurtrey et al. 1998).

So far, there is now general agreement that VEGFR-2 is the major mediator of mitogenic, angiogenic, and permeability-enhancing effects of VEGF.

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. Neuropilin-1 (NP1), as its name suggest, is a molecular that implicated in neuronal guidelince and had been previously shown to bind the collapsin/semaphorin family. NP1 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 mannar that potentiates VEGFR-2 signalling (Soker, Takashima et al. 1998). This result shows that neuropilin stabilizes the VEGF/VEGFR-2 signalling complex when expressed on adjacent cells.

1.4 Angiogenesis inhibitors

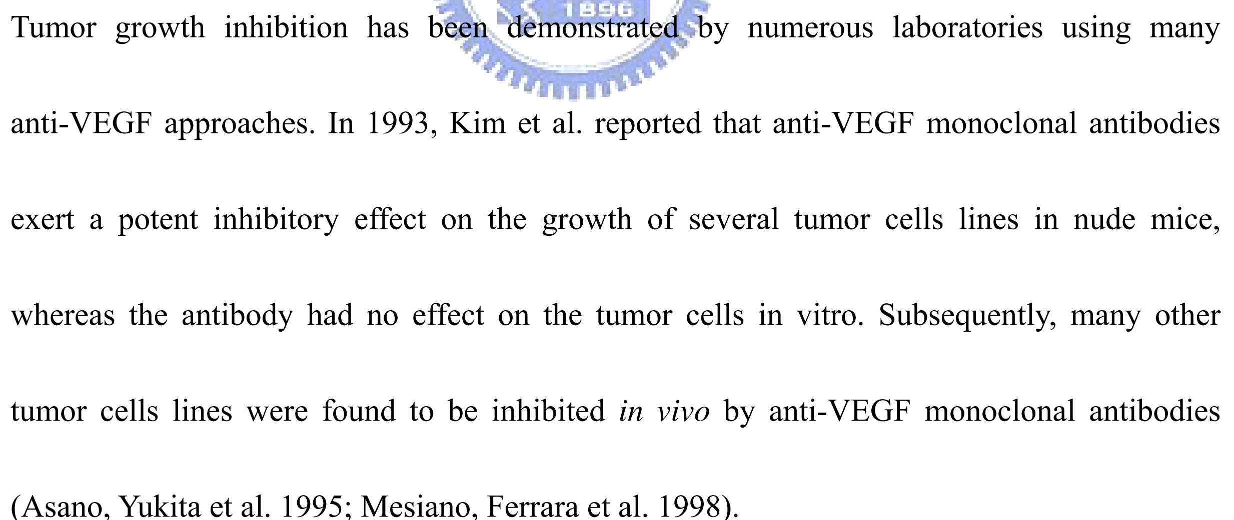
At present, patients diagnosed with cancer typically rely on surgery or radiation to eradicate the tumor and on follow-up radiation or chemotherapy, or both, to try to destroy tumors. In 1971, Folkman proposed that inhibition of angiogenesis was a strategy to treat cancer, and ever since than it initiates the inhibitors that associated with angiogenesis. In 1989, interferon alpha, the first clinical trail of an antiangiogenic agent which decreases production of the angiogenic protein FGF made by tumors, began for the treatment of life-theatening hemangioma. By the following years, TNP-470, a low molecular weight agent which selectively inhibits proliferation and migration of endothelials, was approved by the Unite States Food and Drug Administration (FDA) in clinical trails for a wide variety of cancers in clinics. Also, there are many strategies toward this field, for example, inhibition of the influx of calcium into cells to suppress proliferation of endothelial cells (Faehling, Kroll et al. 2002) or inducement of inflammation in tumors, which destroying growing capilliaries (Yakes, Wamil et al. 2000).

Antiangiogenesis therapy does not attempt to target tumors. Instead, its by interfering with the expanding network of blood vessels and by attacking blood supply in tumors. So far, many angiogenesis inhibitors are proved successfully in vivo and in clinical trails (for an extensive details on the current status of anti-angiogenesis therapy in clinical trails, see National Cancer Institute official website: http://www.cancer.gov/). In this thesis, we focus on

a number of strategies as well as details to target VEGF signalling.

1.5 Strategies to inhibit VEGF signaling

VEGF is now general considered a central in the process of angiogenesis. Apart from the molecular interations that are discussed above, several different strategies have been designed to target VEGF/VEGFR signal trasduction, including a humanized anti-VEGF monoclonal antibody, anti-VEGFR-1 antibody, anti-VEGFR-2 antibody, small molecules inhibiting VEGFR-2 signal transduction, and a VEGFR chimeric protein (Ferrara and Kerbel 2005).



1.5.1 Anti-VEGF antibody

Tumor growth inhibition has been demonstrated by numerous laboratories using many anti-VEGF approaches. In 1993, Kim et al. reported that 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. Subsequently, many other tumor cells lines were found to be inhibited *in vivo* by anti-VEGF monoclonal antibodies (Asano, Yukita et al. 1995; Mesiano, Ferrara et al. 1998).

Clinically, the most successful so far is the humanized monoclonal anti-VEGF antibody, bevacizumab or Avastin, which was approved by the FDA in early 2004 for the treatment of clororectal cancer. Furthermore, Willett and collaborates have shown that VEGF blockade

with bevacizumab decreases tumor perfusion, vascular volumn, microvascular density, interstitial fluide pressure and the number of viable circulating endothelial and progenitor cells in colorectal cancer patients (Willett, Boucher et al. 2004).

Anti-VEGF approches act by blocking tumor associated angiogenesis, which appears to be widely required by many different types of tumors. Thus, these approaches may be usefully against a wide assortment of cancers.

1.5.2 Anti-VEGFR-2 antibody

Besides bevacizumab, several other anti-VEGFR-2 inhibitors are being clinically pursued. In 1999, Prewett and their collaborates reported anti-Flk (fetal liver kinase 1, VEGFR-2 in mouse) monoclonal antibody. Their results show that blockade of the Flk-1 receptor by systemic administration of the monoclonal antibody inhibits angiogenesis in *in vivo* models and the growth of several mouse and human tumors (Prewett, Huber et al. 1999). Furthermore, 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.

Altogether, these finding are consistent with the reduction in tumor vessel density in response to anti-VEGFR-2 antibodies treatment. Furthermore, the use of anti-VEGFR-2 therapy in combination with conventional chemotherapy, radiotherapy or immunotherapy may

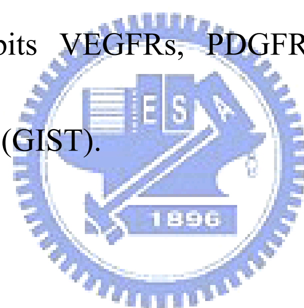
improve the efficacy of these antibody. Because VEGF has been shown to act as a survival factor for endothelial cells in response to radiation (Katoh, Tauchi et al. 1995).

1.5.3 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 monoclonol antibody highlights the need to optimize blockade of this pathway. In 2002, Holash and collaborates 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 varient, 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 (Holash, Davis et al. 2002). 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 (Economides, Carpenter et al. 2003). Luckily, VEGF-trap is also undergoing clinical development as an anti-cancer agent and is in Pase II/III trails for ovarian cancer.

1.5.4 Small molecular drugs

A variety of small-molecule receptor tyrosine kinase inhibitors targeting the VEGF receptors have been developed. These receptor tyrosine kinase inhibitors generally target several kinases in addition to VEGFR-2, possibly directly inhibiting tumor cell proliferation or survival. The most advanced are Sunitinib and Sorafenib (Smith, Mamoon et al. 2004). Sorafenib (BAY43-9006) is a multitargeted kinase inhibitor, including VEGFR-2, PDGFR-beta, Flt-3 and c-kit, which have been tested in large phase III clinical trails for treatment of metastatic kidney cencer. The FDA-approved Sunitinib (SU11248) also targets multple kinases, which inhibits VEGFRs, PDGFR, c-kit and Flt-3, for treatment of gastro-intestinal stromal tumor (GIST).



ES
1896

1.5.5 Others

In 1998, Ruckman proposed that an aptamer that interacts with the heparin-binding domain of VEGFA165. Aptamers were nucleic acids that can be highly potent antagonists of enzyme catalysis or of specific protein-protein interactions. In their studies, they have isolated and modified 2'F-pyrimidine RNA neucleotide ligands by using the SELEX (systematic evolution of ligands by expotential enrichment) process. The results show that these aptamers bind equally well 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 Antibody based therapy

Monoclonal antibodies have become the most rapidly expanding class of pharmaceuticals for treating a wide variety of human disease. Antibodies are Y shaped dimeric structure with two identical heavy (H) and light (L) chains. The variable (V) regions of the H and L chains form a antigen-binding site that engage an epitope on the target antigen. At present, more than one quarter of pharmacological agents that are currently under development are based on antibodies (Glennie and van de Winkel 2003). And most of these clinically approved drugs are directly targeting tumor cells, including experimental antibodies.

Currently, key therapeutic antibody technologies include murine antibodies, chimeric antibodies, humanized antibodies, and human antibodies. Murine antibodies are derived from hybridoma technology; chimeric antibodies are obtained by joing the antigen-binding variable domains of a mouse mAb to human constant domain; humanized antibodies are created by grafting the complementarity-determing regions (CDRs), an antigen binding loop, from a mouse mAb into a human IgG. About high affinity human antibodies, they are obtained from

transgenic mice that have had their endogenous antibody genes replaced by the equivalent human sequences. Therefore, immunization elicts the production of human antibodies recoverable using hybridoma technology (Carter 2001).

Humanization and human antibodies are now the prefered technologies for developing antibodies as therapeutics because they display long half-life and show little, if any, immunogenicity (Glennie and van de Winkel 2003). Humanization is a clinically well-validated technology that might be favoured if a well-characterized mouse mAb is avaliable. By contrast, direct routes to human antibodies offer faster preclinical development in cases with no existing mouse mAb. However, the choice of different human antibody technologies will depend on their availability.

For cancer therapies, promising and potentially alternative strategies to direct tumor targeting are focus on targeting tumor vascular, angiogenic growth factors and their receptor as discussed previously.

1.6.1 Immune responses induced by Fc fragement

Numerous strategies for improving the killing capacity of mAbs have been investigated. Human antibodies of IgG1 and IgG3 isotypes can potentially support the effector functions of antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Tumor cell killing by ADCC is triggered by the interation between the Fc region of an

antibody bound to a tumor cell, and the Fcγ receptors on immune effector cells, such as neutrophils, macrophages and natural killer (NK) cells. Among these receptors, FcγRIIIa is predominant FcγR on NK cells (Clynes, Towers et al. 2000; Cartron, Dacheux et al. 2002). 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 recruting the immune system. This is because the Fc regions of IgG1 interact strongly with all types of FγR on lymphoid and myeloid effectors and are strong complement activators (Cragg, French et al. 1999; van Dijk and van de Winkel 2001).

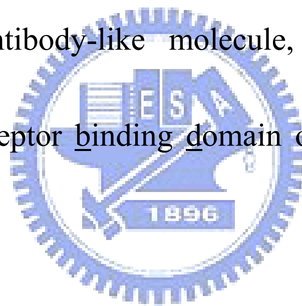
Several mouse and clinical studies indicate that ADCC is an important therapeutic mechanism of clinically effective antibodies. Supporative evidence for this interpretation is found in the studies of FDA approved Herceptin (trastuzumab), a humanized IgG1 antibody targeted at the growth factor HER-2/neu for the treatment of HER-2 positive breast cancer. Clynes et al. showed that engagement of Fcγ receptor on the effector cells is a dominatant component of the in vivo activity of antibody against tumors, because the antitumor activity of Herceptin was greatly reduced in mice that lack the activation receptors FcγRI and FcγRIII, whereas disruption of the gene that encodes the inhibitory receptor FcγRIIB subtantially enhanced antitumor activity (Clynes, Towers et al. 2000). Also, Kirschfink indicated that the essential mechanisms responsible for the antitumor activities of Herceptin and its mouse parent IgG antibody against HER2 *in vivo*, are due to receptor-ligand blockade (Kirschfink

2001). Similar mechanisms were found in the ability of Rituxan (rituximab), an antibody against the CD20 antigen on B cells, which arrests the growth of non-Hodkin's lyphoma (Press, Leonard et al. 2001).

These results demonstrate that Fc receptor dependent mechanisms contribute subtantially to the action of cytotoxic antibodies against tumors and indicate that an optimal antibody against tumors would bind preferentially to activation Fc receptors.

1.7 Research Rationale and Objectives

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



VEGF is highly specific mitogen promoting the formation of blood vessels in pathological angiogensis of tumor growth. This biological properties make VEGF 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 (Keyt, Nguyen et al. 1996; Muller, Li et al. 1997), which amino acids lined between 8 and 109 of full VEGFA165.

VEGFA165, a antiparallel homodimer, engages VEGFR-2 using two symmetrical

binding sites located at opposite ends of the molecule; each site is composed of two “hot spot” for binding that consist of residues presented for binding both subunits of the homodimer (**Figure 1**). These two hydrophobic hot spots are resudues Phe-17, Ile-43, Ile-46, Ile-83, and Pro-85, as well as the three important polar residues, Glu-64, Glu-79, and Lys-84. In addition, the crystal structural of VEGF8-109 has been determined in a complex with a soluble VEGFR-1 loop-2 fragment (Wiesmann, Fuh et al. 1997). Furthermore, structural and functional studies have yielded insights into how VEGF displays different receptor binding sites for VEGFR-1 and VEGFR-2 (as shown in **Figure 2**). The A site, composed of basic residues (amino acids 82-86), mediates the binding to VEGFR-2. The B site, composed of acidic residues (amino acids 63-67), mediates the interaction with VEGFR-1. This diagram illustrutes bivalent epitopes at opposite ends of VEGF, suggesting a possible mechanism for ligand-induced dimerization of receptors (Keyt, Berleau et al. 1996).

In the present study, we generated the RBDV-IgG1 Fc fusion proteins**.** This involves the conjugation of the receptor binding domain of VEGF (amino acids 8-109, including leader peptide, amino acid 1-7) to the Fc domain of immunoglobulin. Here, the IgG1 subclass was selected because it is arguably the most active in the immune systems, being able to engage receptors on cytotoxic effector cells, called FcγR, and to activate complement and destroy target cells, effectively (Gorter and Meri 1999). The Fc component contains the hinge region, CH2 domain, and CH3 domain, but not the CH1 domain of IgG. In particular, the hinge and

Fc regions form a convential scaffold and flexibility for presenting functional RBDV domain. Furthermore, Fc provides a ready means for detection of the expression of chimeric receptors. Typically, an anti-human IgG antibody against the IgG Fc is used. Importantly, loss of the heparin-binding domain, the amino acids 111-165, results in a reduction in the mitogenic activity of VEGF (Keyt, Berleau et al. 1996).

Hence, RBDV-IgG1 Fc is designed as an antagonist, which blocks the interaction of natural VEGF ligands to its receptors, VEGFR-1 and VEGFR-2.

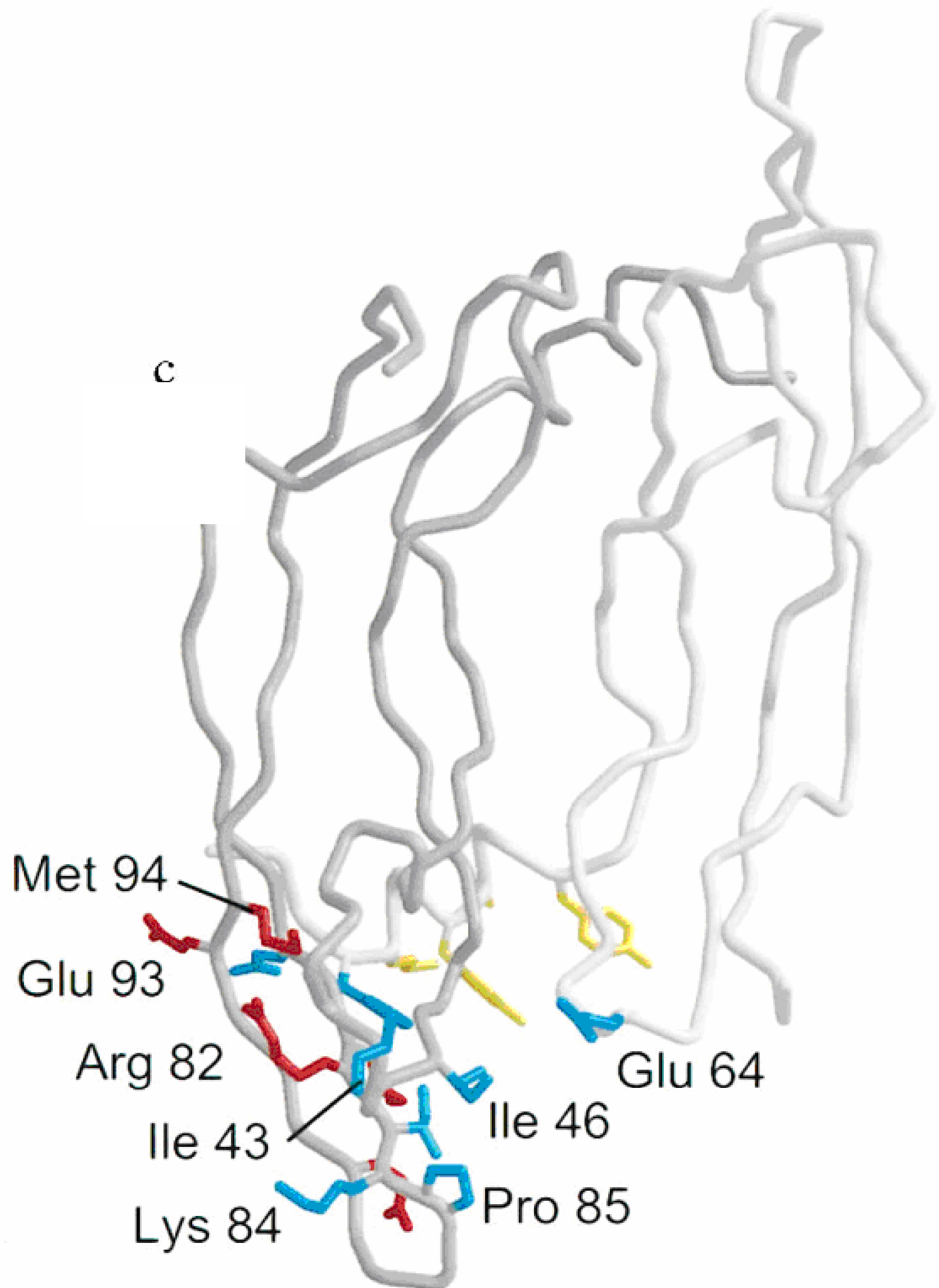


Figure 1. Tube representation of the dimeric structure of the receptor binding domain of VEGF 8–109. (Source: Muller, et al. 1997)

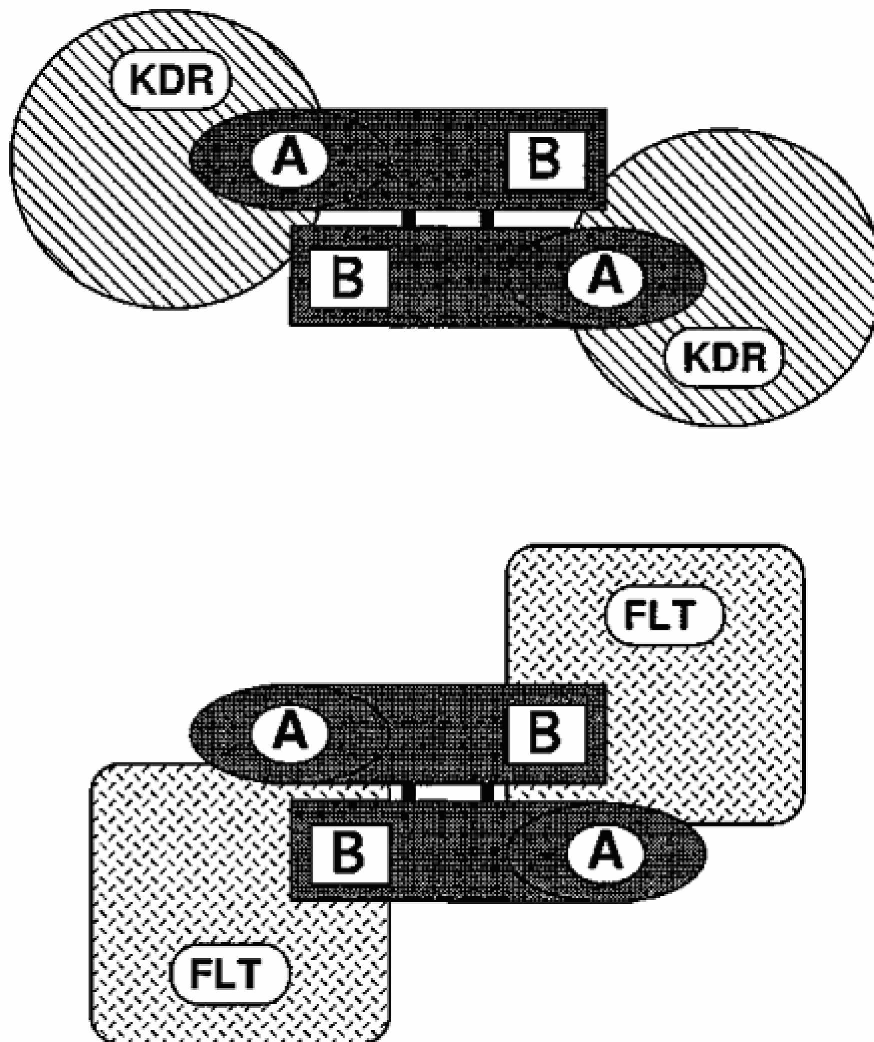


Figure 2. Diagram of different receptor binding sites of VEGF for KDR (VEGFR-2) and FLT-1 (VEGFR-1). The A site, composed of basic residues (residues 82–86), mediates the binding to KDR. The B site, composed of acidic residues (residues 63–67), mediates the interaction with FLT-1 receptor. (Source: Keyt, et al. 1995)

CHAPTER TWO

MATERIALS & METHODS

2.1 Materials

2.1.1 Chemicals

Table 1: List of all chemicals used in the experiments

Chemical	Company	Catalog #
100 bp DNA ladder	Protech	M1-100T
1kb DNA ladder	Protech	M1-1KB
Acetic acid	TEDIA	AS-1102
Acrylamide:N,N'-Methylenebisacrylamide 29:1 solution	Fluka	01708
Agar	AMRESCO	J637
Albumin bovine Fraction V (BSA)	MP	160069
Ammonium persulfate	SIGMA	A9164
Ampicillin	AMRESCO	0339
*Apa*I (restriction enzyme)	Promega	R6361
ATP	EPICENTRE	L0805
*Bam*HI (restriction enzyme)	Fermentas	ER0051
Beta-mercaptoethanol	AMERSCO	0482
Bromophenol blue	ICN biochemicals	101123
Coomssie Brilliant blue	AMERSCO	0472
DMSO	MP	196055
dNTP	Promega	01151
Dulbecco's modified Eagle's medium (DMEM)	SIGMA	D5648
Endothelial cell growth supplement (ECGS)	SIGMA	E 0760
EDTA	Tedia	ER-0531
EtBr	AMRESCO	3434B14
Ethanol	SIGMA	E7023

Fetal Bovine Serum	Biological industries	04-001-1A
Folic acid	SIGMA	F8758-5G
Gelatin	SIGMA	G9391
Glycerol	Showa	0706-0150
Glycine	AMERSCO	0167-1KG
Haparin sodium salt	SIGMA	H3149
HCl	Scharlau	AC0741
*Hin*dIII	Fermentas	ER0501
HisTrap affinity column	Amersham Biosciences	17-5247-01
Horse serum	GIBCO	
Imidazole	Acros Organics	122025000
Isopropanol	C-Echo	PH-3101
Luria Bertani (LB) agar	AMRESCO	J637
Luria Bertani (LB) broth	Scharlau	02-385
Matrigel (growth factor reduced)	BD Biosciences	354230
Medium 199	Gibico	12350039
Minimum Essential Medium Eagle	SIGMA	M0894
Methanol	ECHO chemical	MA-1101
MTT	SIGMA	522333
MTS	Promega	G3580
Myo-inositol	SIGMA	I7508-50G
NaCl	AMRESCO	0241
$NaHCO_3$	MP	194847
NaH_2PO_4	Showa	1932-8250
Na_2HPO_4	scharlau	SO 0329

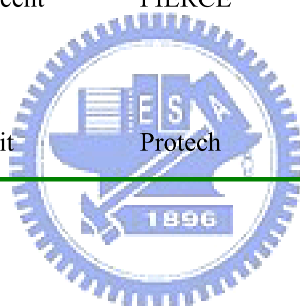
NaOH	Showa	1943-0150
Penicillin-streptomycin amphotericin B (PSA)	Biological Industries	03-033-1B
Prestained protein ladder	Fermantas	SM0617
Pfu polymerase	MDBio	826049
Propium iodide (PI)	SIGMA	P4170
Protein G agarose	Upstate	16-266
Protein molecular weight marker	Fermantas	SM0431
RPMI	GIBCO	31800-022
VEGF	Upstate	01-185
VEGF R1/Flt-1 (aa 27-328/Fc chimera)	R&D systems	3516-FL
VEGF receptor 2 D1-D7	Merck-Calbiochem	676490
Sephadex G-25	Amersham	17-0033
Sodium dodecylsulfate (SDS)	SIGMA	L4390-250G
SuperSignal West Pico Chemiluminescent Substrate	PIERCE	34080
T4 ligase (10U)	Epicentre	L0805H
Taq polymerase	BioKit	Bio Taq
Taq DNA polymerase XL	Protech	P6a
TEMED	Bio-Rad	161-0800
Tris base	MDBio	101-77-86-1
Tris-HCl	MDBio	316029
TMB substrates	KPL	
Trypan blue stain	GIBCO	0759
Trypsin	GIBCO	27250-018
Tryptone	Pronadisa	1612.00
Tween 20	MP	194724

Yease extract	Pronadisa	1702.00
*Xho*I (restriction enzyme)	Fermentas	ER0691

2.1.2 Kits

Table 2: Kits used in this study

Kit	Company	Catalog #	Used in
Geneaid gel/PCR DNA fragments extraction kit	Geneaid	DF 300	DNA extraction, clean-up
Centricon YM-3	Millipore	4202	Protein concentration
SuperSignal West Pico Chemiluminescent Substrate	PIERCE	34080	Western blot
Gene-Spin^{TM} Miniprep Purification Kit	Protech	PT-MP530XLO-V2	DNA extraction



2.1.3 Primers

Table 3: List of all primers used in this study

Name	Primer Sequence (5′ to 3′)
hGH poly (A)	AAG GCT GGT GGG CAC TGG
β-globin intron	ACA GCT CCT GGG CAA CG
Exp 1101 His-Tag linker F	CGC ATC ATC ACC ATC ACC ATT GAA
Exp 1101 His-Tag linker R	AGC TTT CAA TGG TGA TGG TGA TGA TGC GGG CC

2.1.4 Antibodies

Table 4: Antibodies used in this study

Antibody	Description	Company	Catalog #
Anti-human IgG HRP		SeroTec	STAR106P
Anti-human IgG FITC		Acris antibodies	R1337F
Anti-his tag HRP		Novus Biologicals	NB 600-32

2.1. 5 Cells

Table 5: Cells used in this study

Cell line	Description	ATCC #	Used in (section)
HEK-293	Human kidney epithelial cell	CRL-1573	2.2.7
NK-92MI	Human natural killer cell	CRL-2408	2.2.15
HUVEC	Human umbilical vein endothelial cell Primary cell culture	Kindly provided by Dr. Ko-Jiunn Liu , National Health Reserch Institutes, Taiwan	2.2.12；2.2.13；2.2.14；2.2.15

2.1.6 Buffers and Media

Table 6: List of all buffers and media used in this experiments

Buffer	Description	Used in
1X PBS	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH7.4	
1.0 M Tris-HCl, pH6.8	12.1 g Tris base in ddwater, adjusted to pH6.8 with 12N HCl	SDS-PAGE
1.5 M Tris-HCl, pH8.8	18.2 g Tris base in ddwater, adjusted to pH8.8 with 12 N HCl	SDS-PAGE

5% Blocking buffer	1 g non-fat powdered milk dissolved in 20 ml 1X PBS buffer	ELISA
10% APS	0.1 g ammonia persulfate disoved in 1mM ddwater	10% APS
10%Running (Separating) gel	12% acryamide/bisacrymide 29:1, 375 mM Tris-HCl, pH8.8, 0.1% SDS, 0.16% APS, 0.1% TEMED	SDS-PAGE
10% Stacking gel	5% acryamide/bisacryamide 29:1, 125MmM Tris-HCl, pH6.8, 0.1% SDS, 0.16% APS, 0.1% TEME	SDS-PAGE
50X TAE buffer	48.4 g Tris base, 0.5 M EDTA (pH8.0) 20 ml, 11.42 ml acetic acid. dd H_2O was added to 200 ml.	Gel electrophoresis
Binding buffer	20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH7.4	HisTrap column
Buffer S1	50 mM Tris-HCl, 10mM EDTA, 100µg/ml RNase A, pH8.0	Midi prep.
Buffer S2	200 mM NaOH, 1% SDS	Midi prep.
Buffer S3	2.8 KAc, pH 5.1	Midi prep.
Buffer N2	100 mM Tris,15% ethanol, 900 mM KCl, 0.15% Triton X100, adjusted to pH 6.3 with H_3PO_4	Midi prep.
Buffer N3	100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH6.3 with H_3PO_4	Midi prep.
Buffer N5	100 mM Tris, 15% ethanol, 1M KCl, adjusted to pH 8.5 with H_3PO_4	Midi prep.

Destain solution	30% methanol, 10% acetic acid	SDS-PAGE
DMEM medium	10% FBS, 1% PSA in Dulbecco's Modified Eagle's Medium	Cell culture
EDTA-trysin	2.5 g trypsin, 0.1 M EDTA (pH8.0) in 1L 1X PBS, pH7.4, 0.2 µm filtered	Cell culture
Elution buffer	50 mM Glycine-HCl, pH 2.7	Protein G column
Elution buffer	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH7.4	HisTrap column
LB (Luria-Bertani) broth	1% tryptone, 0.5% yeast extract, 1% NaCl	Cloning
LB (Luria-Bertani)/Ampicillin broth	1% tryptone, 0.5% yeast extract, 1% NaCl, 50µg/ml ampicillin	Cloning
LB (Luria-Bertani)/Ampicillin agar	1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50µg/ml ampicillin	Cloning
Neutralization buffer (NB)	1M Tris-HCl, 1.5 M NaCl, 1mM EDTA, pH8.0	protein G column
PBST	0.5% Tween 20 in 1x PBS	ELISA
Running Buffer	0.3% Tris base, 1.44% glycine, 0.1% SDS	SDS-PAGE
Sample buffer	0.05 M Tris-HCl, pH6.8, 2%SDS.1% glycerol, 1.1%	SDS-PAGE

	beta-mercaptoethanol, 0.01% (w/v) bromophenol blule	
Staining buffer	1% BSA, 0.05% NaN_3 in 1x PBS	Flow cytometry
Staining solution	0.3 % Coomassie Brilliant Blue, 10% acetic acid, 50% methanol	SDS-PAGE
Stop solution	1 N HCl	ELISA
Transfer buffer	48 mM Tris base, 39mM glycine,10%SDS 3.75ml and 20% methanol	Western blot
Versene	0.2g EDTA in 1L 1X PBS	Cell culture
TBS buffer	50 mM Tris-HCl, 150 mM NaCl in 1L d.d. water, pH7.4	Protein G column

2.2 Methods

2.2.1 Construction of the pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc (summary)

The human vascular endothelial growth factor (VEGF) cDNA fragment was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total cellular RNA extracted of A431 cells, using the primers 5′-TGG TGA GAG ATC TGG TTC CCG AAA-3′, and 5′-TTT CGG GAA CCA GAT CTC TCA CCA-3′. A pair of primers was designed to amplify the receptor binding domain of human VEGF (RBDV, amino acids 1-109) cDNA sequence containing *Bam*HI site and another primer containing *Xho*I site (in bold) were as follows: forward primer: 5′–A**GG ATC C**AT GAA CTT TCT GCT GTC TTG G-3′; reverse

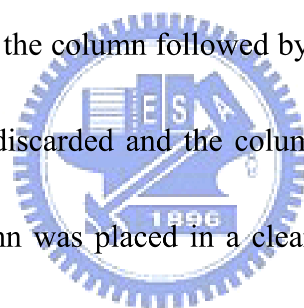
primer: 5′-A**CT CGA G**TT AGA TCC GCA TAA TCT GCA TGG T-5′. The human Fc effector domain contained an IL-2 signal sequence on the pcDNA3.1 expression vector (Invitrogen, USA) was obtained from our lab. The PCR fragments of RBDV were cut with *Bam*HI and *Xho*I, and then ligated to the N terminal of the Fc portion of IgG1, lacking the IL-2 signal sequence, in the pcDNA3.1 expression vector (this had been done by Wan-I Wang) . The fulllength of the fusion constructions, digested with *Bam*HI and *Apa*I, were then sub-cloned into the pAAV/MCS vector (Stratagene, USA), within the poly his-tagged linker at the C terminals, in frame. The poly his tag linkers were designed as follows: forward primer: 5′-CGC ATC ATC ACC ATC ACC ATT GAA-3′; reverse primer: 5′-AGC TTT CAA TGG TGA TGG TGA TGA TGC GGG CC-3′. The full sequence of pAAV/MCS-RBDV-IgG1 Fc was confirmed by sequencing (Protech Technology, Taipei, Taiwan;). For control group, the Fc portion of human IgG1 contained poly his tag in pAAV/MCS was cloned.

2.2.2 IgG1 Fc and RBDV-IgG1 Fc fragements Preparation

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 (Fermantas), supplied with the enzyme and incubated at 37°C. Digestion of plasmid DNA was fractionated by electrophoresis through a 0.8 or 1.5% agarose gel and was

analyzed on a UV table (EZlab, USA) after ethidium bromide (EtBr) staining.

DNA fragment, digested by the dual restriction enzymes, was extracted from a 1% agarose gel containing EtBr with a Geneaid gel/PCR DNA fragment extraction kit with the buffers supplied in the kit according to the manufacturer's instructions. Briefly, the DNA fragment was excised from the gel and weighed (300 mg). 500 μl binding buffer was added toin the gel/buffer mixture and was incubated at 55°C for 15 min with repeated inverting of the tube. The sample was loaded into a DF column, which was placed on a 2 ml collection tube and centrifuged for 30 sec at 13,000 rpm. After discarding the flow-through, 600 μl of washing solution was added to the column followed by centrifugation. This step was repeated twice. The flow-through was discarded and the column was centrifuged for an additional 3 min at 13.000 rpm. The column was placed in a clean 1.5 ml eppendorf and the DNA was eluted with 30 μl Elution buffer by centrifugation for 1 min at maximum speed.

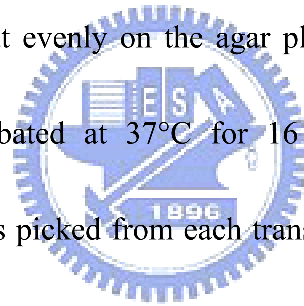


2.2.3 Ligation and transformation

The amount of clean DNA fragment, as a insert, was estimated (150 ng) to ligate with pAAV-MSC vector and His-Tag linker, which is annealed by a gradually decreasing temperature gradient, for a 1:1:1, 3:1:3 insert:vector:linker ratio, respectively. The ligation reaction was a mixture of fresh insert, 10x ligation buffer, vector, T4 DNA ligase (10U/μl) and dd water in a total volumn of 10μl. Finally, the ligation reaction was carried out at 14°C

overnight in a waterbath.

The competent *E. coli* Top 10 strain (Invitrogen, USA) was used for transformation. One vial of stored competent cells (100 μl) for each ligation was thawed and kept on ice. One μl of the ligation reaction were mixed into each vial containing the competent cells by stirring and incubated for 30 minutes on ice. Then, the vials were heat shocked for exactly 90 seconds in a 42°C waterbath and placed on ice for additional 2 min. 250 μl of LB broth medium were added to each tube and the vials were then incubated at 37°C for 50 minutes at 225 rpm shaking incubator; the vials with the transformed cells were placed on ice. 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. For further mimi-preparation experiments, signal colony was picked from each transformation and placed into 3 ml of LB broth medium containing ampicillin (50 μg/ml).



2.2.4 Polymerase chain reaction (PCR) and bacterial *E.Coli* colony PCR

Colony PCR was used to determine the correct ligation products from transformation. A bacterial single colony was picked with an autoclaved pipette tip, transferred it into 3 ml of LB broth, which contains Ampicillin (50 μg/ml), and incubated at 37°C for approximately 14~16 hours. One μl β-globin intron primer and 1μl hrGH poly(A) primer (10μM each) were used for bacterial colony PCR, along with 0.2 μl of bacterial solution, 5 μl 10x Tag

buffer, 1µl dNTPs (25mM), 0.5µl Tag polymerase (5U/µl), and filled with double-distilled (dd) water to 50 µl. PCR cycle conditions were 95°C, 5 min; 34x (94°C, 30 sec; 51°C, 30 sec; 72°C, 2 min); 72°C, 5min. The amplification yields were then checked in an agarose gel using 5 µl of the reaction as a sample. As for PCR screening of the constructs, 20 ng of DNA from Midi-preparation was used. The following steps were the same as described above.

2.2.5 Mini-preparation

Mini-preps of plasmid DNA were carried out with a Gene-Spin^{TM} Miniprep Purification Kit (Protech, Taipei, Taiwan) according to the protocol supplied by the manufacturer. Briefly, two ml of bacteria culture (as described in section 2.2.3) were recovered by centrifugation at 13,000 rpm for 1 min. the bacterial pellet was resuspended in 200 µl Solution I. 200 µl of lysis buffer, Solution II, was added and mixed gently. 200 µl of ice-cold neutralization buffer, Solution III, was added, mixed gently and centrifuged at 13,000 rpm for 5 min. The lysate was transferred to the Mini spin column and spun at 13,000 rpm for 30 sec. 700 µl of Washing solution was added, and washed twice. After the filtrate was discarded, the column was centrifuged at 13,000 rpm for 3 min again, and incubated at 65 °C for further 5 min to remove residue trace of ethanol. Plasmids were eluded with pre-warmed 30µl elution buffer and centrifuged at 13,000 rpm for 1 min.

2.2.6 Midi-preparation

For further midi-preparation experiments, signal colony was picked from each transformation, placed into 100 ml of LB broth medium containing ampicillin (50 μg/ml) and incubated at 37°C for 16 hours at 225 rpm shaking incubator. Then, bacteria culture were centrifuged at 8,000 rpm for 15 min, the supernatant was blotted off, and the bacterial pellet was resuspended in 4 ml of Buffer S1. Four ml of Buffer S2 lysis buffer was added, the sample was gently mixed by inverting 6-8 times, and the bacteria were allowed to lyse for 5 min at room temperature. Four ml of ice-cold Buffer S3 neutralization solution was added. The sample was gently mixed and incubated on ice for 10 min. Then, the mixture was cleared by centrifugation at 10,000 rpm for 25 min at 4°C. The clear lysate was applied onto NucleoBond ion-exchange resin, which was pre-equilibrated with 4 ml Buffer N2. The column was washed 2x with 10 ml Buffer N3. Plasmid DNA was eluted from the column with 5 ml high-salt Buffer N5. The plasmid DNA was precipitated with 0.7 volumes isopropanol, kept on ice for 10 min and centrifugation at 13,000 rpm for 30 min at 4°C in 1.5 ml eppendorf. The DNA pellets were washed with ice-cold 70% ethanol, air-dried and dissolved in 20 μl dd water. The concentration of the DNA was determined by measuring the absorbance at 260/280 nm.

2.2.7 Cell Culture

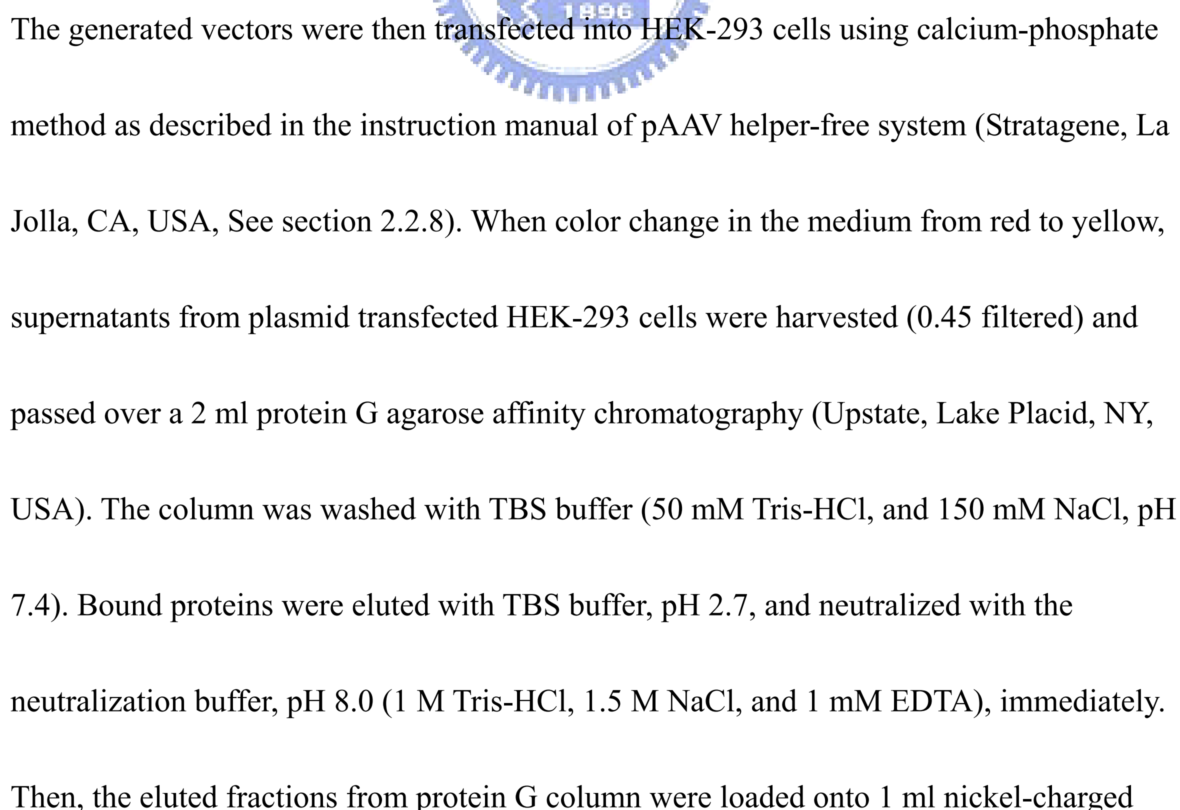
Primary cultures of human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Ko-Jiunn Liu (Nation Health Research Institute, Taipei, Taiwan) and were grown in Medium 199 (Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS; Biological industries, USA), 25 units/ml heparin (Sigma, St Louis, MO, USA) and 30 μg/ml endothelial cell growth supplements (ECGS; Sigma), according to the instructions of the American Type Culture Collection (Manassas, VA). Passages between 3 and 7 were used for the subsequent experiments. Human epithelial kidney (HEK) 293T cells, and human epidermoid carcinoma cells (A431) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Gaithersburg, MD), supplemented with 10% FBS and 1% penicillin-streptomycin amphotericin B (PSA; Biological industries, USA). NK-92MI were grown in minimum essential medium eagle alpha modification (MEM alpha; Sigma) supplemented with 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol(Sigma), 0.02 mM folic acid (Sigma), 12.5% horse serum (Gibco) and 12.5% fetal bovine serum. All the cells were incubated in tissue culture incubator with 5% CO_2 at 37℃.

2.2.8 Transfecting HEK-293 cell

HEK 293 cells were plated and distributed evenly at 5×10^5 cells per 100 mm tissue culture plate in 10 ml of DMEM growth medium. After 48 hours of incubation, 70-80% confluent of

the plates were selected. Pipet 10 μl of desired plasmid solution (1μg/ml) into a 15 ml conical tube containg 1 ml of 0.3M $CaCl_2$, and then mix gently. Meanwhile, peptite 1ml of 2×HBS (280 mM NaCl, 1.5 mM Na_2HPO_4 and 50 mM HEPES, pH7.1) into a second 15 ml conical tube. Then, pipete the 1.03 ml plasmids /$CaCl_2$ mixure into the second tube containing 2×HBS solution dropwise and mix by repeated pipeting. Immediately apply the plasmids/$CaCl_2$/HBS mixture to the plate of cell in a dropwise fashion, swirling gently to distribute the plasmid suspension evenly in the growth medium. After 6 hours of incubation, remove the medium from the plate and replace it with 25 ml of fresh DMEM growth medium.

2.2.9 Expression and purification of chimeric proteins



The generated vectors were then transfected into HEK-293 cells using calcium-phosphate method as described in the instruction manual of pAAV helper-free system (Stratagene, La Jolla, CA, USA, See section 2.2.8). When color change in the medium from red to yellow, supernatants from plasmid transfected HEK-293 cells were harvested (0.45 filtered) and passed over a 2 ml protein G agarose affinity chromatography (Upstate, Lake Placid, NY, USA). The column was 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 with the neutralization buffer, pH 8.0 (1 M Tris-HCl, 1.5 M NaCl, and 1 mM EDTA), immediately. Then, 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 NaH_2PO_4, 0.5 M NaCl, 30 mM imidazole, pH7.4), and eluted with elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH7.4). Buffer was desalted using sephadex G-25 (Amersham Biosciences). The recombinant proteins in PBS were concentrated by the Centricon centrifugal filter unit (Millipore, MA, USA). Protein concentractions were determined by comparing to a BSA standard curve.

2.2.10 SDS-PAGE and Western blot

The supernatants from plasmid-transfected HEK-293 cells of culture medium were collected, and clarified by centrifugation (1500 rpm for 5 min at 4°C), and resulting supernatants were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane (PVDF). Membranes were blocked with 5% nonfat milk in phosphate buffered saline (PBS). Immunoactive bands were all visualized by using horseradish peroxidase-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 (0.5% Tween-20 in PBS) were used for all washing steps. As for purify validation, columns purified proteins were assay by the use of Quantity One software (Bio-Rad, Hercules,

CA, USA).

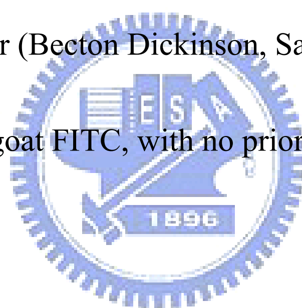
2.2.11 In vitro receptor binding assay

Sandwich enzyme link immunosobant assays (ELISA) were carried out to examine the binding activities of the RBDV-IgG1 Fc. Four μg/ml of the extracellular domain 1-3 of VEGFR-1s (R&D systems, MN, USA) or the extracellular domain 1-7 of VEGFR-2 (Merck-Calbiochem, CA, USA) in PBS were first coated onto the 96-well microtiter plate (Nunc, Denmark) at room temperature overnight, followed by incubating with blocking buffer (1% bovine serum albumin, and 0.05% Tween-20 in PBS) for 1 hour. The plates were washed with PBST three times, and then the purified proteins were applied onto the coated plates. The plates were then incubated with HRP-conjugated anti his-tagged antibodies (Novus Biologicals, Littleton, CO, USA) at room temperature for additional 1 hour, after which the plates were washed three times with PBST. 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.

2.2.12 Cell surface binding assay

Flow cytometry was used to analyze the cell surface binding ability. HUVECs were seeded at a density of 1×10^6 on T75 flask. Viable cell counts were determined by trypan blue dye

exclusion. HUVECs were washed with PBS, detached by 0.2% EDTA in PBS, washed, and resuspended in flow cytometry buffer (1% bovine serum albumin, and 0.05% sodium azide in PBS, pH 7.4). Aliquots of 100 μl containing 3×10^5 cells were distributed in 15 ml centrifuge tube (Corning Inc., Corning, NY, USA). Cells were pre-incubated with 5 μg/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 cytomer buffer and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Negative controls were cells incubated with anti-goat FITC, with no prior exposure to recombinant proteins.



2.2.13 HUVECs proliferation assay

Five thousand human umbilical vein endothelial cells were seeded onto 1% gelatin-coated flat-bottomed well of a 96-well plate (TPP, Switzerland) in assay medium (M199 plus 20% FBS, 25U heparin and 1% PSA), allowed to settled for 16 hour, and incubated with recombinant proteins for 2 hour, then challenged for 70 hr with human $VEGF_{165}$ (Upstate Inc., Lake Placid, NY, USA) at a final concentration of 8 ng/ml. The proliferative response was measured by adding 1.9 mg/ml of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carb-oxymethoxy phenyl)-2- (4-sulfophenyl)-2H-tetrazolium) (Promega, WI, USA) to each well with a further 2

hour of 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.

2.2.14 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 (TPP, Trasadingen, Switzerland), and allowed to polymerized for 2 hours at 37℃. A suspension of 1.5×10^4 HUVECs were incubated with recombinant proteins for 1 hour and passed into each well containing 16 ng/ml of VEGF. As the positive control, HUVECs were incubated with 16 ng/ml of VEGF (Upstate Inc., Lake Placid, NY, USA) alone. Cells were incubated for further 16 hours at 37°C and photographed using a Olympus microscope (40× magnification). Total number of network formations was counted.

2.2.15 NK killing assay

Human umbilical vein endothelial cells were washed with PBS, detached by 0.2% EDTA in PBS, washed, and resuspended in assay medium (M199 with 20% FBS and 25U heparin). Then, target cells were incubated with recombinant proteins at a final concentration of 4 μg/ml for 1 hour. A total of 10,000 target cells per well was used in the assay. During this

incubation time, NK 92-MI effector cells were washed, resuspended at 10,000 cells per well in 100µl of MEM alpha growth medium (as described in section 2.2.7). One hour later, effector cells were mixed with target cells at E/T ratio of 1:1, and distributed on 1% gelatin coated 96-well round-bottom plates (Corning, NY, USA). Plates were centrifuged for 4 minutes at 250×g , and incubated for 5 hours. Specific lysis was determined by MTS. Briefly; plates were washed with M199 medium three times and incubated with 100 µl assay medium with 1.9 mg/ml MTS for further 2 hour. To determine control, target cells were cultured in the absence of effector cells or cultured with NK92-MI cells without recombinant proteins.

2.2.16 Statistic analysis

Statistical analyses were done using SPSS statistics software (SPSS Inc., Chicago, IL, USA). Nonparametric Wilcoxon Man-Whitney test was used when comparing two independent samples and nonparametric Kruskal-Wallis test was used when comparing multiple samples. A value of $p < 0.05$ was considered significant (Asym. 2-tailed).

CHAPTER THREE

RESULTS

3.1 Construction of pAAV-MCS/IgG1 Fc expression plasmid

The IgG1 Fc fragment was obtained by digesting with restriction enzymes *Bam*HI and *Apa*I from IL-2LS IgGr1/pcDNA3.1 (**Figure 4A**). The above DNA fragment (830 bp) was further extracted from 1% agarose (**Figure 4B**) before it was ligated with His-Tag linker and pAAV-MCS vector. The construct was then transformed into *E. coli* Top 10 strain, and ten randomly clones were picked. The bacterial colony PCR reaction with the primers β-globin intron and hGH-poly(A) showed that a fragment of the predicted size (1050 bp) was obtained (**Figure 6**). Then, the clone #3 was identified by sequencing with the results according with what was expected (see **Appendices**). The clone# 3 DNA was extracted by Midi-praparation as describe in 2.2.5. The recombinatant plasmid was amplified by PCR using β-globin and hGH-poly(A) primers and the resulting PCR product was a 1050 bp fragment, consistent with the predicted size (**Figure 8, lane 2**) . Also, the obtained plasmid was confirmed by restriction enzyme digestion (**Figure 9, lanes 1-3**).

The transgene in pAAV-MCS/IgG1 Fc vector contains a human cytomegalovirus (CMV)-driven promoter immediately upstream of the cassette, and represents an IL-2 leader sequence followed by the Fc portion of the human IgG1 (hing-CH2-CH3 domain). In addiction, a 6x his tag is attached at the carboxyl terminal end of the Fc fragment.

3.2 Construction of pAAV-MCS/RBDV-IgG Fc expression plasmid

The RBDV-IgG1 Fc fragment was obtained by digesting with restriction enzyme *Bam*HI and *Apa*I from RBDV IgGr1/pcDNA3.1(**Figure 5A**). The above DNA fragment (1070bp) was further separated and extracted from 1% agarose (**Figure5B**) before it was ligated with His-Tag linker and pAAV-MCS vector. The construct was then transformed into *E. coli* Top 10 strain and thirty-one randomly clones were picked. The bacterial colony PCR reaction with the primers β-globin and hGH-poly(A) showed that a fragment of the predicted size (1270bp) was obtained (**Figure7**). Then, the clone #24 was identified by sequencing with the results according with what was expected (see **Appendices**), and extracted by Midi-praparation as describe in 2.2.5. The recombinatant plasmid was confirmed by PCR using β-globin and hGH-poly(A) primers and the resulting PCR product was a 1270 bp fragment, consistent with the predicted size (**Figure 8**). Also, the obtained plasmid was confirmed by restriction enzyme digestion (**Figure 9**).

The transgene in pAAV-MCS/RBDV-IgG1 Fc vector contains CMV-driven promoter immediately upstream of the cassette, and represents a leader peptide and a targeting domain, the receptor binding domain of human VEGF (RBDV; amino acids 8-109), followed by the Fc portion of the human IgG1 (Hing-CH2-CH3 domain). In addition, a 6x his tag was attached at the carboxyl terminal end of the Fc fragment and the complete sequence has been submitted to GenBank (**accession no. EF490666**).

3.3 Expression and characterization of chimeric gene

Figure 3 shows a diagram of the chimeric genes, IgG1 Fc and RBDV-IgG1 Fc. RBDV-IgG1 Fc encodes a receptor binding domain of VEGF (amino acids 8-109) in the front followed by the Fc regions (Hing-CH2-CH3 domains) of the human immunoglobulin G1. The chimeric genes were cloned into the pAAV-MCS expression vector. The resulting constructs, pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc, were transiently transfected into HEK-293 cells by calcium phosphate based methods as described in section 2.2.8. The efficiency of transfection of this method had been confirmed by pAAV-hrGFP vector (Strategene, USA; **Figure 10**). 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 2.2.10. A protein band that was immunoreactive against both an anti-his tag antibody (**Figure 11A**) or an anti-human IgG antibody (**Figure 11B**) was detected, where no signal could be detected in the vehicle group. To further examine the structure of chimeric proteins, we also harvested culture supernatant to nickel-charged HisTrap affinity chromatography followed by a reducing and non-reducing SDS-PAGE , and was transferred and blotted by anti-human IgG antibody (**Figure 15**). In the presence of 2-ME, the immunoactive bands appeared with a molecular weight of ~38 kDa for the IgG1 Fc with glycosylation and apparent bands of ~48 kDa for RBDV-IgG1 Fc with glycosylation. On the contrast, in the absence of 2-ME, there

appeared an ~76 kDa for dimeric IgG1 Fc, and an ~98 kDa for dimeric RBDV-IgG1 Fc fusion protein. Furthermore, there was an additional band at approximately ~196 kDa, which was predicted as tetramer.

3.4 Chimeric proteins purification

To purify the IgG1 Fc and RBDV-IgG1 Fc fusion proteins, the culture supernatants were harvested after the initial transfection. The result supernatants were first applied onto protein G affinity chromatography. Protein G elutes were subsequently loaded onto a nickel-charged HisTrap affinity chromatography and were desalted using sephadex G-25. The procedure of the purification is given in **Figure 12**. Our first attempts to purify the chimeric proteins by protein G column alone, HisTrap column alone or HisTrap column followed by potein G column, however, were not successful for obtaining highly purity recombinatant proteins. (**Figure 13**). To determine the purity, coomassie-stained SDS-PAGE was performed . After reduction condictions, the protein migrated as a single band at ~38 kDa for IgG1 Fc and at ~48 kDa for RBDV-IgG1 Fc with >95% of purity (**Figure 14**). Also, the yield of purified protein in this preparation was obtained as high as 750 μg/liter of culture supernatants.

3.5 The activity of RBDV-IgG1 Fc binding to human VEGF receptor

To directly examine RBDV-IgG1 Fc binding to its receptors, sanwich enzyme-linked

immunosorbent assay (ELISA) was used in this study. Immobilized VEGFR-1 Ig domain 1–3 Fc fusion (VEGFR1$_{D1–3}$-Fc), and VEGFR2 extracellular domain with seven Ig-like domains (VEGFR2$_{D1–7}$) were incubated with RBDV-IgG1 Fc fusion protein, respectively. The results showed that strong binding of RBDV-IgG1 Fc were observed in immobilized VEGFR-1 (**Figure 16A)** and VEGFR-2 (**Figure 16B**), respectively. Human IgG1 Fc, however, could not binding to VEGF receptors, either in VEGFR-1 or VEGFR-2.

3.6 The activity of RBDV-IgG1 Fc binding to HUVECs cell surface

RBDV-IgG1 Fc was shown to retain binding to VEGF receptors activity by ELISA. To further characterize the *in vitro* properties of the chimeric proteins, we tested its ability to target the VEGF receptors expressed on the cell surface of HUVECs. HUVECs were probed with RBDV-IgG1 Fc and assayed by flow cytometry. Because HUVECs has been clearly demonstrated to express two major receptors, VEGFR-1 and VEGFR-2 (Waltenberger, Claesson-Welsh et al. 1994). As shown in **Figure 17** , FACS analysis of RBDV-IgG1 Fc binding to HUVECs cell surface using FITC-labeled anti-human IgG.

3.7 *In vitro* potency and efficacy of RBDV-IgG1 Fc in inhibiting HUVECs proliferation.

It is well known that VEGF stimulates the proliferation of endothelial cells through the

VEGFR-2. Using *in vitro* proliferation assay as described in section 2.2.13, though by slightly modification, we first examined the effect of RBDV-IgG1 Fc on HUVECs to confirm the specificity of binding could induce post-receptor signal that we observed in specific binding to VEGFR-1 and VEGFR-2 *in vitro* (**Figure 18A**). HUVECs (2×10^4 cells/well in a 24-well plate) were in M199 medium with 20% FBS and heparin (25 units/ml), incubated in the presence of IgG1 Fc, RBDV-IgG1 Fc or simply PBS for 72 hours and proliferation assay was determined by MTT. The results show that addiction of VEGF (8 ng/ml) to HUVECs for 72 hours caused an increase in cell proliferation compared with no VEGF supplement group. In fact, in the presence of RBDV-IgG1 Fc (2.5 μg/ml), HUVECs did not show any significance of proliferation, as well as IgG1 Fc (2.5 μg/ml) alone. RBDV-IgG1 Fc had the ability of binding to its receptor in ELISA and flow cytometry, we therefore decided to test whether RBDV-IgG1 Fc might also suppress endothelial cell proliferation in the presence of VEGF. As expected, the proliferative response of endothelial cells was significantly enhanced by the addition of 8 ng/ml of VEGF (**Figure 18B**). In contrast, when VEGF was co-incubated with RBDV-IgG1 Fc (1 μg/ml), cellular proliferation was reduced by ~50% when RBDV-IgG1 Fc was present at a concentration of 1 μg/ml of 72 hours incubation, whereas an equivalent concentration of the control, IgG1 Fc, had no significant effect on the cell growth, indicating this response was specific to VEGF and an antagonistic action of RBDV-IgG1 Fc. As shown in **Figure 18B**, RBDV-IgG1 Fc significantly suppressed the mitogenic response of HUVECs

to VEGF in a dose-dependent manner. Furthermore, addition within the concentration range of 10 to 15 μg/ml RBDV-IgG1 Fc to HUVECs lead to a decrease in VEGF-stimulated HUVECs proliferation. These analyses revealed that the proliferation of HUVECs was not induced by RBDV-IgG1 Fc, however, it had the ability to block the interaction between VEGF and VEGF receptors.

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

To investigate angiogenesis mechanism *in vitro*, by which VEGFR-1 promotes capillary morphogenesis, we examined the effect of RBDV-IgG1 Fc on capillary-like structures in HUVECs. HUVECs were exposed to IgG1 Fc (10 μg/ml), RBDV-IgG1 Fc (10 μg/ml) or PBS for 60 min before stimulation with VEGF (8 ng/ml), and allowed to incubate for 16 hours. RBDV-IgG1 Fc inhibited VEGF-induced total tube formation compared with VEGF alone or IgG1 Fc within VEGF stimulation, and capillary connections were also blocked as shown in **Figure 19D**. In the presence of RBDV-IgG1 Fc, HUVECs remained in isolated islands, and accumulated in aggregates. In contrast, IgG1 Fc alone did not cause a significant decrease in VEGF-induced tube and network formation (**Figure 19C**). At high concentration (15 μg/ml) tested, RBDV-IgG1 Fc caused strongly reduced in capillary connection (data not shown). These data showed that capillary connections were apparently blocked by RBDV-IgG1 Fc. The number of capillary network connections in each group was also evaluated (**Figure 20**)

3.9 IL-2 activitated human NK cytotoxicity

In clinical cancer therapy, the humanized antibody, rituximab or Rituxan, has been shown that its efffectiveness relied on the cytotoxicity function of NK cells. (Eisenbeis, Grainger et al. 2004; Gluck, Hurst et al. 2004). We therefore wanted to explore the activities of the Fc portion of RBDV-IgG1 Fc fusion protein, whereas adhensive properties of HUVECs were as a target cells. As effector cells, we choosed to use NK-92MI, which was shown to contain, and express the human IL-2. IL-2 have been determined as growth factor for enhancing NK lytic activity (Rosenberg, Lotze et al. 1993). To determine targets killed by NK cells, we used MTT tetrazolium assay, which sensitivity and accuracy has been determined (Malich, Markovic et al. 1997). Data from **Figure 21** showed that RBDV-IgG1 Fc fusion proteins trigger lysis of HUVECs ($P<0.05$) in the presence of NK cells, whereas NK cells alone could not sufficiently process the lytic response. Killing activities was proportional to the percent of HUVECs survival.

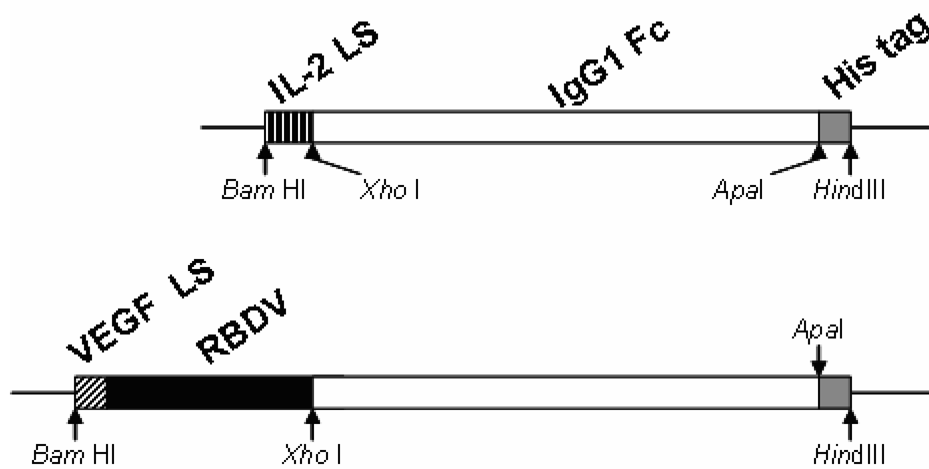


Figure 3. Scheme of the chimeric gene construction. The receptor binding domain of human vascular endothelial growth factor was fused to the N terminal of the Fc portion of IgG1, followed by the poly his tag. LS refers to the leader sequence; RBDV, the receptor binding domain of vascular endothelial growth factor; IgG1 Fc, the constant region (Hing, CH2 and CH3 domains) of the Fc domain of human immunoglobulin G1. As a control, a human IgG1 Fc molecule was also created.

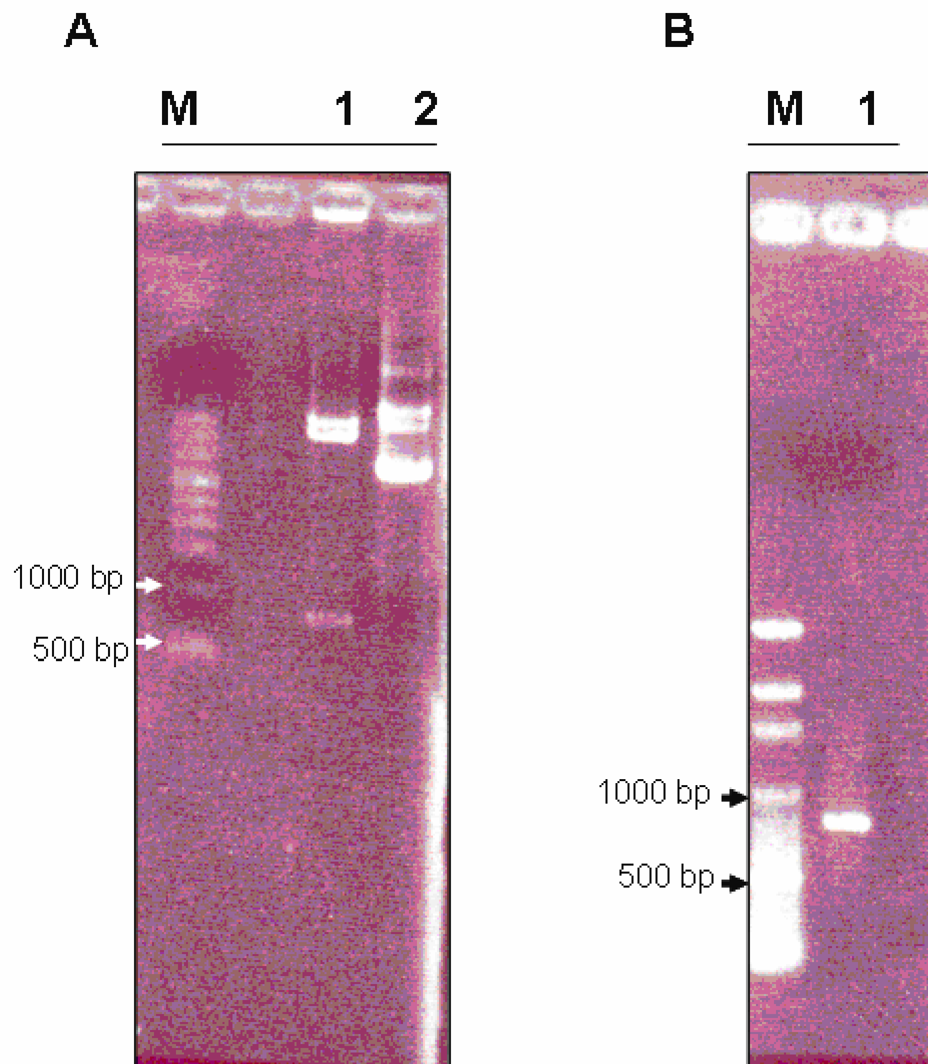


Figure 4. Restriction enzyme digestion of the IL-2 IgGr1/ pcDNA3.1 plasmid. (A) Lane1: *Bam*HI and *Apa*I digestions of IL-2 IgGr1/ pcDNA3.1. Lane 2 control, IL-2 IgGr1/ pcDNA3.1Fc alone. The expected fragments of 830 bp are obtained. (B) A clear band can be observed after extraction from agarose gel. M: 100 bp ladder marker.

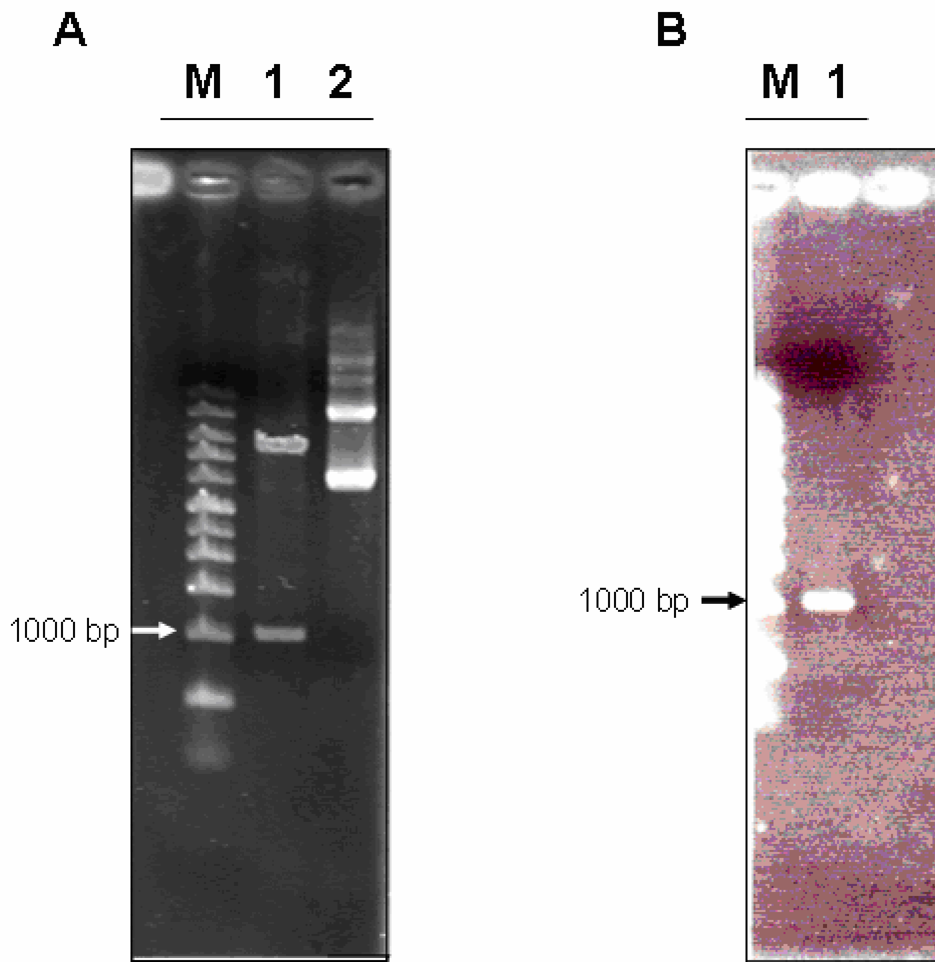


Figure 5. Restriction enzyme digestion of the RBDV-IgGr1/pcDNA3.1 plasmid. (A) Lane1: *Bam*HI and *Apa*I digestions of RBDV-IgGr1/pcDNA3.1. Lane 2 control, RBDV-IgGr1/pcDNA3.1 alone. The expected fragments of 1070 bp are obtained. (B) A clear band can be observed after extraction from agarose gel. M: 100 bp ladder marker.

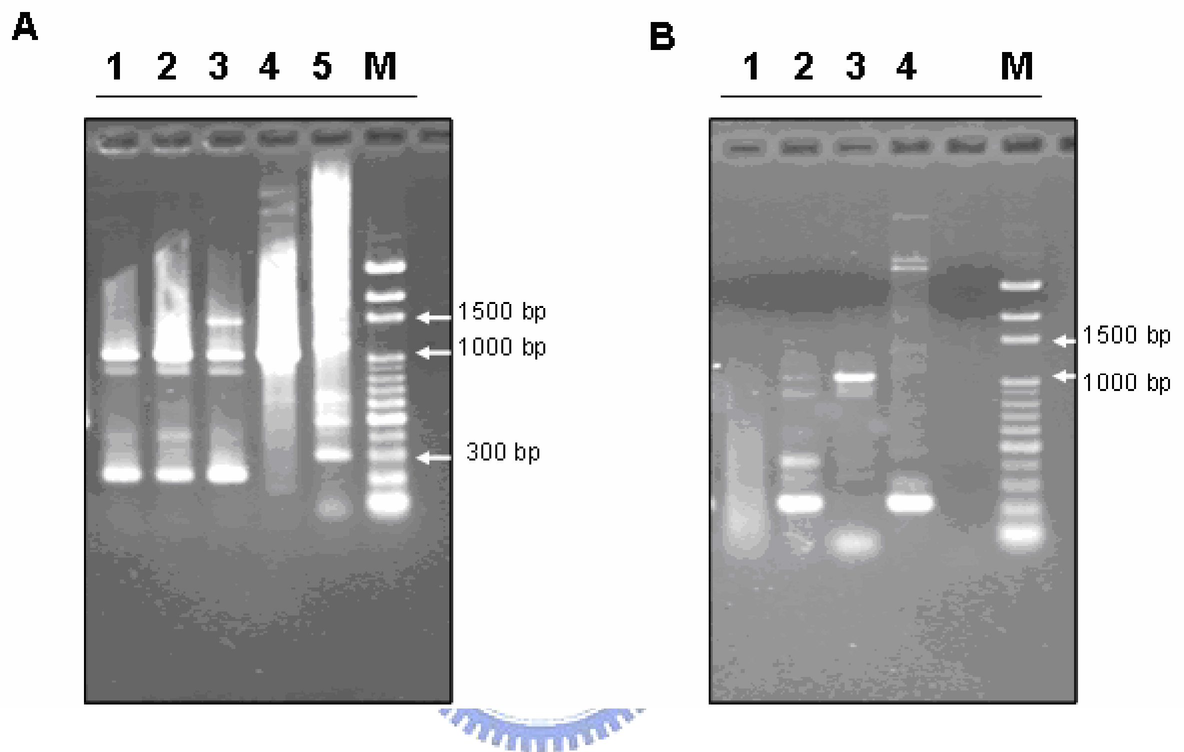


Figure 6. Bacterial colony PCR assay for pAAV-MCS/IgG1 Fc contruct. (A) The IgG1 Fc fragement was gel purified, and cloned with pAAV-MCS vector and poly-His tag linker. Single colony was picked and processed as described in section 2.2.5. Lane 1, colones 1~3; lane 2, colones #4~6; lane 3, colones #7~10; lanes 4 and 5, control. (B) Lane 1, colon #1; lane 2, colones #2; lane 3, colon #3; lane 4, control. The expected PCR product of 1050 bp was obtained (lane 3). Colon #3 was picked and sequenced.

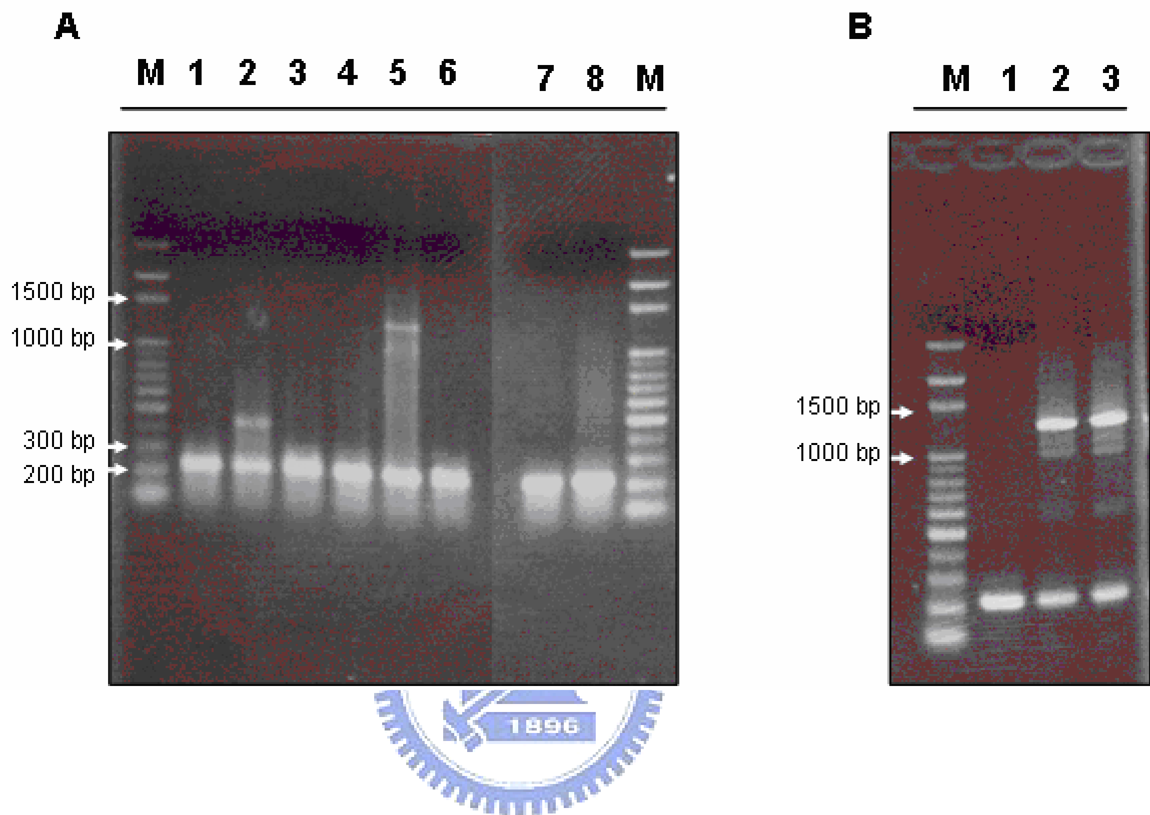


Figure 7. Bacterial colony PCR assay for pAAV-MCS/RBDV-IgG1 Fc contruct. (A) The RBDV-IgG1 Fc fragement was gel purified, and cloned with pAAV-MCS vector and poly-His tag linker. Single colony was picked and processed as described in section 2.2.5. Lane 1, colones #11~13; lane 2, colones #14~16; lane 3, colones #17~19; lane 4, colones #20~22; lane 5, colones #23~25; lane 6, colones #26~28; lane 7, colones #29~31; lane 8, control. The expected PCR product of 1270 bp was obtained in lane 5. (B) Lane 1, colon #23; Lane 2, colon #24; Lane 3, colon #25. The expected PCR product of 1270 bp was obtained (lanes 2 and 3). Colony #24 was then picked and sequenced.

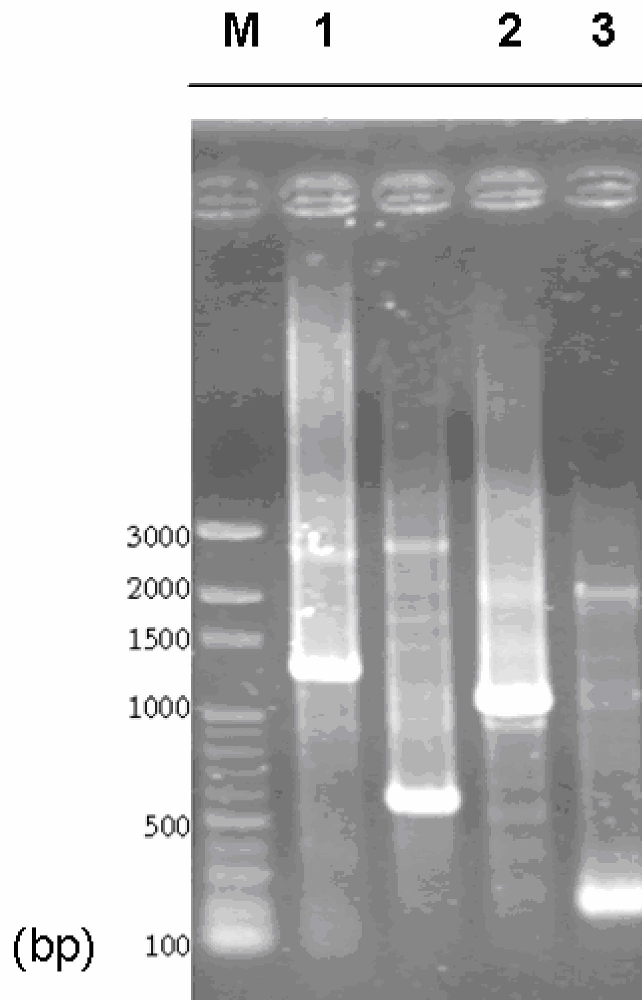


Figure 8. PCR screening of pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc plasmids. Recombinatant plasmid DNA was amplified by PCR analysis using RBDV Fc and hrGH-poly A primers and the product was identified by running it on the agarose gel. Lane 1, pAAV-MCS/RBDV-IgG1 Fc with a fragment of the predicted size (1270 bp). Lane 2, pAAV-MCS/IgG1 Fc with a fragment of the predicted size (1050 bp). Lane 3 control.

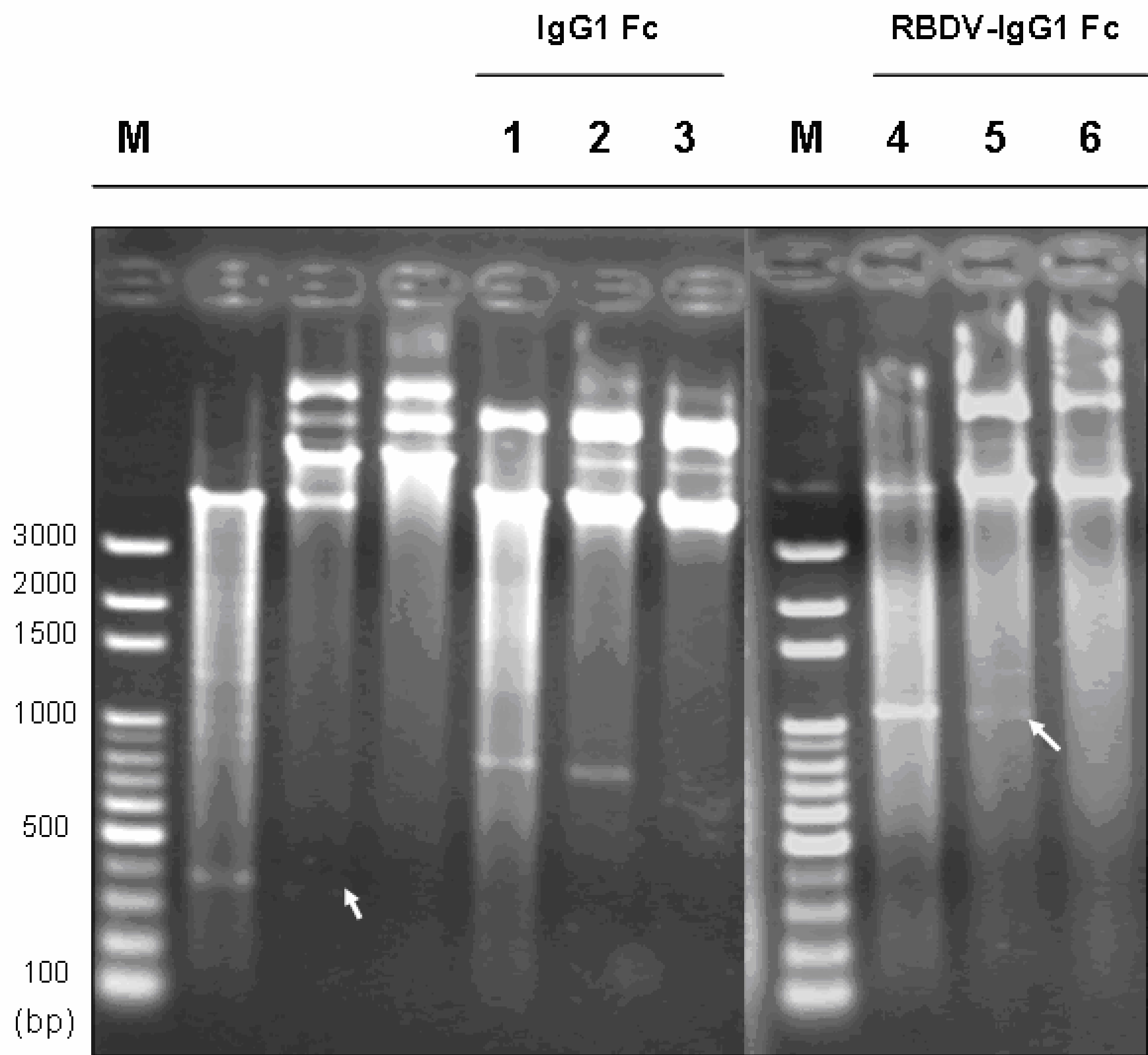


Figure 9. Restriction enzyme digestion of the pAAV-MCS/IgG1 Fc and pAAV-MCS/RBDV-IgG1 Fc. Lanes 1 and 4: *Bam*HI and *Hin*dIII digestion and the expected fragments of 855 and 1080 bp are obtained, respectively. Lanes 2 and 5: *Bam*HI and *Apa*I digestion and the expected fragments of 830 and 1050 pb (arrow indicated) can be observed, respectively. M: 100 bp ladder marker.

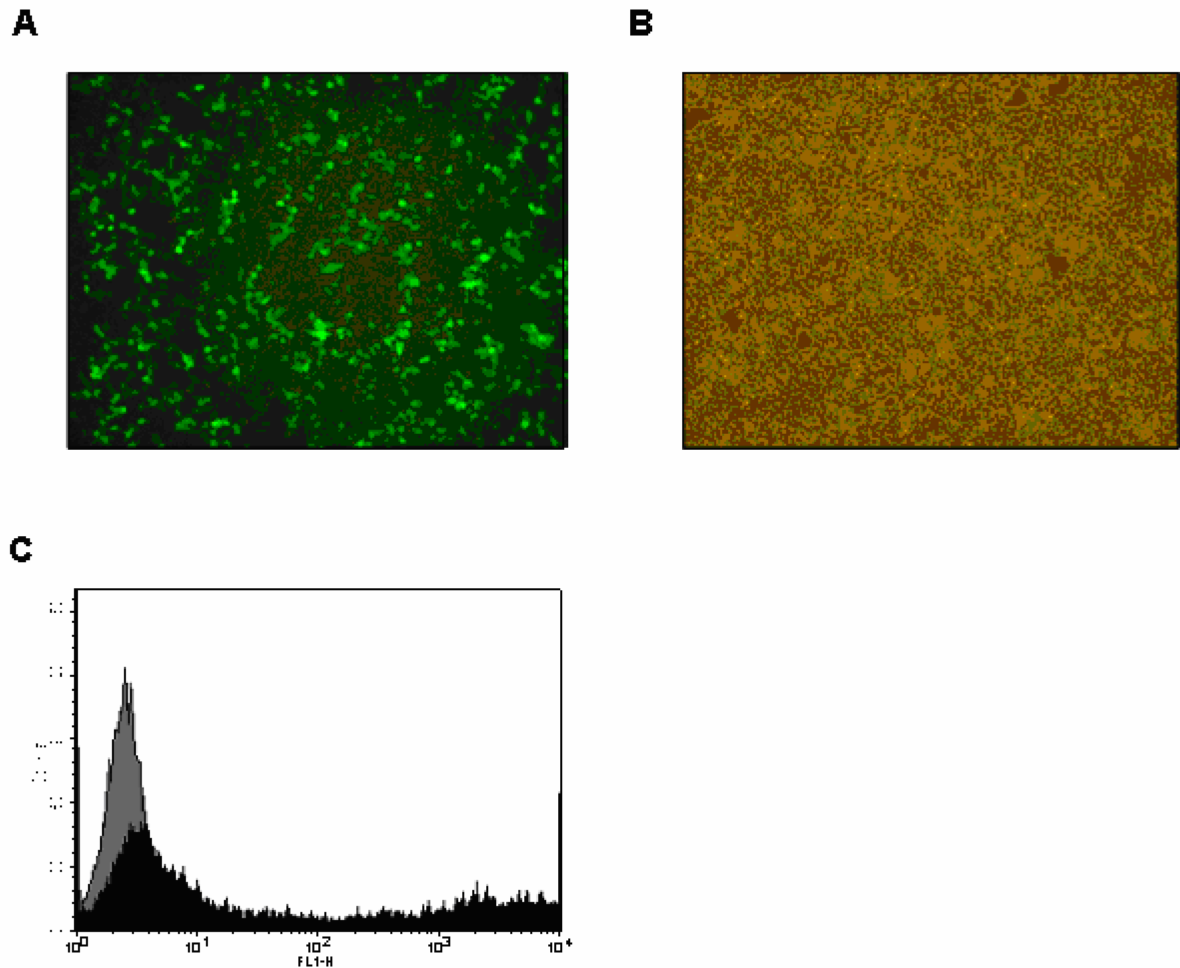


Figure 10. The fluorescence expression in HEK-293 cell. HEK-293 cells were transfected with pAAV-MCS-hrGFP vector using calcium phosphate based transfection methods. (A) Photograph of the hrGFP-transfected HEK-293 cell obtained under a fluorescence microscope, 100X magnification. (B) Light microscope picture of hrGFP-transfected HEK 293 cells, 100X magnification. (C) Histograms of the hrGFP-transfected cells from 10,000 events measured on a flow cytometer. (Transfection effeciency: >50% compared with negative control).

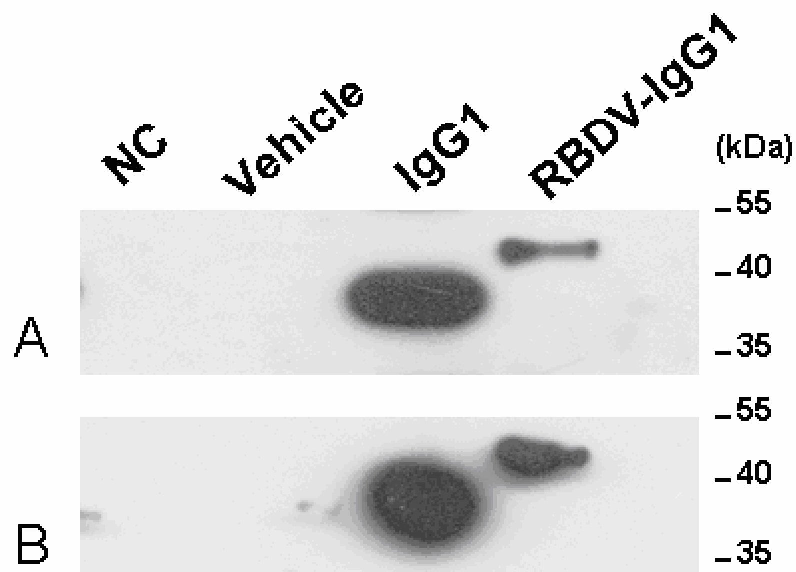


Figure 11. Detection of the chimeric proteins. Plasmid pAAV-MCS/IgG1 Fc or pAAV-MCS/RBDV-IgG1 Fc, driven by a human cytomegalovirus (CMV) immediate-early promoter, was transfected into human kidney epithelial (HEK) 293 cells. When the color of cultured medium change to orange, cultured medium of transfectants were collected, run on reducing 10% SDS-polyacryamide gel electrophoresis, and then analyzed by Western blotting with horseradish phosphatase-labeled goat anti- His tag antibody (A), or anti- human IgG antibody (B). The membrane was visualized with chemiluminescence kit. NC, the culture media; Vehicle, 293T cells transfected with pAAV-MCS vector

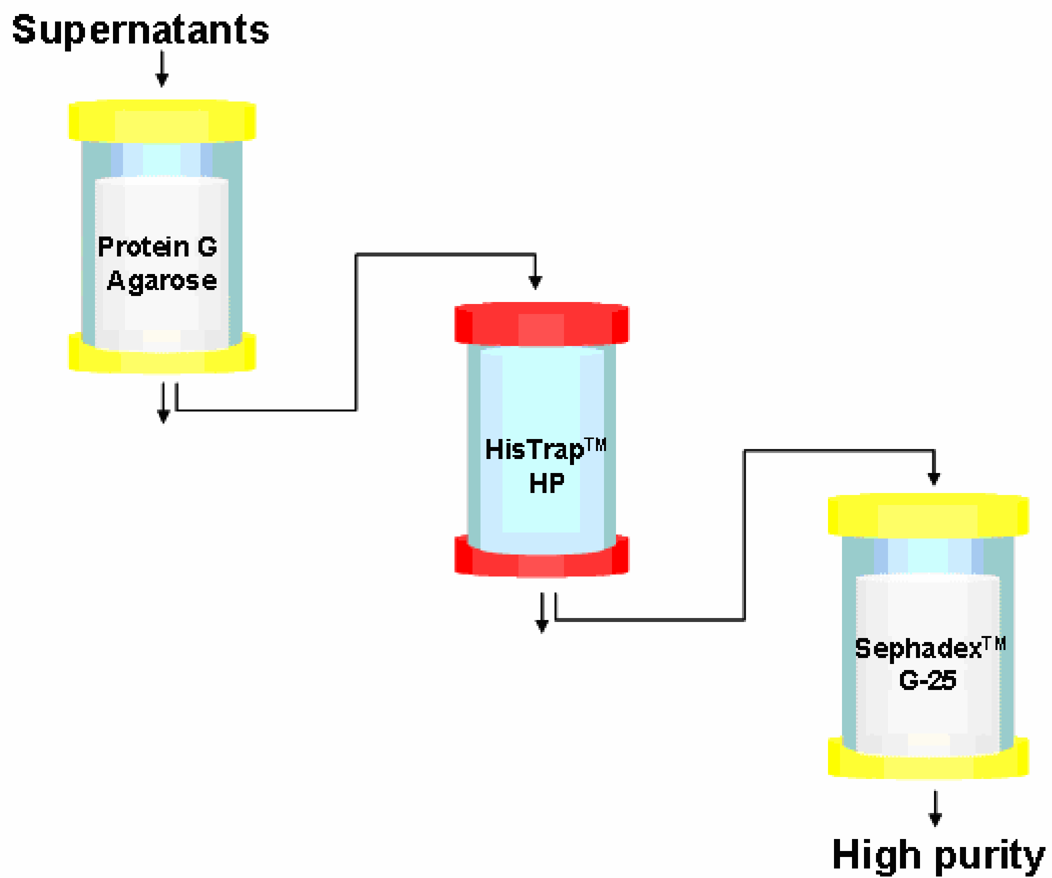


Figure 12. Diagram of procedure for the purification of chimeric proteins in this study. The culture supernatants were harvested after the initial transfection. The result supernatants were first applied onto protein G affinity chromatography. Protein G elutes were subsequently loaded onto a nickel-charged HisTrap affinity chromatography and were desalted using sephadex G-25.

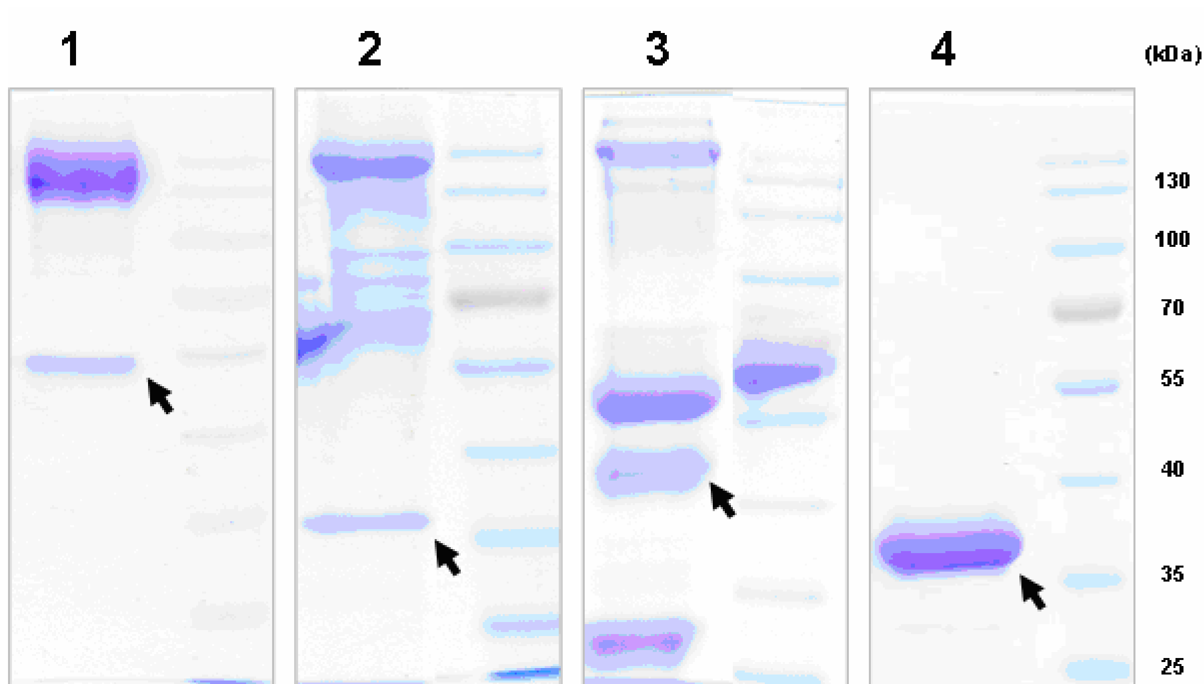


Figure 13. SDS-PAGE analysis of recombinatant proteins showing the purification results. Lane 1, IgG 1 Fc eluted from protein G affinity chromatography alone (arrow), 10% non-reducing SDS-PAGE. Lane 2, IgG1 Fc eluted from HisTrap affinity chromatography alone (arrow), 10% reducing SDS-PAGE. Lane 3, RBDV-IgG1 Fc, arrow indicated, was fisrt purified by HisTrap affinity chromatography, followed by protein G affinity chromatography, 10% reducing SDS-PAGE. Lane 4, IgG1 Fc, arrow indicated, was purified by protein G affinity chromatography, followed by HisTrap affinity chromatography, 10% reducing SDS-PAGE. A strong single band was detected in lane 4, where many visible bands were occurred in others. The molecular markers in kilodaltons (kDa) are showm in the right.

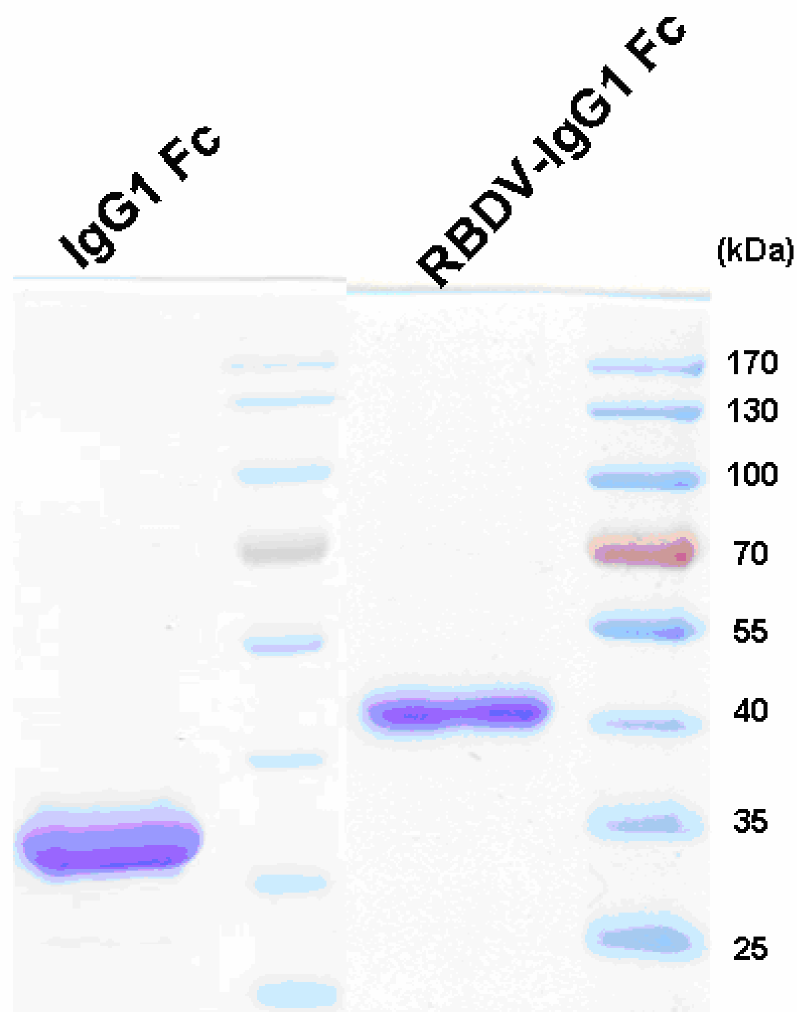


Figure 14. SDS-PAGE analysis of the purified chimeric proteins. IgG1 Fc and RBDV-IgG1 Fc proteins were expressed and purified as described in section 2.2.9 and 2.2.10 . Purified proteins were resolved by 10% SDS-PAGE under reducing conditions and stained with Coomassie blue. The molecular markers in kilodaltons (kDa) are showm in the right.

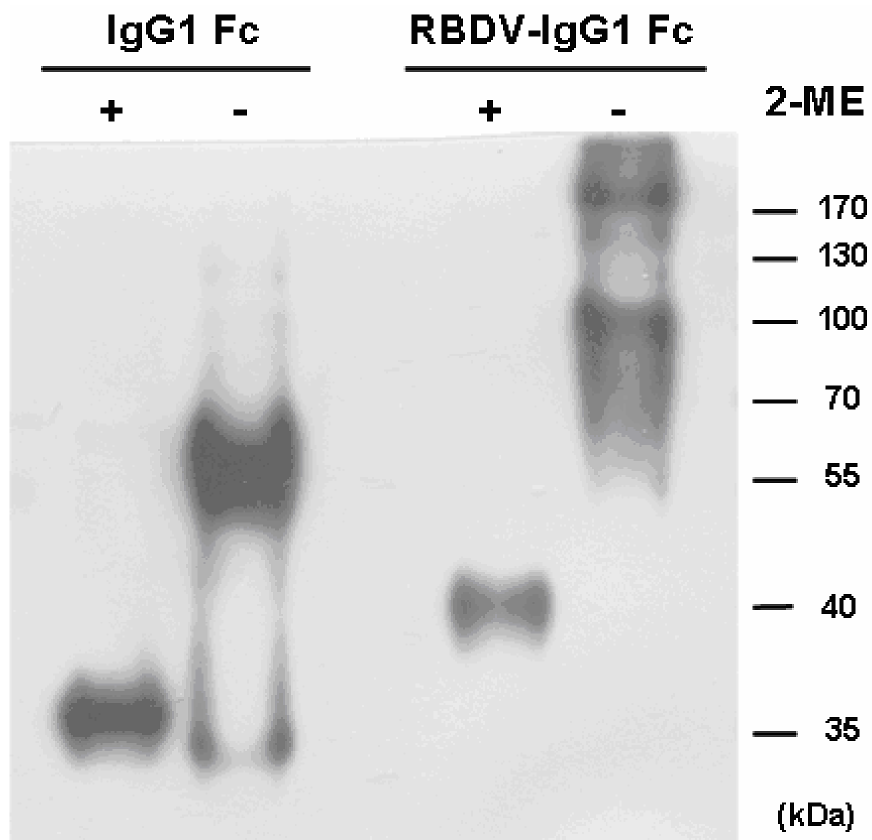


Figure 15. Characterization of HisTrap column purified chimeric proteins. As for the characterization of chimeric proteins, SDS-PAGE was further transferred onto PVDF membrane, analyzed by Wesern blotting with the antibody to human IgG. The molecular markers in kilodaltons (kDa) are shown in the right. 2-ME, beta-mecaptoethanol.

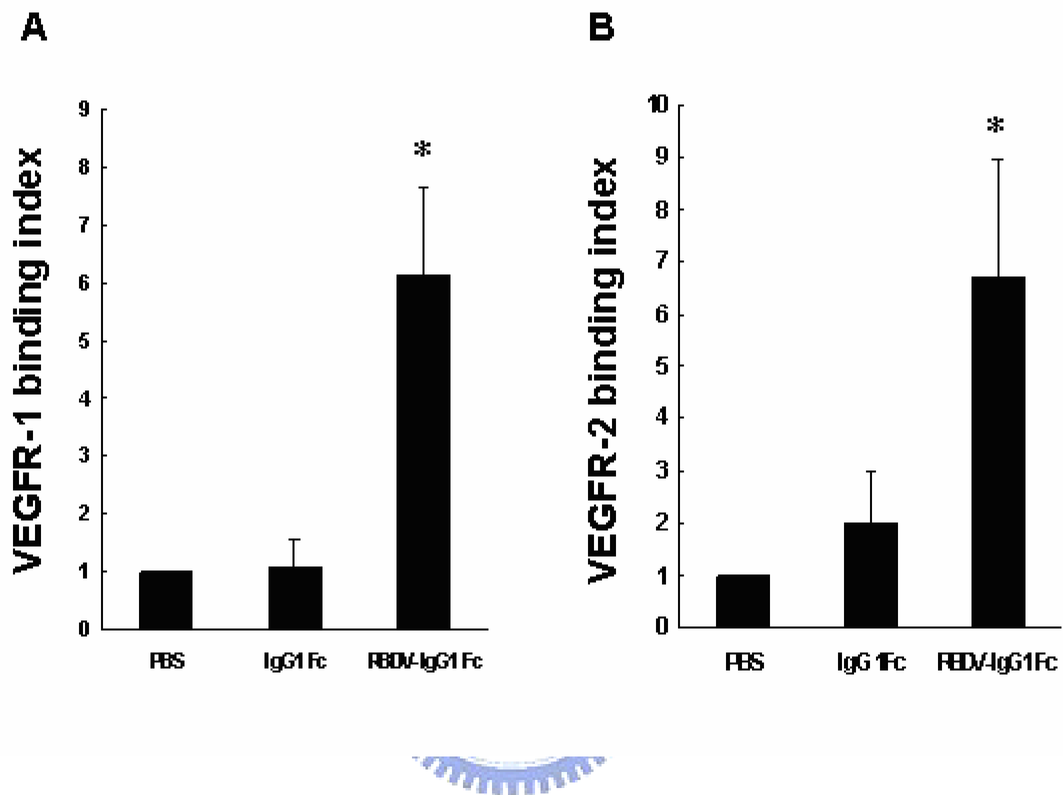


Figure 16. Receptor binding activities of purified RBDV IgG1 Fc. (A) The binding activity of the purified RBDV-IgG1 Fc to immobilized hVEGFR-1. Ten µg/ml of column purified protein were incubated in 96-well plates precoated with hVEGFR$_{D1-3}$- Fc (4µg/ml) at room temperature overnight, followed by incubation with HRP conjugate anti-his tag antibody for an additional 1 hour. The plates were then incubated with a peroxidase TMB substrate, followed by reading of the absorbance at 450 nm. (*P<0.05). (B) The binding activity to immobilized hVEGFR-2 by the purified RBDV-IgG1 Fc protein. Ten µg/ml of column purified protein were incubated in 96-well plates precoated with hVEGFR$_{D1-7}$ (4 µg/ml) at room temperature overnight, the plates were then incubated with an anti-human IgG antibody-HRP conjugate, followed by development of the plates as described above.($^{*}P$<0.05). The data performed here are the mean ±SD of two independent experiments (n=4) corresponding to PBS or IgG1 Fc control.

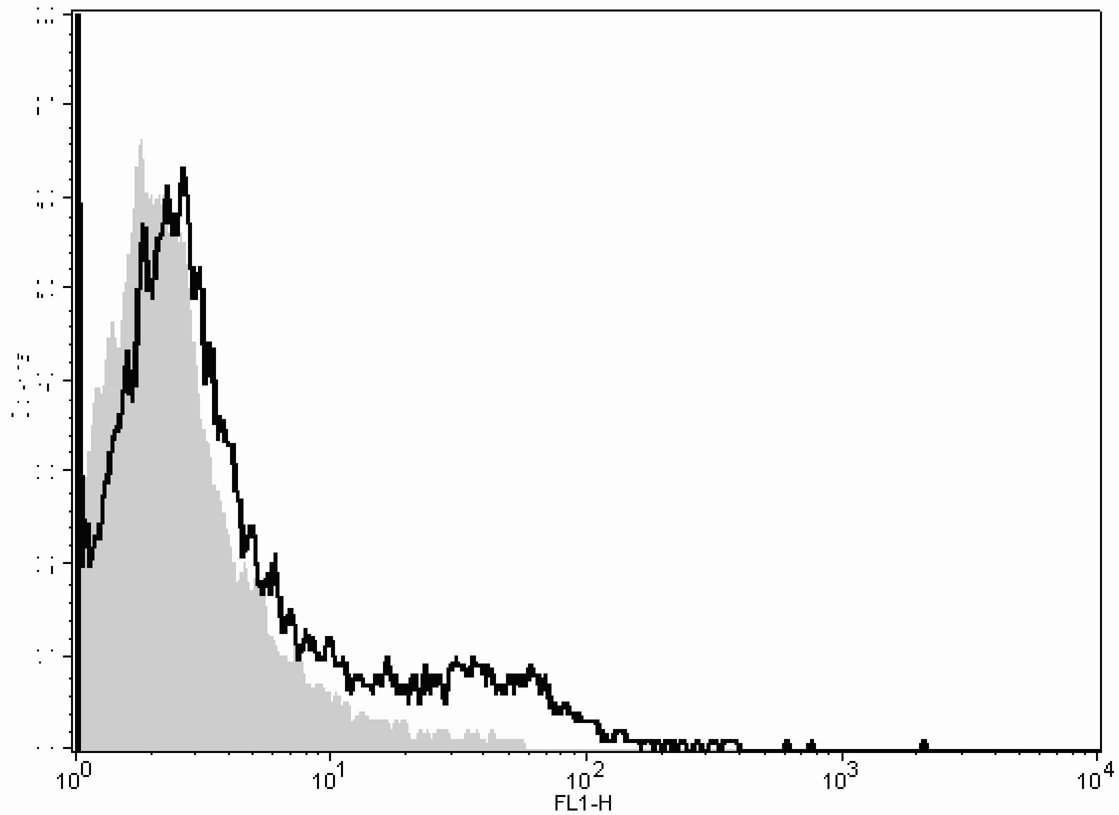


Figure 17. Cell surface binding ability. RBDV-IgG1 Fc chimeric protein was detected by flow cytometric analysis using HUVECs. The HUVECs were incubated with chimeric proteins at 4 °C for 1 hour, followed by incubation with FITC-labeled goat anti-human IgG antibody for an additional hour (solid line). Cells stained with FITC-labeled goat anti-human IgG antibody were used as negative control (gray area).

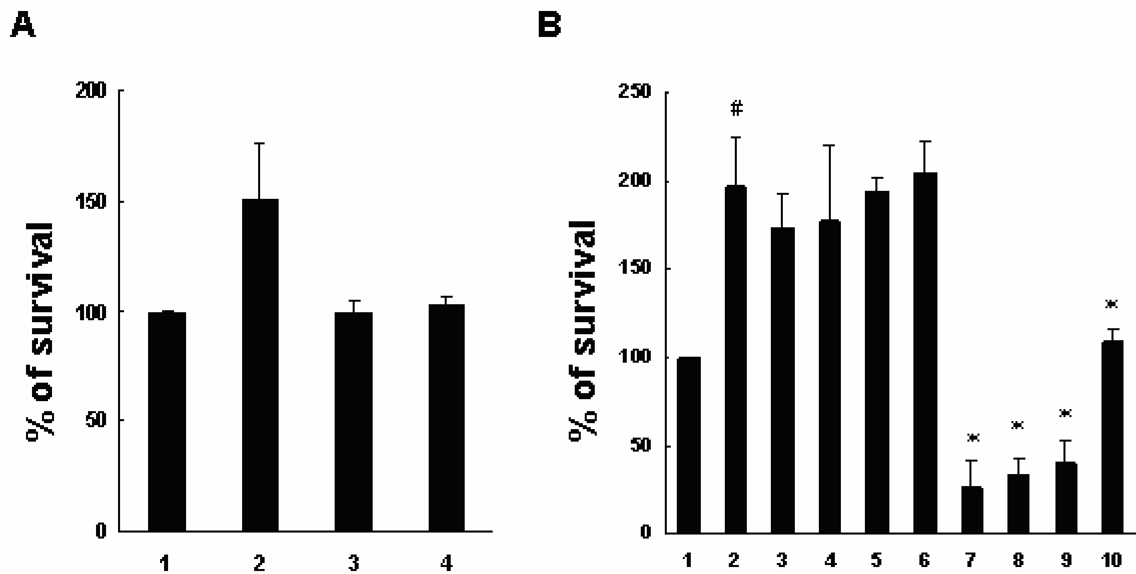


Figure 18. RBDV-IgG1 Fc inhibits the VEGF-induced proliferation of HUVECs in a dose-dependent manner. (A) HUVECs were incubated with chimeric proteins for 72 hours and proliferation profile was determined by 5 mg/ml MTT**.** Lane 1, PBS. Lane 2, VEGF (10ng/ml). Lane 3, IgG1 Fc (2.5 μg/ml). Lane 4, RBDV-IgG1 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 ±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).

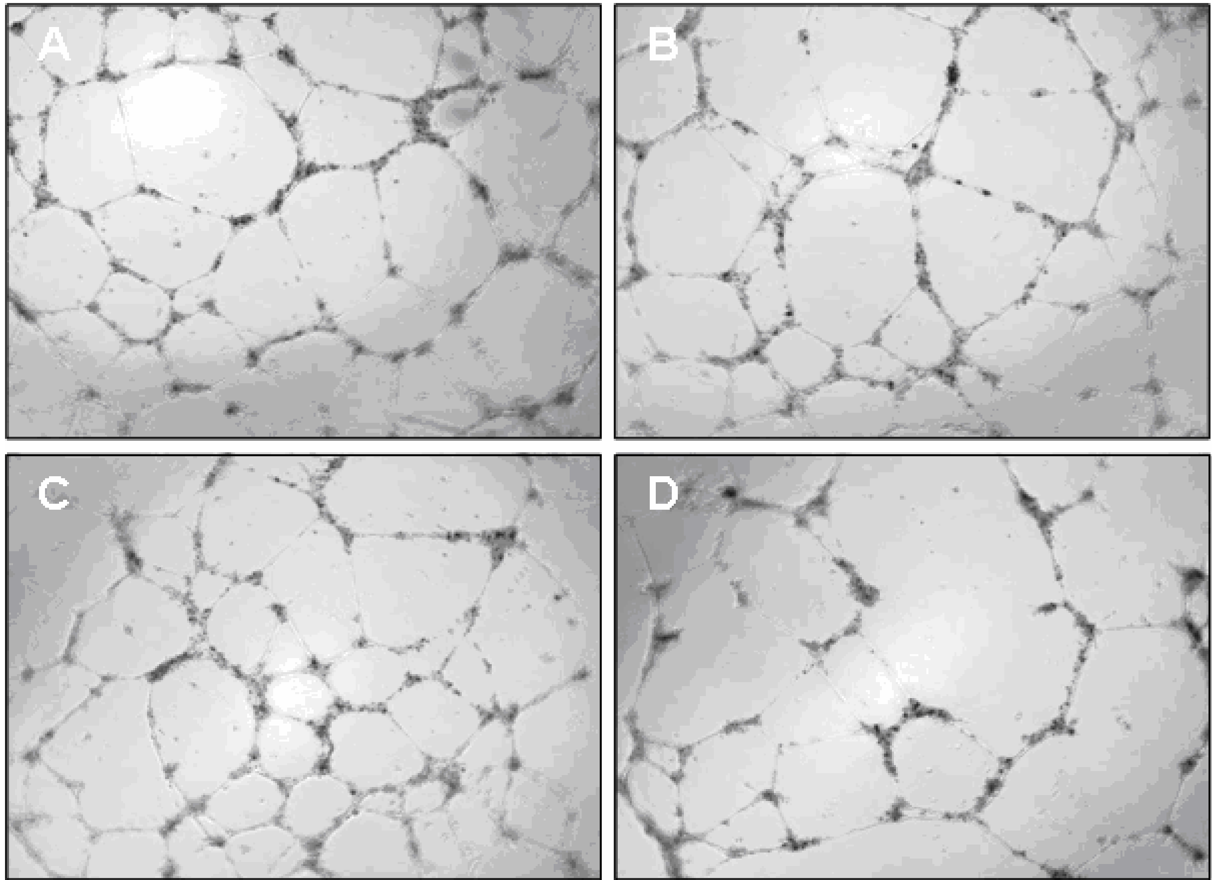


Figure 19. Effect of RBDV-IgG1 Fc on in vitro tube formation, 40X magnification. To determine the angiogenic activity, HUVECs (1.5×10^4 cells/well) were pre-incubated with IgG1 Fc (10 μg/ml) or RBDV-IgG1 Fc (10 μg/ml) for 1 hour, and were added onto growth factor-reduced Matrigel with VEGF (15 ng/ml) in M199 containing 20% fetal bovine serum and heparin (25 units/ml) for 16 hours at 37°C. Under normal conditions, HUVECs formed a network of tubes (B) that was enhanced when stimulated with VEGF (15 ng/ml). (C)HUVECs were treated with IgG1 Fc. (D) Tube formation was apparently inhibited with RBDV-IgG1 Fc. (A) PBS.

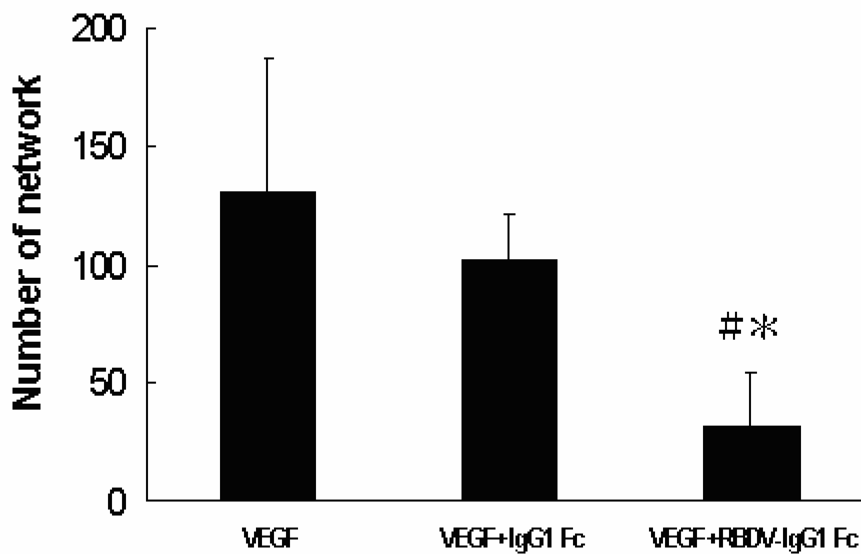


Figure 20. Statistics of HUVECs networks formation. Total numbers of HUVECs networks formation were counted. The data represent a mean ±SD of two independent experiments (n=4).#*P*< 0.05 when compared with VEGF group.**P*< 0.05 when compared with VEGF+IgG1 Fc group.

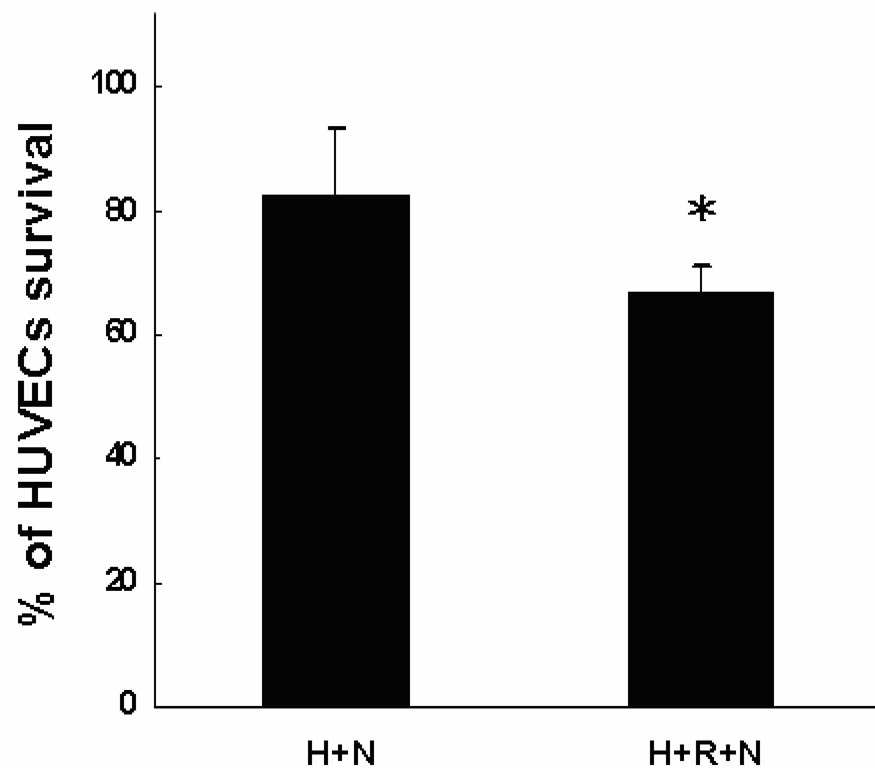


Figure 21. IL-2 activated human NK cytotoxicity. IL-2 activated human NK-92MI cells were co-incubated with HUVECs in the presence of RBDV-IgG1 Fc at an E/T ratio of 1 for 5 hours. Specific lysis was determined by MTS as described in materials and methods. H: HUVECs. N: NK-92MI cells. R: RBDV-IgG1 Fc. The data represent a mean ±SD of two independent experiments (n=4). **P*<0.05 when compare with H+N group.

CHAPTER FOURE

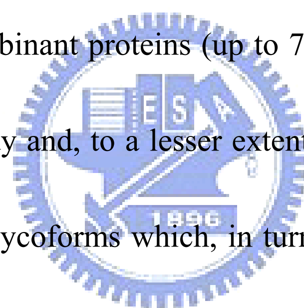
DISCUSSION

.

Inhibition of VEGF/VEGF receptors signal pathway has been shown to suppress pathological angiogenesis in a wide variety of models, including genetic models of cancer, leading to clinical development of a variety of VEGF inhibitors. Here, we created a novel chimeric protein by fusion the receptor binding domain of human VEGFA165 ligands to the Fc portion of human IgG1 as an affibody, which is designed as antagonist that aborgate the physiological interactions between VEGF ligands and its 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 (Keyt, Nguyen et al. 1996; Muller, Li et al. 1997), which amino acids lined between 8 and 109 of full VEGFA165. Thus, to construct the RBDV-IgG1 Fc fusion protein as an antagonist, the receptor binding domain of VEGF (amino acids 8-109), contains the epitope that binds with receptors, was coupled with the Fc portion of human IgG.. Usually, the structural and biochemical properties of the fusion partner are retained in chimeric proteins, perhaps as a result of the spatial separation from Fc, conbined with flexibility of the hinge. Fc fusion proteins have many biological characters in common with IgG, including phamacokinetic properties and target-recognition capacity. Therefore, they are often considered as an alternative IgGs, both in research and in therpeutics (Chamow and Ashkenazi 1996).

In this study, we also described a method for the non-serum-free production and

column purification of recombinant RBDV-IgG1 Fc proteins, which is convenient and inexpensive as compared with lipid-mediated transfection such as lipofetamine. This method utilizes calcium phosphate-mediated plasmid transfection for HEK-293 cells in growth medium followed by collection and purification of RBDV-IgG1 Fc via a combination of a two-steps affinity chromatography. Traditionally, recombinant proteins, expressed in mammalian cell system are purified from serum-free medium. This method of production is cumbersome in that only limited yields can be obtained at one time due to low life-span of production host. Here, with the combination of two affinity columns, we can obtain large amounts of functionally recombinant proteins (up to 750 μg/L) with high-purity. In addition, the cells producing the antibody and, to a lesser extent, curture conditions, can significantly affect the resulting antibody glycoforms which, in turn, can influence the ability of antibody to participate in ADCC (Hosaka, Takase et al. 2006). Moreover, glycosylation of IgG molecule at Asn297 helps to maintain the tertiary structure of their CH-2 domain (Arnold, Wormald et al. 2007).



The stimulation of endothelial cells by growth factors, such as VEGF, is required for the process of angiogenesis (Shibusa, Shijubo et al. 1998). HUVECs express two major VEGF receptors on cell surface: VEGFR-1 is expressed at a few thousand copies per cell, while VEGFR-2 is more abundant on the cell surface (Waltenberger, Claesson-Welsh et al. 1994). VEGFR-2 is a receptor tyrosine kinase, which is responsible for the angiogenic

activity of VEGF. The binding of VEGF to this receptor leads to its activation through the phosphorylation of a tyrosine residue (Heldin 1995). Since RBDV-IgG1 Fc fusion proteins can binds to immoblized VEGF receptors in ELISA. We, therefore, demonstrated this receptor binding domain containing fusion protein would induce any signal transduction cacades in HUVECs. Clearly, in the course of our study, we discovered that this format does not induce any proliferation of HUVECs when compared with their natural interactions with VEGF alone. This can be supported by the research of the caarboxyl terminal of VEGF. The heparin binding region (amino acid 110-165) has been strongly indicated that the heaprin, through simultaneous binding to VEGF and its receptors, increase in signal amplitude and duration. Here we show that the RBDV-IgG1 Fc inhibits VEGF-stimulated receptor activation and endothelial cell mitogenesis. Our results suggest that the mechanism of endothelial cell growth inhibition mediated by the chimeric proteins may due to directing block of VEGF/VEGFR-2 interaction, resulting in inhibition of VEGF-induced VEGFR-2 activation. Moreover, it may directly or indirectly induce HUVECs apoptosis, when dosage were increased.

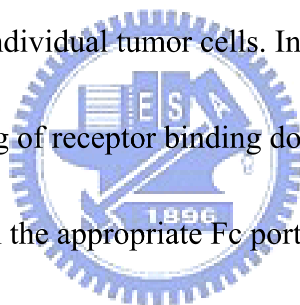
Hiratsuka recently proposed that disturbance of VEGFR-1 activity results in vascular malformation (Hiratsuka, Kataoka et al. 2005), though the role of VEGFR-1 in angiogenesis is still the object of debate. In our study, however, VEGF-driven, capillary-like tube formation in HUVEC was strikingly decreased by RBDV-IgG1 Fc. Morphological changes of HUVEC,

induced by VEGF, were inhibited by RBDV-IgG1 Fc.

Several strategies have previously been reported to function as VEGF binding antagonists, including anti-VEGF antibody (Asano, Yukita et al. 1995; Mesiano, Ferrara et al. 1998), anti-VEGFR-2 receptor antibody(Prewett, Huber et al. 1999), RNA-based aptamers (Ruckman, Green et al. 1998), and various peptides (Bae, Gho et al. 2000; Binetruy-Tournaire, Demangel et al. 2000). 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. Further, IgG-like fusion proteins may prove to be better choices over smaller molecule substances or fragments such as peptide for other in vivo applications by providing the Fc domain that not only confers long phamacokinetic half-life but also supports secondary immune functions, such as ADCC and CDC (complement dependent cytotoxicity). In our study, we further show that RBDV-IgG1 Fc can induce cell-mediated cell cytotoxicity by NK cells. The structure determined by non-reduced SDS-PAGE showed that RBDV-IgG1 Fc can be in a form of tetramer, which is ligated by the intra-disulfide bound bwtween the receptor binding domains of VEGF. Caron et al. proposed that IgG homodimerization by chemical coupling increases the antitumor activity of antibodies against secveral different tumor antigens by mechanisms that include more potent ADCC (Gorter and Meri 1999) or CDC (Caron, Laird et al. 1992), direct killing (Wolff, Schreiber et al. 1993). Significantly, IgG homodimerization might enhance in vivo antitumor activity. Thus, we suppose that this

structure may have the similar immune responses induced by the RBDV-IgG1 Fc *in vivo*.

Our data indicate that 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(Plate, Breier et al. 1993), therefore, antagonists against the receptor may offer higher specificity compared to other agents. Further, tumor vessel endothelial cells are in direct contact with the blood. Hence, they have greater accessibility compared to antibodies against markers expressed on individual tumor cells. In summary, our study indicate an effective strategy of engineering of receptor binding domain of ligands against specific targeted cells, which along with the appropriate Fc portion of IgG for their therapeutic capacity. Also, this novel concept can be used in the treatment of human cancer cell line and other angiogenesis-dependent disease

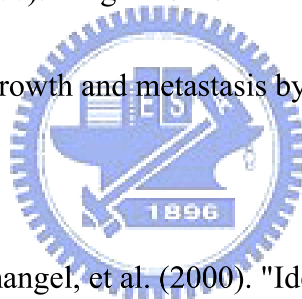


Reference

Arnold, J. N., M. R. Wormald, et al. (2007). "The impact of glycosylation on the biological function and structure of human immunoglobulins." Annu Rev Immunol **25**: 21-50.

Asano, M., A. Yukita, et al. (1995). "Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121." Cancer Res **55**(22): 5296-301.

Bae, D. G., Y. S. Gho, et al. (2000). "Arginine-rich anti-vascular endothelial growth factor peptides inhibit tumor growth and metastasis by blocking angiogenesis." J Biol Chem **275**(18): 13588-96.



Binetruy-Tournaire, R., C. Demangel, et al. (2000). "Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis." Embo J **19**(7): 1525-33.

Byzova, T. V., C. K. Goldman, et al. (2000). "A mechanism for modulation of cellular responses to VEGF: activation of the integrins." Mol Cell **6**(4): 851-60.

Carmeliet, P. (2000). "Mechanisms of angiogenesis and arteriogenesis." Nat Med **6**(4): 389-95.

Carmeliet, P., L. Moons, et al. (2001). "Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in

pathological conditions." Nat Med **7**(5): 575-83.

Caron, P. C., W. Laird, et al. (1992). "Engineered humanized dimeric forms of IgG are more effective antibodies." J Exp Med **176**(4): 1191-5.

Carter, P. (2001). "Improving the efficacy of antibody-based cancer therapies." Nat Rev Cancer **1**(2): 118-29.

Cartron, G., L. Dacheux, et al. (2002). "Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene." Blood **99**(3): 754-8.

Chamow, S. M. and A. Ashkenazi (1996). "Immunoadhesins: principles and applications." Trends Biotechnol **14**(2): 52-60.

Clynes, R. A., T. L. Towers, et al. (2000). "Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets." Nat Med **6**(4): 443-6.

Cohen, T., D. Nahari, et al. (1996). "Interleukin 6 induces the expression of vascular endothelial growth factor." J Biol Chem **271**(2): 736-41.

Cragg, M. S., R. R. French, et al. (1999). "Signaling antibodies in cancer therapy." Curr Opin Immunol **11**(5): 541-7.

de Vries, C., J. A. Escobedo, et al. (1992). "The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor." Science **255**(5047): 989-91.

Dias, S., K. Hattori, et al. (2000). "Autocrine stimulation of VEGFR-2 activates human

leukemic cell growth and migration." J Clin Invest **106**(4): 511-21.

Economides, A. N., L. R. Carpenter, et al. (2003). "Cytokine traps: multi-component, high-affinity blockers of cytokine action." Nat Med **9**(1): 47-52.

Eisenbeis, C. F., A. Grainger, et al. (2004). "Combination immunotherapy of B-cell non-Hodgkin's lymphoma with rituximab and interleukin-2: a preclinical and phase I study." Clin Cancer Res **10**(18 Pt 1): 6101-10.

Faehling, M., J. Kroll, et al. (2002). "Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole." Faseb J **16**(13): 1805-7.

Ferrara, N. (2002). "VEGF and the quest for tumour angiogenesis factors." Nat Rev Cancer **2**(10): 795-803.

Ferrara, N. (2004). "Vascular endothelial growth factor: basic science and clinical progress." Endocr Rev **25**(4): 581-611.

Ferrara, N. and T. Davis-Smyth (1997). "The biology of vascular endothelial growth factor." Endocr Rev **18**(1): 4-25.

Ferrara, N., H. P. Gerber, et al. (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-76.

Ferrara, N. and W. J. Henzel (1989). "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells." Biochem Biophys Res Commun

161(2): 851-8.

Ferrara, N., K. J. Hillan, et al. (2004). "Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer." Nat Rev Drug Discov **3**(5): 391-400.

Ferrara, N., K. Houck, et al. (1992). "Molecular and biological properties of the vascular endothelial growth factor family of proteins." Endocr Rev **13**(1): 18-32.

Ferrara, N. and R. S. Kerbel (2005). "Angiogenesis as a therapeutic target." Nature **438**(7070): 967-74.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nat Med **1**(1): 27-31.

Folkman, J. (1996). "Fighting cancer by attacking its blood supply." Sci Am **275**(3): 150-4.

Folkman, J. and M. Klagsbrun (1987). "Angiogenic factors." Science **235**(4787): 442-7.

Folkman, J., E. Merler, et al. (1971). "Isolation of a tumor factor responsible for angiogenesis." J Exp Med **133**(2): 275-88.

Fong, G. H., J. Rossant, et al. (1995). "Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium." Nature **376**(6535): 66-70.

Fong, G. H., L. Zhang, et al. (1999). "Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice." Development **126**(13): 3015-25.

Fuh, G., B. Li, et al. (1998). "Requirements for binding and signaling of the kinase domain

receptor for vascular endothelial growth factor." J Biol Chem **273**(18): 11197-204.

Fukumura, D., R. Xavier, et al. (1998). "Tumor induction of VEGF promoter activity in stromal cells." Cell **94**(6): 715-25.

Gerber, H. P., V. Dixit, et al. (1998). "Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells." J Biol Chem **273**(21): 13313-6.

Gerber, H. P., K. J. Hillan, et al. (1999). "VEGF is required for growth and survival in neonatal mice." Development **126**(6): 1149-59.

Gerber, H. P., J. Kowalski, et al. (2000). "Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor." Cancer Res **60**(22): 6253-8.

Gerber, H. P., A. McMurtrey, et al. (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation." J Biol Chem **273**(46): 30336-43.

Glennie, M. J. and J. G. van de Winkel (2003). "Renaissance of cancer therapeutic antibodies." Drug Discov Today **8**(11): 503-10.

Gluck, W. L., D. Hurst, et al. (2004). "Phase I studies of interleukin (IL)-2 and rituximab in B-cell non-hodgkin's lymphoma: IL-2 mediated natural killer cell expansion

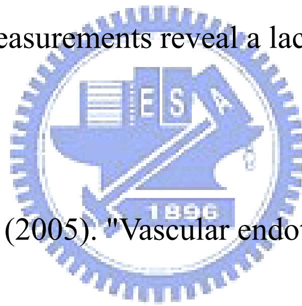
correlations with clinical response." Clin Cancer Res **10**(7): 2253-64.

Gorter, A. and S. Meri (1999). "Immune evasion of tumor cells using membrane-bound complement regulatory proteins." Immunol Today **20**(12): 576-82.

Gospodarowicz, D., N. Ferrara, et al. (1987). "Structural characterization and biological functions of fibroblast growth factor." Endocr Rev **8**(2): 95-114.

Heldin, C. H. (1995). "Dimerization of cell surface receptors in signal transduction." Cell **80**(2): 213-23.

Helmlinger, G., F. Yuan, et al. (1997). "Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation." Nat Med **3**(2): 177-82.



Hiratsuka, S., Y. Kataoka, et al. (2005). "Vascular endothelial growth factor A (VEGF-A) is involved in guidance of VEGF receptor-positive cells to the anterior portion of early embryos." Mol Cell Biol **25**(1): 355-63.

Holash, J., S. Davis, et al. (2002). "VEGF-Trap: a VEGF blocker with potent antitumor effects." Proc Natl Acad Sci U S A **99**(17): 11393-8.

Hosaka, T., K. Takase, et al. (2006). "Deletion analysis of the subunit genes of V-type Na+-ATPase from Enterococcus hirae." J Biochem (Tokyo) **139**(6): 1045-52.

Katoh, O., H. Tauchi, et al. (1995). "Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on

apoptotic cell death caused by ionizing radiation." Cancer Res **55**(23): 5687-92.

Kendall, R. L. and K. A. Thomas (1993). "Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor." Proc Natl Acad Sci U S A **90**(22): 10705-9.

Keyt, B. A., L. T. Berleau, et al. (1996). "The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency." J Biol Chem **271**(13): 7788-95.

Keyt, B. A., H. V. Nguyen, et al. (1996). "Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis." J Biol Chem **271**(10): 5638-46.

Kirschfink, M. (2001). "Targeting complement in therapy." Immunol Rev **180**: 177-89.

Li, J., C. Yen, et al. (1997). "PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer." Science **275**(5308): 1943-7.

Malich, G., B. Markovic, et al. (1997). "The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines." Toxicology **124**(3): 179-92.

Maniotis, A. J., R. Folberg, et al. (1999). "Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry." Am J Pathol **155**(3): 739-52.

Mesiano, S., N. Ferrara, et al. (1998). "Role of vascular endothelial growth factor in ovarian

cancer: inhibition of ascites formation by immunoneutralization." Am J Pathol **153**(4): 1249-56.

Mesri, E. A., H. J. Federoff, et al. (1995). "Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice." Circ Res **76**(2): 161-7.

Muller, Y. A., B. Li, et al. (1997). "Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site." Proc Natl Acad Sci U S A **94**(14): 7192-7.

Okada, F., J. W. Rak, et al. (1998). "Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells." Proc Natl Acad Sci U S A **95**(7): 3609-14.

Olsson, A. K., A. Dimberg, et al. (2006). "VEGF receptor signalling - in control of vascular function." Nat Rev Mol Cell Biol **7**(5): 359-71.

Pajusola, K., O. Aprelikova, et al. (1992). "FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines." Cancer Res **52**(20): 5738-43.

Pertovaara, L., A. Kaipainen, et al. (1994). "Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells." J Biol

Chem **269**(9): 6271-4.

Plate, K. H., G. Breier, et al. (1993). "Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis." Cancer Res **53**(23): 5822-7.

Press, O. W., J. P. Leonard, et al. (2001). "Immunotherapy of Non-Hodgkin's lymphomas." Hematology Am Soc Hematol Educ Program: 221-40.

Prewett, M., J. Huber, et al. (1999). "Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors." Cancer Res **59**(20): 5209-18.

Rosenberg, S. A., M. T. Lotze, et al. (1993). "Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer." J Natl Cancer Inst **85**(8): 622-32.

Ruckman, J., L. S. Green, et al. (1998). "2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain." J Biol Chem **273**(32): 20556-67.

Semenza, G. L. (2003). "Angiogenesis in ischemic and neoplastic disorders." Annu Rev Med **54**: 17-28.

Shalaby, F., J. Rossant, et al. (1995). "Failure of blood-island formation and vasculogenesis in

Flk-1-deficient mice." Nature **376**(6535): 62-6.

Shibusa, T., N. Shijubo, et al. (1998). "Tumor angiogenesis and vascular endothelial growth factor expression in stage I lung adenocarcinoma." Clin Cancer Res **4**(6): 1483-7.

Smith, J. K., N. M. Mamoon, et al. (2004). "Emerging roles of targeted small molecule protein-tyrosine kinase inhibitors in cancer therapy." Oncol Res **14**(4-5): 175-225.

Soker, S., S. Takashima, et al. (1998). "Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor." Cell **92**(6): 735-45.

Tsuzuki, Y., D. Fukumura, et al. (2000). "Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha--> hypoxia response element--> VEGF cascade differentially regulates vascular response and growth rate in tumors." Cancer Res **60**(22): 6248-52.

van Dijk, M. A. and J. G. van de Winkel (2001). "Human antibodies as next generation therapeutics." Curr Opin Chem Biol **5**(4): 368-74.

Waltenberger, J., L. Claesson-Welsh, et al. (1994). "Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor." J Biol Chem **269**(43): 26988-95.

Wiesmann, C., G. Fuh, et al. (1997). "Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor." Cell **91**(5): 695-704.

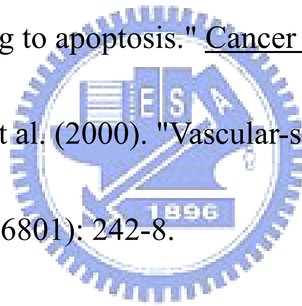
Willett, C. G., Y. Boucher, et al. (2004). "Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer." Nat Med **10**(2): 145-7.

Wolff, E. A., G. J. Schreiber, et al. (1993). "Monoclonal antibody homodimers: enhanced antitumor activity in nude mice." Cancer Res **53**(11): 2560-5.

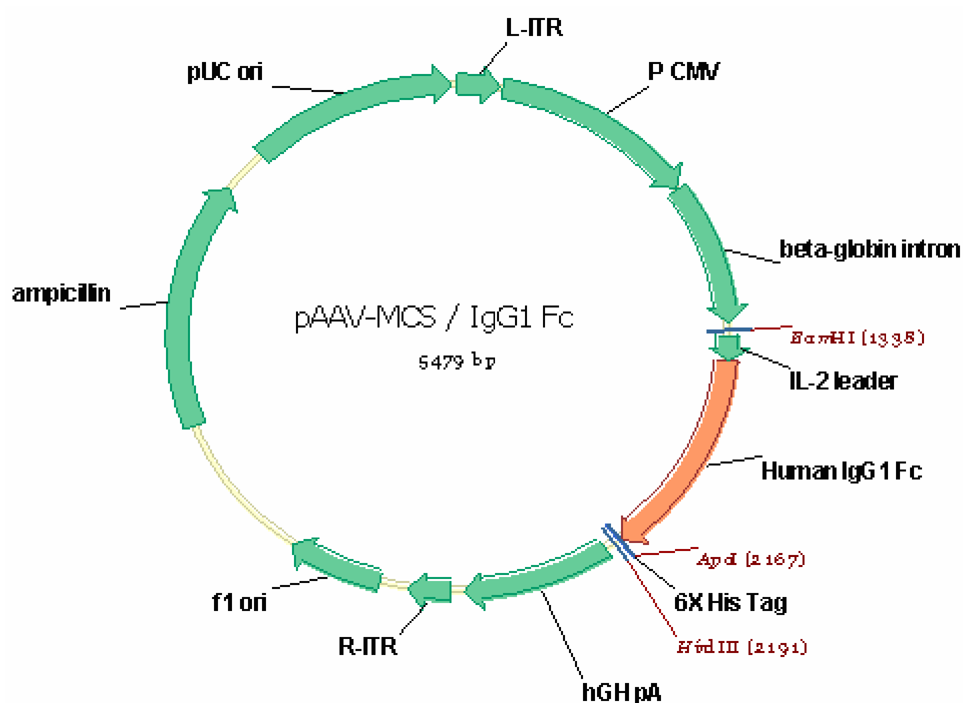
Wu, Y., A. T. Hooper, et al. (2006). "The vascular endothelial growth factor receptor (VEGFR-1) supports growth and survival of human breast carcinoma." Int J Cancer **119**(7): 1519-29.

Yakes, F. M., B. D. Wamil, et al. (2000). "CM101 treatment overrides tumor-induced immunoprivilege leading to apoptosis." Cancer Res **60**(20): 5740-6.

Yancopoulos, G. D., S. Davis, et al. (2000). "Vascular-specific growth factors and blood vessel formation." Nature **407**(6801): 242-8.



Appendices

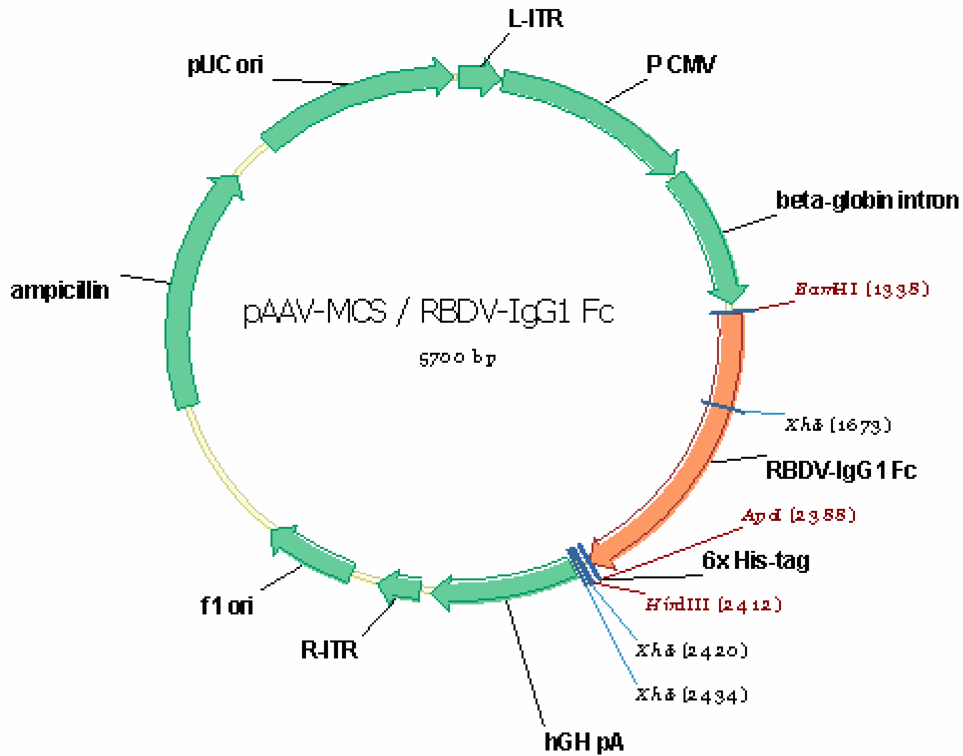


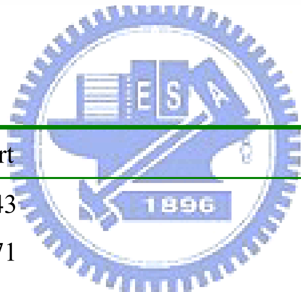
Description	start	end
IL-2 leader sequence	1357	1440
Human IgG1 Fc	1441	2166
L-ITR	1	141
CMV	150	812
Beta globin intron	820	1312
6X His Tag	2169	2189
Hgh Pa	2224	2702
R-ITR	2742	2882
F1 ori	2974	3280
Ampicillin	3799	4656
pUC ori	4807	5474
Apa1	2167	
BamH1	1338	
HindIII	2191	
XhoI	1452	

1 CCTGCAGGCA GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG
51 CCCGGGCGTC GGGCGACCTT TGGTCGCCCG GCCTCAGTGA GCGAGCGAGC
101 GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC TGCGGCCGCA
151 CGCGTGGAGC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT
201 AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC
251 TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG
301 TTCCCATAGT AACGTCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAG
351 TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
401 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
451 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC
501 GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA
551 TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA
601 TTGACGTCAA TGGGAGTTTG TTTTGCACCA AAATCAACGG GACTTTCCAA
651 AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA
701 CGGTGGGAGG TCTATATAAG CAGAGCTCGT TTAGTGAACC GTCAGATCGC
751 CTGGAGACGC CATCCACGCT GTTTTGACCT CCATAGAAGA CACCGGGACC
801 GATCCAGCCT CCGCGGATTC GAATCCCGGC CGGGAACGGT GCATTGGAAC
851 GCGGATTCCC CGTGCCAAGA GTGACGTAAG TACCGCCTAT AGAGTCTATA
901 GGCCCACAAA AAATGCTTTC TTCTTTTAAT ATACTTTTTT GTTTATCTTA
951 TTTCTAATAC TTTCCCTAAT CTCTTTCTTT CAGGGCAATA ATGATACAAT
1001 GTATCATGCC TCTTTGCACC ATTCTAAAGA ATAACAGTGA TAATTTCTGG
1051 GTTAAGGCAA TAGCAATATT TCTGCATATA AATATTTCTG CATATAAATT
1101 GTAACTGATG TAAGAGGTTT CATATTGCTA ATAGCAGCTA CAATCCAGCT
1151 ACCATTCTGC TTTTATTTTA TGGTTGGGAT AAGGCTGGAT TATTCTGAGT
1201 CCAAGCTAGG CCCTTTTGCT AATCATGTTC ATACCTCTTA TCTTCCTCCC
1251 ACAGCTCCTG GGCAACGTGC TGGTCTGTGT GCTGGCCCAT CACTTTGGCA
1301 AAGAATTGGG ATTCGAACAT CGATTGAATT CCCCGGGGAT CCACTAGTCC
1351 AGTGTGGTGG AATTGCCCTT ATGTACAGGA TGCAACTCCT GTCTTGCATT
1401 GCACTAAGTC TTGCACTTGT CACAAACAGT ATCCAGCACA GTGGCGGCCG
1451 CTCGAGTGAG CCCAAATCTT GTGACAAAAC TCACACATGC CCACCGTGCC
1501 CAGCACCCGA ACTCCTGGGG GGACCGTCAG TCTTCCTCTT CCCCCCAAAA
1551 CCCAAGGACA CCCTCATGAT CTCCCGGACC CCTGAGGTCA CATGCGTGGT
1601 GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC TGGTACGTGG
1651 ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA GGAGCAGTAC
1701 AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC ACCAGGACTG
1751 GCTGAATGGC AAGGAGTACA AGTGCAAGGT CTCCAACAAA GCCCTCCCAG
1801 CCCCCATCGA GAAAACCATC TCCAAAGCCA AGGGGCAGCC CCGAGAACCA

1851 CAGGTGTACA CCCTGCCCCC ATCCCGGGAT GAGCTGACCA AGAACCAGGT
1901 CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC ATCGCCGTGG
1951 AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC CACGCCTCCC
2001 GTGCTGGACT CCGACGGCTC CTTCTTCCTC TACAGCAAGC TCACCGTGGA
2051 CAAGAGCAGG TGGCAGCAGG GGAACGTCTT CTCATGCTCC GTGATGCATG
2101 AGGCTCTGCA CAACCACTAC ACGCAGAAGA GCCTCTCCCT GTCTCCGGGT
2151 AAAGTCGACG AGGGCCCGCA TCATCACCAT CACCATTGAA AGCTTGCCTC
2201 GAGCAGCGCT GCTCGAGAGA TCTACGGGTG GCATCCCTGT GACCCCTCCC
2251 CAGTGCCTCT CCTGGCCCTG GAAGTTGCCA CTCCAGTGCC CACCAGCCTT
2301 GTCCTAATAA AATTAAGTTG CATCATTTTG TCTGACTAGG TGTCCTTCTA
2351 TAATATTATG GGGTGGAGGG GGGTGGTATG GAGCAAGGGG CAAGTTGGGA
2401 AGACAACCTG TAGGGCCTGC GGGGTCTATT GGGAACCAAG CTGGAGTGCA
2451 GTGGCACAAT CTTGGCTCAC TGCAATCTCC GCCTCCTGGG TTCAAGCGAT
2501 TCTCCTGCCT CAGCCTCCCG AGTTGTTGGG ATTCCAGGCA TGCATGACCA
2551 GGCTCAGCTA ATTTTTGTTT TTTTGGTAGA GACGGGGTTT CACCATATTG
2601 GCCAGGCTGG TCTCCAACTC CTAATCTCAG GTGATCTACC CACCTTGGCC
2651 TCCCAAATTG CTGGGATTAC AGGCGTGAAC CACTGCTCCC TTCCCTGTCC
2701 TTCTGATTTT GTAGGTAACC ACGTGCGGAC CGAGCGGCCG CAGGAACCCC
2751 TAGTGATGGA GTTGGCCACT CCCTCTCTGC GCGCTCGCTC GCTCACTGAG
2801 GCCGGGCGAC CAAAGGTCGC CCGACGCCCG GGCTTTGCCC GGGCGGCCTC
2851 AGTGAGCGAG CGAGCGCGCA GCTGCCTGCA GGGGCGCCTG ATGCGGTATT
2901 TTCTCCTTAC GCATCTGTGC GGTATTTCAC ACCGCATACG TCAAAGCAAC
2951 CATAGTACGC GCCCTGTAGC GGCGCATTAA GCGCGGCGGG TGTGGTGGTT
3001 ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT
3051 CGCTTTCTTC CCTTCCTTTC TCGCCACGTT CGCCGGCTTT CCCCGTCAAG
3101 CTCTAAATCG GGGGCTCCCT TTAGGGTTCC GATTTAGTGC TTTACGGCAC
3151 CTCGACCCCA AAAAACTTGA TTTGGGTGAT GGTTCACGTA GTGGGCCATC
3201 GCCCTGATAG ACGGTTTTTC GCCCTTTGAC GTTGGAGTCC ACGTTCTTTA
3251 ATAGTGGACT CTTGTTCCAA ACTGGAACAA CACTCAACCC TATCTCGGGC
3301 TATTCTTTTG ATTTATAAGG GATTTTGCCG ATTTCGGCCT ATTGGTTAAA
3351 AAATGAGCTG ATTTAACAAA AATTTAACGC GAATTTTAAC AAAATATTAA
3401 CGTTTACAAT TTTATGGTGC ACTCTCAGTA CAATCTGCTC TGATGCCGCA
3451 TAGTTAAGCC AGCCCCGACA CCCGCCAACA CCCGCTGACG CGCCCTGACG
3501 GGCTTGTCTG CTCCCGGCAT CCGCTTACAG ACAAGCTGTG ACCGTCTCCG
3551 GGAGCTGCAT GTGTCAGAGG TTTTCACCGT CATCACCGAA ACGCGCGAGA
3601 CGAAAGGGCC TCGTGATACG CCTATTTTTA TAGGTTAATG TCATGATAAT
3651 AATGGTTTCT TAGACGTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA
3701 CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG
3751 AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT

3801 GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT
3851 GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT
3901 GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG
3951 CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA
4001 GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC
4051 GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT
4101 TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
4151 GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC
4201 TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA
4251 CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA
4301 ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGTAGCAATG
4351 GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC
4401 CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC
4451 TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA
4501 GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG
4551 TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA
4601 TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG
4651 CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT
4701 AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA
4751 ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA
4801 GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG
4851 CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT
4901 GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC
4951 AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG
5001 CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA
5051 TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG
5101 TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC
5151 GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC
5201 TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG
5251 AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG
5301 CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG
5351 GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG
5401 GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT
5451 GGCCTTTTGC TGGCCTTTTG CTCACATGT





Dscription	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
Apa1	2388	
BamH1	1338	
HindIII	2412	
XhoI	1673	

1 CCTGCAGGCA GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG
51 CCCGGGCGTC GGGCGACCTT TGGTCGCCCG GCCTCAGTGA GCGAGCGAGC
101 GCGCAGAGAG GGAGTGGCCA ACTCCATCAC TAGGGGTTCC TGCGGCCGCA
151 CGCGTGGAGC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT
201 AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC
251 TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG
301 TTCCCATAGT AACGTCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAG
351 TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
401 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
451 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC
501 GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA
551 TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA
601 TTGACGTCAA TGGGAGTTTG TTTTGCACCA AAATCAACGG GACTTTCCAA
651 AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA
701 CGGTGGGAGG TCTATATAAG CAGAGCTCGT TTAGTGAACC GTCAGATCGC
751 CTGGAGACGC CATCCACGCT GTTTTGACCT CCATAGAAGA CACCGGGACC
801 GATCCAGCCT CCGCGGATTC GAATCCCGGC CGGGAACGGT GCATTGGAAC
851 GCGGATTCCC CGTGCCAAGA GTGACGTAAG TACCGCCTAT AGAGTCTATA
901 GGCCCACAAA AAATGCTTTC TTCTTTTAAT ATACTTTTTT GTTTATCTTA
951 TTTCTAATAC TTTCCCTAAT CTCTTTCTTT CAGGGCAATA ATGATACAAT
1001 GTATCATGCC TCTTTGCACC ATTCTAAAGA ATAACAGTGA TAATTTCTGG
1051 GTTAAGGCAA TAGCAATATT TCTGCATATA AATATTTCTG CATATAAATT
1101 GTAACTGATG TAAGAGGTTT CATATTGCTA ATAGCAGCTA CAATCCAGCT
1151 ACCATTCTGC TTTTATTTTA TGGTTGGGAT AAGGCTGGAT TATTCTGAGT
1201 CCAAGCTAGG CCCTTTTGCT AATCATGTTC ATACCTCTTA TCTTCCTCCC
1251 ACAGCTCCTG GGCAACGTGC TGGTCTGTGT GCTGGCCCAT CACTTTGGCA
1301 AAGAATTGGG ATTCGAACAT CGATTGAATT CCCCGGGGAT CCATGAACTT
1351 TCTGCTGTCT TGGGTGCATT GGAGCCTTGC CTTGCTGCTC TACCTCCACC
1401 ATGCCAAGTG GTCCCAGGCT GCACCCATGG CAGAAGGAGG AGGGCAGAAT
1451 CATCACGAAG TGGTGAAGTT CATGGATGTC TATCAGCGCA GCTACTGCCA
1501 TCCAATCGAG ACCCTGGTGG ACATCTTCCA GGAGTACCCT GATGAGATCG
1551 AGTACATCTT CAAGCCATCC TGTGTGCCCC TGATGCGATG CGGGGGCTGC
1601 TGCAATGACG AGGGCCTGGA GTGTGTGCCC ACTGAGGAGT CCAACATCAC
1651 CATGCAGATT ATGCGGATCA TCTCGAGTGA GCCCAAATCT TGTGACAAAA
1701 CTCACACATG CCCACCGTGC CCAGCACCCG AACTCCTGGG GGGACCGTCA
1751 GTCTTCCTCT TCCCCCCAAA ACCCAAGGAC ACCCTCATGA TCTCCCGGAC

1801 CCCTGAGGTC ACATGCGTGG TGGTGGACGT GAGCCACGAA GACCCTGAGG
1851 TCAAGTTCAA CTGGTACGTG GACGGCGTGG AGGTGCATAA TGCCAAGACA
1901 AAGCCGCGGG AGGAGCAGTA CAACAGCACG TACCGTGTGG TCAGCGTCCT
1951 CACCGTCCTG CACCAGGACT GGCTGAATGG CAAGGAGTAC AAGTGCAAGG
2001 TCTCCAACAA AGCCCTCCCA GCCCCCATCG AGAAAACCAT CTCCAAAGCC
2051 AAGGGGCAGC CCCGAGAACC ACAGGTGTAC ACCCTGCCCC CATCCCGGGA
2101 TGAGCTGACC AAGAACCAGG TCAGCCTGAC CTGCCTGGTC AAAGGCTTCT
2151 ATCCCAGCGA CATCGCCGTG GAGTGGGAGA GCAATGGGCA GCCGGAGAAC
2201 AACTACAAGA CCACGCCTCC CGTGCTGGAC TCCGACGGCT CCTTCTTCCT
2251 CTACAGCAAG CTCACCGTGG ACAAGAGCAG GTGGCAGCAG GGGAACGTCT
2301 TCTCATGCTC CGTGATGCAT GAGGCTCTGC ACAACCACTA CACGCAGAAG
2351 AGCCTCTCCC TGTCTCCGGG TAAAGTCGAC GAGGGCCCGC ATCATCACCA
2401 TCACCATTGA AAGCTTGCCT CGAGCAGCGC TGCTCGAGAG ATCTACGGGT
2451 GGCATCCCTG TGACCCCTCC CCAGTGCCTC TCCTGGCCCT GGAAGTTGCC
2501 ACTCCAGTGC CCACCAGCCT TGTCCTAATA AAATTAAGTT GCATCATTTT
2551 GTCTGACTAG GTGTCCTTCT ATAATATTAT GGGGTGGAGG GGGGTGGTAT
2601 GGAGCAAGGG GCAAGTTGGG AAGACAACCT GTAGGGCCTG CGGGGTCTAT
2651 TGGGAACCAA GCTGGAGTGC AGTGGCACAA TCTTGGCTCA CTGCAATCTC
2701 CGCCTCCTGG GTTCAAGCGA TTCTCCTGCC TCAGCCTCCC GAGTTGTTGG
2751 GATTCCAGGC ATGCATGACC AGGCTCAGCT AATTTTTGTT TTTTTGGTAG
2801 AGACGGGGTT TCACCATATT GGCCAGGCTG GTCTCCAACT CCTAATCTCA
2851 GGTGATCTAC CCACCTTGGC CTCCCAAATT GCTGGGATTA CAGGCGTGAA
2901 CCACTGCTCC CTTCCCTGTC CTTCTGATTT TGTAGGTAAC CACGTGCGGA
2951 CCGAGCGGCC GCAGGAACCC CTAGTGATGG AGTTGGCCAC TCCCTCTCTG
3001 CGCGCTCGCT CGCTCACTGA GGCCGGGCGA CCAAAGGTCG CCCGACGCCC
3051 GGGCTTTGCC CGGGCGGCCT CAGTGAGCGA GCGAGCGCGC AGCTGCCTGC
3101 AGGGGCGCCT GATGCGGTAT TTTCTCCTTA CGCATCTGTG CGGTATTTCA
3151 CACCGCATAC GTCAAAGCAA CCATAGTACG CGCCCTGTAG CGGCGCATTA
3201 AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA CACTTGCCAG
3251 CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT CTCGCCACGT
3301 TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC TTTAGGGTTC
3351 CGATTTAGTG CTTTACGGCA CCTCGACCCC AAAAAACTTG ATTTGGGTGA
3401 TGGTTCACGT AGTGGGCCAT CGCCCTGATA GACGGTTTTT CGCCCTTTGA
3451 CGTTGGAGTC CACGTTCTTT AATAGTGGAC TCTTGTTCCA AACTGGAACA
3501 ACACTCAACC CTATCTCGGG CTATTCTTTT GATTTATAAG GGATTTTGCC
3551 GATTTCGGCC TATTGGTTAA AAAATGAGCT GATTTAACAA AAATTTAACG
3601 CGAATTTTAA CAAAATATTA ACGTTTACAA TTTTATGGTG CACTCTCAGT
3651 ACAATCTGCT CTGATGCCGC ATAGTTAAGC CAGCCCCGAC ACCCGCCAAC
3701 ACCCGCTGAC GCGCCCTGAC GGGCTTGTCT GCTCCCGGCA TCCGCTTACA

3751 GACAAGCTGT GACCGTCTCC GGGAGCTGCA TGTGTCAGAG GTTTTCACCG
3801 TCATCACCGA AACGCGCGAG ACGAAAGGGC CTCGTGATAC GCCTATTTTT
3851 ATAGGTTAAT GTCATGATAA TAATGGTTTC TTAGACGTCA GGTGGCACTT
3901 TTCGGGGAAA TGTGCGCGGA ACCCCTATTT GTTTATTTTT CTAAATACAT
3951 TCAAATATGT ATCCGCTCAT GAGACAATAA CCCTGATAAA TGCTTCAATA
4001 ATATTGAAAA AGGAAGAGTA TGAGTATTCA ACATTTCCGT GTCGCCCTTA
4051 TTCCCTTTTT TGCGGCATTT TGCCTTCCTG TTTTTGCTCA CCCAGAAACG
4101 CTGGTGAAAG TAAAAGATGC TGAAGATCAG TTGGGTGCAC GAGTGGGTTA
4151 CATCGAACTG GATCTCAACA GCGGTAAGAT CCTTGAGAGT TTTCGCCCCG
4201 AAGAACGTTT TCCAATGATG AGCACTTTTA AAGTTCTGCT ATGTGGCGCG
4251 GTATTATCCC GTATTGACGC CGGGCAAGAG CAACTCGGTC GCCGCATACA
4301 CTATTCTCAG AATGACTTGG TTGAGTACTC ACCAGTCACA GAAAAGCATC
4351 TTACGGATGG CATGACAGTA AGAGAATTAT GCAGTGCTGC CATAACCATG
4401 AGTGATAACA CTGCGGCCAA CTTACTTCTG ACAACGATCG GAGGACCGAA
4451 GGAGCTAACC GCTTTTTTGC ACAACATGGG GGATCATGTA ACTCGCCTTG
4501 ATCGTTGGGA ACCGGAGCTG AATGAAGCCA TACCAAACGA CGAGCGTGAC
4551 ACCACGATGC CTGTAGCAAT GGCAACAACG TTGCGCAAAC TATTAACTGG
4601 CGAACTACTT ACTCTAGCTT CCCGGCAACA ATTAATAGAC TGGATGGAGG
4651 CGGATAAAGT TGCAGGACCA CTTCTGCGCT CGGCCCTTCC GGCTGGCTGG
4701 TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC GCGGTATCAT
4751 TGCAGCACTG GGGCCAGATG GTAAGCCCTC CCGTATCGTA GTTATCTACA
4801 CGACGGGGAG TCAGGCAACT ATGGATGAAC GAAATAGACA GATCGCTGAG
4851 ATAGGTGCCT CACTGATTAA GCATTGGTAA CTGTCAGACC AAGTTTACTC
4901 ATATATACTT TAGATTGATT TAAAACTTCA TTTTTAATTT AAAAGGATCT
4951 AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC TTAACGTGAG
5001 TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC
5051 TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC
5101 CACCGCTACC AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT ACCAACTCTT
5151 TTTCCGAAGG TAACTGGCTT CAGCAGAGCG CAGATACCAA ATACTGTCCT
5201 TCTAGTGTAG CCGTAGTTAG GCCACCACTT CAAGAACTCT GTAGCACCGC
5251 CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC TGCCAGTGGC
5301 GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT TACCGGATAA
5351 GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC GTGCACACAG CCCAGCTTGG
5401 AGCGAACGAC CTACACCGAA CTGAGATACC TACAGCGTGA GCTATGAGAA
5451 AGCGCCACGC TTCCCGAAGG GAGAAAGGCG GACAGGTATC CGGTAAGCGG
5501 CAGGGTCGGA ACAGGAGAGC GCACGAGGGA GCTTCCAGGG GGAAACGCCT
5551 GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT TGAGCGTCGA
5601 TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA ACGCCAGCAA
5651 CGCGGCCTTT TTACGGTTCC TGGCCTTTTG CTGGCCTTTT GCTCACATG