약학석사학위논문

Mechanism study of Doppel protein in human colorectal cancer

인간 대장암에서 도펠 단백질의 메커니즘 규명

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이소정의 석사 학위논문을 인준함

2018년 8월

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Abstract

Mechanism study of Doppel protein in human colorectal cancer

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Angiogenesis is a biological process that is essential not only for normal tissue growth and development but also for cancer growth and metastasis. Although anticancer agents that inhibit angiogenesis have been actively developed, there is a limit in that the target of these agents are commonly expressed in both normal tissues and cancers, so that the angiogenesis is inhibited without any distinction. Herein, this study targeted the colorectal cancer and identified the mechanism of protein 'Doppel,' which is expressed much more in tumor endothelial cells than normal endothelial cells.

Experimental results show that ‘Doppel’ antibody reduced phosphorylation of VEGFR-2 & FGFR-1, the two most essential receptors involved in angiogenesis. Also, ‘Doppel’ was located from 10 nm range with FGFR-1 on the cell membrane.

Furthermore, ‘Doppel’ antibody also affected β-catenin and STAT5a which are related to VEGF-A expression and sensitivity of chemotherapeutic agents to colorectal cancer. In animal models of colorectal cancer, Doppel’ antibody showed more than 50% anticancer effect. Taken together, ‘Doppel’ is associated with both angiogenesis and chemotherapeutic sensitivity in colorectal cancer. Studying of Doppel's role in colorectal cancer for the first time, gives a great novelty to this paper. The detailed mechanism will allow the colorectal cancer patients to use the ‘Doppel’ antibodies as a new way to overcome the limitations of cancer recurrence, resistance, and side effects associated with conventional anticancer agents.

Keywords : Doppel, Human colorectal cancer, Angiogenesis, VEGFR-2, FGFR-1, Resistance to anticancer drugs, Anticancer antibody treatment

Student Number : 2016-27629

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1. Introduction

1.1 Doppel

Doppel (Dpl; also known as PRND protein; UniProtKB-Q9UKY0) is prion-like protein. It has 25% sequence homology with normal prion protein. Doppel is composed of 176 amino acids and the length of the chain excluding the signal peptide and the propeptide is 127. The NMR structure of the recombinant human Doppel protein hDpl (24-152) revealed that the Doppel is composed of the globular region and the flexible N-terminal tail portion. The globular domain consists of four α-helixes and short two-strand anti-parallel β-sheets. Doppel is also attached to the plasma membrane(PM) via the GPI anchor at the C-terminus [1] **(Figure 1).** The molecular weight of Doppel is 20.293 kDa. However, it can be larger due to post-translational modifications which are N- and O-glycosylation. Doppel is expressed in neonatal brain endothelium, or in testicular cells in adults. Also, previous studies have shown that Doppel gene is expressed in solid Tumor Endothelial Cell (TEC)[2] and Doppel is ten times more expressed in TEC than in Normal Endothelial Cells [3]. Doppel screening in various types of mouse solid tumor endothelial cell and solid tumor cell was performed in our lab and found that Doppel is expressed at different degrees depending on the type of solid tumors [4]. In addition to a solid tumor, Doppel is also expressed in bone marrow cells of patients with acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS) [5]. Therefore, Doppel is emerging as a new target for

tumor. This paper investigated the role of Doppel in a human colorectal cancer (CRC), especially focused on human colonic tumor endothelial cell (HCTEC) and conducted study related to angiogenesis.

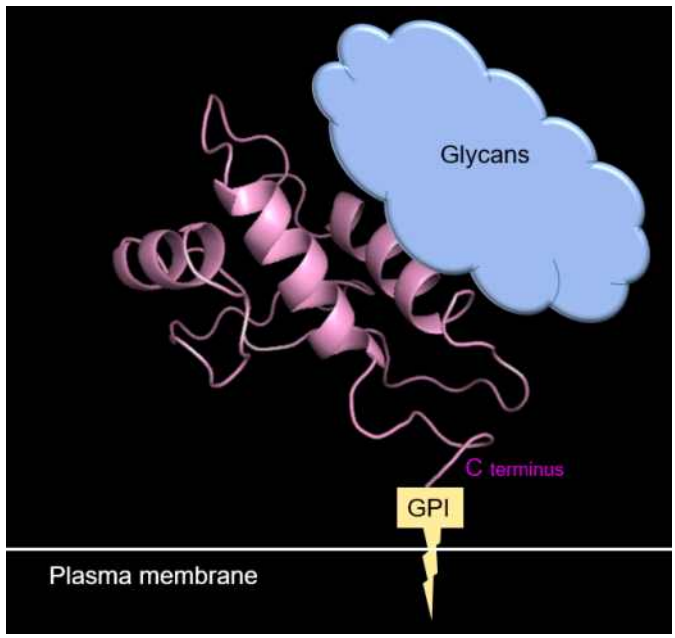


Figure 1. Structure of Doppel. For simplicity, the flexible tail at the N-terminus is not represented in the figure.

1.2 Tumor angiogenesis

Angiogenesis is a vital process that provides oxygen and nutrients to normal tissues for growth and development. Also it is important in tumor for proliferation and metastasis. Various precursors and angiogenic factors control this process. Angiogenic factors include angiogenesis inducer and angiogenesis inhibitor. Angiogenesis inducer includes various soluble growth factors such as VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) and PDGF (Platelet-derived growth factor), etc. They induce Endothelial Cell (EC) proliferation, migration, and differentiation. Angiogenesis inhibitor includes Angiostatin, Endostatin, Vasostatin, etc. They Inhibit EC proliferation/migration and induce EC apoptosis (Table 1). For our body to work in harmony, balance between the angiogenic inducer and the inhibitor is essential.

Several diseases are caused by an imbalance of angiogenesis such as insufficient or excessive angiogenesis. Insufficient angiogenesis can result in myocardial ischemia, peripheral ischemia, cerebral ischemia, insufficient wound healing, peptic ulcer, and diabetic foot ulcer disease. Studies have been actively undertaken to overcome these diseases by stimulating angiogenesis. For example, In some preclinical studies and Phase I clinical studies, orally administrated bFGF induced angiogenesis and healed gastrointestinal ulceration [6]. In contrast, excessive angiogenesis leads to various diseases such as hemangiomas, Kaposi's sarcoma, ocular neovascularization, rheumatoid arthritis, and atherosclerosis. Furthermore, tumor growth and metastasis are typical phenomena of uncontrolled angiogenesis-related

diseases (Table 2) [6]. Especially, angiogenesis is essential when the tumor needs to keep growing and proliferating over the $2-3mm^2$ size. There are two types of angiogenesis, Sprouting angiogenesis and intussusceptive angiogenesis (Figure 2). This paper will explain these two types of angiogenesis thoroughly.

Table 1. Overview of the different angiogenic factors.

Category	Names	Major functions
Angiogenesis inducers	Vascular endothelial growth factor family (VEGF-A or VEGF, P1GF, VGGF-B, VEGF-C, VEGF-D, orf virus VEGF or VEGF-E), Fibroblast growth factor family (aFGF, bFGF, etc.), Angiopoietin 1 (Ang-1), Transforming growth factor-alpha/beta (TGF α/β), Platelet-derived growth factor (PDGF), Hepatocyte growth factor/ scatter factor (HGF/SF), Tumor necrosis factor-alpha (TNFα), Interleukin-1/8, angiogenin, ephrins, integrins $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha 5\beta 1$, Cyclooxygenase-2 (COX-2)	(i) Induction of EC proliferation, migration, and differentiation (ii) TGF-β shows opposite effect in some contexts
Angiogenesis inhibitors	Thrombospondin-1/2 (TSP-1/2), Angiostatin(plasminogen fragment), Endostatin (collagen XVIII fragment), Vasostatin (calreticulin fragment), Tumstatin, Platelet factor-4 (PF4), anti-angiogenic antithrombin III, kringle 5 (plasminogen fragment), Prolactin 16-kD fragment, fragment of SPARC, 2-methoxyestradiol, Metalloproteinase inhibitors (TIMPs),Interferon-alpha/beta/gamma (IFN $\alpha/\beta/\gamma$), Interleukin-12 (IL-12), IP-10, Ang-2	(i) Inhibit EC proliferation/migration (ii) Induce EC apoptosis (iii) TIMPs: inhibit MMP or uPA activity (iv) Ang-2: inhibit blood vessels maturation, antagonist of Ang-1

* Ref: So Young Yoo and Sang Mo Kwon. Mediators of Inflammation, Volume 2013 (2013), Article ID 127170, 11 pages

Table 2. Several diseases caused by an imbalance of angiogenesis.

Cause	Diseases
Insufficent angiogenesis	Induction of collateral vessel formation (Myocardial ischemia, Peripheral ischemia, Cerebral ischemia), In sufficient Wound healing, Diabetic foot ulcer, Peptic ulcer
Excessive angiogenesis	Hemangiomas, Psoriasis, Kaposi' s sarcoma, Ocular neovascularization, Rheumatoid arthritis, Endometriosis, Atherosclerosis, Tumor growth and metastasis

*Ref: So Young Yoo and Sang Mo Kwon. Mediators of Inflammation, Volume 2013 (2013), Article ID 127170, 11 pages

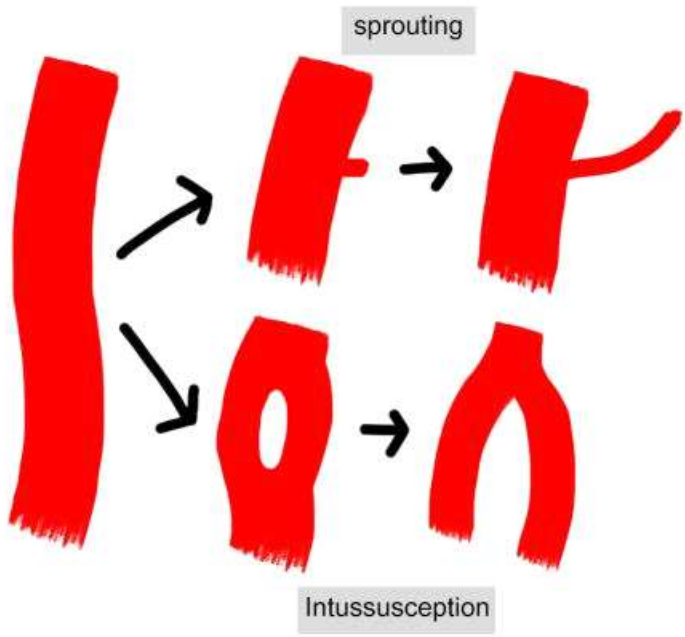


Figure 2. Two different types of angiogenesis.

1) Sprouting angiogenesis

Sprouting angiogenesis is the formation of entirely new blood vessels from existing blood vessels. This type of angiogenesis consists of four steps. First, Angiogenic factors (VEGFs, FGFs etc) bind to the respective receptors of endothelial cells in pre-existing blood vessels, activating the receptor, resulting in lower signal transduction. Second, as the receptor is activated, the protease is secreted, the basement membrane and extracellular matrix surrounding the endothelial cells are degraded. Thus, the endothelial cells can escape from the existing blood vessels and invade the surrounding matrix. Third, Endothelial cells escaped from the existing blood vessels proliferate and migrate and form vessel lumens with the help of integrin, adhesion molecules. Eventually, an immature blood vessel is formed. Finally, Immature blood vessels are stabilized by pericyte cells, smooth muscle cells and various molecules [7]. The major angiogenic factors and receptors involved in sprouting angiogenesis are VEGF-A / VEGFR-2 and bFGF / FGFR2.

2) Intussusceptive angiogenesis

Intussusceptive angiogenesis, also known as splitting angiogenesis, is the creation of new blood vessels by splitting the existing blood vessels into two. This process involves only endothelial cell migration and vascular remodeling which does not require endothelial cell proliferation. Therefore, it can be rapidly progressed within hours or minutes and is particularly crucial in embryonic development. Not only Sprouting angiogenesis but also Intussusceptive angiogenesis consists of four steps. First, Endothelial cells of two opposing capillary walls contact and a translumbar bridge is created. Second, the endothelial cell junctions are reconstituted and the vascular bilayer is pierced, allowing growth factors and cells to penetrate into the lumen. Third, a core is formed between two new blood vessels in the contact area. The pericytes and myofibroblasts invade and cover the newly formed core. Finally, two separate blood vessels are formed. Changes in blood flow in the arterial branch, changes in shear stress due to endothelium and perivascular stress, platelet-derived growth factor-B (PDGF-B), angiopoietins (Ang-2), and their receptors (Tie) cause intussusceptive angiogenesis [7].

1.3 VEGF and VEGF receptor

Receptor Tyrosine Kinases (RTKs) are transmembrane proteins that act as receptors for growth factors, neurotrophic factors, and other cell signaling molecules. When the ligand binds to the receptor, phosphorylation occurs at the tyrosine residue of the tyrosine kinase located in the intracellular domain of the receptor. Due to this event, the signal transduction starts through a downstream signal, and it is critically related to cell proliferation, survival, and motility. If not adequately controlled, it causes various diseases including cancer. There are more than 50 different RTKs, but the five most important cancer-related RTKs are Vascular Endothelial Growth Factor Receptor (VEGFR), Fibroblast Growth Factor Receptor (FGFR), Platelet-Derived Growth Factor Receptor (PDGFR), Epidermal Growth Factor Receptor (EGFR), and Insulin-like Growth Factor Receptor (IGFR) **(Figure 3)**. These five RTKs affect the process of angiogenesis, proliferation, invasion, metastasis, and apoptosis. Due to its diverse involvement in the cell process, It is essential to regulate these RTKs properly. Uncontrolled RTKs could promote cancer growth. Controlling the RTKs is like a double-edged sword.

Among these five RTKs, VEGFR is the most important RTK which is related to all of these five process (angiogenesis, proliferation, invaison, metastasis, apoptosis). Oftenly, it is over expressed in almost every cancer. Therefore, it is the primary target site in cancer therapy. VEGF receptors (VEGFR) are cell surface receptor tyrosine kinases (RTKs) as receptors for vascular endothelial growth factor (VEGF). There are three

subtypes: VEGFR1 (Flt-1), VEGFR-2 (KDR / Flk-1), and VEGFR3 (Flt-4). The ligand, Vascular endothelial growth factor (VEGF) family consists of six members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor) and plays a vital role in stimulating angiogenesis by binding to VEGF receptors on the cell surface. By activation of VEGFR, transphosphorylation occurs in the kinase domain which stimulates endothelial cell proliferation and migration are stimulated. VEGF-A binds to VEGFR-1 and 2. VEGF-B binds to VEGFR-1, VEGF-C and D bind to VEGFR-2 and 3. Lastly, VEGF-E binds to VEGFR-2 **(Figure 4).** Among these, the most important pair in angiogenesis is known as the interaction between VEGF-A and VEGFR-2.

VEGFR-1 plays either positive or negative regulatory role in angiogenesis according to biological conditions. When it plays a positive regulatory role, it promotes angiogenesis in tumors. In contrast, when it plays a negative regulatory role, VEGFR-1 acts as a decoy receptor that forms a non-productive signaling complex that isolates VEGF-A from VEGFR-2 using the characteristics of higher affinity for VEGF-A than VEGFR-2 [8]. VEGFR-3 plays an important role in lymphangiogenesis. When VEGFR-3 is defective, it affects lymphatic endothelial function and causes lymphatic disorders.

VEGFR-2 is the most important regulator of angiogenesis. It has three different forms. Unglycosylated, immature VEGFR-2 has a molecular weight of approximately 152 kDa. Partially glycosylated VEGFR-2 has a molecular weight of 200 kDa and fully glycosylated VEGFR-2 has a molecular weight of 230 kDa [9]. VEGFR-2 presents as a monomer or

inactivated dimer when the ligand VEGF is not bound and it presents as activated dimer when the ligand is bound [10]. VEGFR-2 consists of extracellular (EC) domain, transmembrane (TM) domain and intracellular (IC) domain. Extracellular domain consist of 7 immunoglobulin (Ig) like domains (D1, D2, D3, D4, D5, D6, D7). D1 is located on the N-terminal side of the receptor which is furthest from the cell membrane, and D7 is located closest to the cell membrane. VEGFs form disulfide-linked anti-parallel homo dimers and bind between D2 and D3 of the VEGFR-2 dimer. Homologous interaction of D4 and D7 between each receptor of VEGFR-2 dimer is essential for VEGFR-2 activation [11]. VEGFR-2 forms a dimer in the plasma membrane even in the absence of the ligand VEGF (inactivated dimer), but the transmembrane domain does not have sufficient orientation to phosphorylate the receptor. A suitable structure of the transmembrane domain stabilizes and activates the VEGFR-2 dimer. The intracellular domain has diverse tyrosine kinase residues such as Tyr801, Tyr951, Tyr1054, Tyr1059, Tyr1175, Tyr1214, Tyr1223, Tyr1305, Tyr1309, and Tyr1319 which ultimately influences angiogenesis by regulating endothelial cell proliferation, migration and survival [8].

Tyr1175 (Y1175) of VEGFR-2 is the major auto-phosphorylation site after VEGF binding and acts as a binding site for phospholipase C-γ (PLC-γ) which causes cell proliferation through the mitogen-activated protein kinase (MAPK) pathway. It also acts as a binding site for Shb and affects endothelial cell migration, cell survival and vascular permeability. The second major auto-phosphorylation site of

VEGFR-2 is Tyr1214 (Y1214), which is involved in the activation of cell division control protein 42 homolog (Cdc42) and p38 mitogen-activated protein kinase (P38 MAPK) and regulates cell motility. In addition, Tyr951 binds to the T-cell-specific adapter (TSAd) and regulates vascular permeability and cell migration by activating the Src pathway. Tyr1054/Tyr1059 controls kinase activity and Tyr801 controls Tyr1054, Tyr1059 phosphorylation. **(Figure 5)** [12].

Because VEGFR-2 is the most important and potent modulator of angiogenesis, many scientists have targeted VEGFs, VEGFR-2 and its sub-signal pathways in cancer drug development. VEGFR-targeting monoclonal antibodies (ramucirumab), VEGF-targeting monoclonal antibodies (bevacizumab, ranibizumab, Ziv-aflibercept), VEGF binding aptamer (pegaptanib), VEGF-Trap, small-molecule tyrosine kinase inhibitors (sorafenib, sunitinib, vatalanib, pazopanib, axitinib, cabozantinib, and regorafenib), antisense and siRNA are examples of targeted therapy [12, 13].

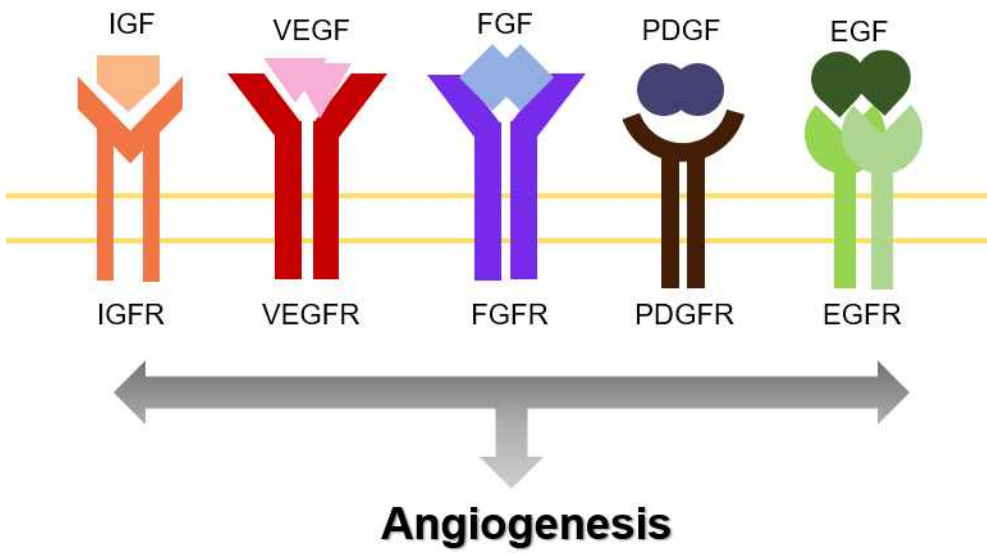


Figure 3. Five most important cancer-related RTKs. IGFR; Insulin-like Growth Factor Receptor, VEGFR; Vascular Endothelial Growth Factor Receptor, FGFR; Fibroblast Growth Factor Receptor, PDGFR; Platelet-Derived Growth Factor Receptor and EGFR; Epidermal Growth Factor Receptor.

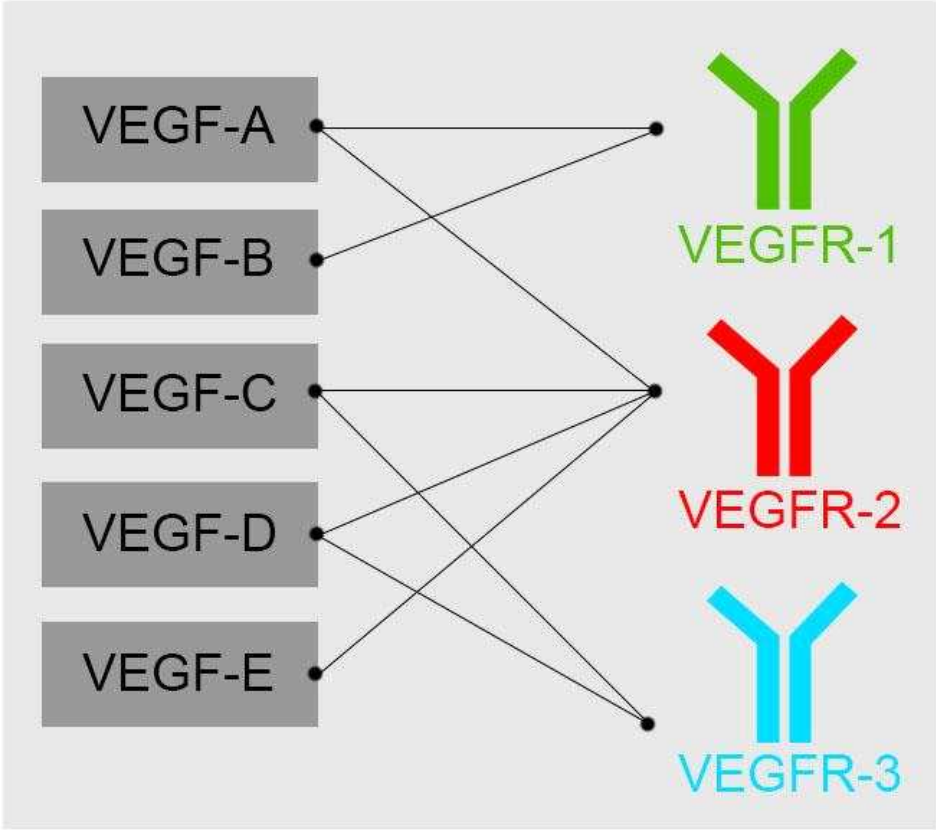


Figure 4. Different types of VEGF and its' receptors

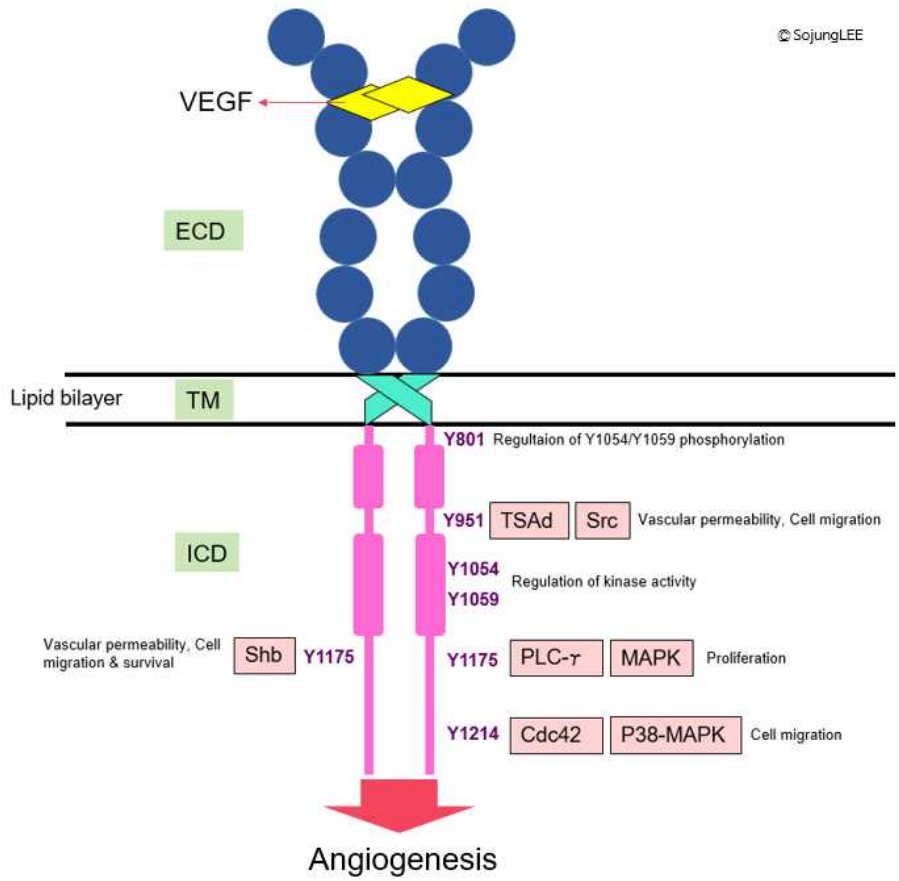


Figure 5. VEGFR-2 structure and signaling pathway.

· Blue circle: Immunoglobulin (Ig) like domains of VEGFR-2, · Cyan square: Transmembrane region of VEGFR-2, · Pink square: Tyrosine kinase of VEGFR-2.

(ECD; Extracellular domain, TM; Transmembrane domain, ICD; Intracellular domain, TSAd; T-cell-specific adapter, PLC-γ; phospholipase C-γ, MAPK; mitogen-activated protein kinase, P38-MAPK; p38 mitogen-activated protein kinase, Cdc42; Cell division control protein 42 homolog.)

1.4 FGF and FGF receptor

Among five RTK, the second most important RTK next to VEGFR is FGF Receptors (FGFR). FGFR is related to four process which are angiogenesis, proliferation, invasion and metastasis in cancer development. FGF receptors (FGFR) are cell surface receptor tyrosine kinases (RTKs) as receptors for fibroblast growth factor (FGF). There are 4 subtypes: FGFR-1, FGFR-2, FGFR-3, and FGFR-4. In endothelial cells, FGFR-1 is the most expressed, followed by FGFR-2. FGFR-3 and 4 are not expressed as much as other two subtypes. FGFR-1 and FGFR2 play a major role in angiogenesis [14]. Fibroblast growth factor (FGF) family consists of 23 members, and FGF-1 and FGF-2 are the best known and influential. FGF-1 is also called as acidic FGF (aFGF) and FGF-2 is also called as basic FGF (bFGF). FGF / FGFR plays a vital role not only in regulating cell differentiation, tissue development, and endothelial cell proliferation but also in migration, extracellular matrix degradation in angiogenesis. They directly participate in angiogenesis and also activate the VEGFR signaling pathway or the PDGFR signaling pathway, thereby indirectly promoting angiogenesis. Gene amplification and activated gene mutation or neither of these two phenomena has the characteristics of over-expressed or unregulated FGF and FGFR in a variety of cancers, of which the bFGF / FGFR-1 is most crucial factors in angiogenesis [18, 19].

FGFR consists of extracellular (EC) domain, transmembrane (TM) domain and intracellular (IC) domain. Extracellular domain consists of 3 immunoglobulin (Ig) like

domains (D1, D2 and D3), the transmembrane domain consists of single α-helix and intracellular domain consist of tyrosine kinase. The FGF receptor forms a dimer in the absence of a ligand, but the ligand FGF must bind to it so that two tyrosine kinases in the transmembrane domain are properly aligned to be transphosphorylated and activate the signal. bFGF (FGF2) causes 20 to 40% higher phosphorylation than FGF1. Thus, FGF2 has involved more in FGFR signaling than FGF1, which contributes more to angiogenesis [15]. FGFR-1 has 19 isoforms, eight potential glycosylation sites, and has a size between 120 kDa (immature form) and 145 kDa (mature form) depending on glycosylation degree [16]. There are also seven tyrosine residues in FGFR-1. (Tyr463, Tyr583, Tyr585, Tyr653, Tyr654, Tyr730 and Tyr766). Of these, Tyr 653/654 is important for activation of FGFR-1 and is essential for signal transduction. In addition, Tyr766 serves as a binding site for one of the SH2 domains of phospholipase Cy and couples FGFR-1 to phosphatidylinositol hydrolysis in several cell types [17]. The remaining tyrosine residues act as docking sites for signaling components such as PLCγ and Crk [17].

In summary, bFGF / FGFR-1 plays an important role in tumor angiogenesis, and in some cancers, bFGF (FGF2) has a greater proangiogenic effect than VEGF-A and synergizes with VEGF-As in angiogenesis [13]. In addition, bFGF/FGFR-1 is not only directly involved in angiogenesis but is also considered to be a complementary angiogenic pathway in angiogenesis resistance mechanisms associated with anti-VEGF therapy. Therefore, it is emerging as a new anti-angiogenesis anticancer therapy strategy for many researchers. FGFR targeted therapies

include nonselective TKIs (lucitanib, lenvatinib, nintedanib, brivanib, NVP-BGJ398; Infigratinib and dovitinib etc.), selective FGFR TKIs (AZD4547, BGJ398, TAS-120, ARQ-087, JNJ 42756493 etc.), FGF-ligand traps (FP-1039), and monoclonal antibodies (MFGR1877S etc.) [14, 20].

1.5 Rationale

Doppel is a membrane protein expressed in endothelial cell. It is expressed more in tumor endothelial cell than in normal endothelial cell. Although the specific expression of Doppel has already been shown in several studies [2,3,4,5], it is still unclear that how Doppel plays the different role in different kinds of cancers. Previously, our lab performed Doppel expression screening in various mouse tumor endothelial cell and found out that colon cancer endothelial cell is most Doppel expressing cancer type **[4].** Therefore, this study focused on the role of Doppel in colorectal cancer by targeting human colonic tumor endothelial cell (HCTEC) for the first time. Conventional anticancer agents targeting angiogenesis-related membrane growth factors and their receptors have limitation because they don't distinguish the normal and tumor endothelial cells leading to inhibition of angiogenesis non-selectively. In addition, resistance to therapy cause relapse, and lead to increasing angiogenesis and worsening the status of cancer. Since Doppel is specific to tumor endothelial cells, it will be a new target to overcome the existing shortcomings.

Colorectal cancer (CRC) is renowned for its difficulty in treatment because of its resistance to conventional chemotherapy. If we can understand the role of Doppel in colorectal cancer by studying its mechanism, Doppel Antibody can be used synergistically with other anticancer agents or replace existing anti-angiogenic anticancer agents and it will open the possibility of effective and novel therapy that reduce side effects or relapse.

2. Materials and Method

2.1 Materials, cell lines

Human colorectal carcinoma (HCT116) were purchased by ATCC (Washington DC, U.S.A). HCT116 were cultured in McCoy's 5a Medium supplemented with 10% FBS (FP-0500-A, Atlas) and 1% antibiotic antifungal agent (15240-062; Thermo Fisher Scientific). Human colon tumor endothelial cell (HC-6203, called as HCTEC in this paper) was purchased by Cell Biologics (Chicago, U.S.A). HCTEC was cultured with ECGM-2 (C-22022, Promocell, Germany) supplemented with supplement mix (C-39226, Promocell, Germany) and 1% antibiotic antifungal agent (15240-062; Thermo Fisher Scientific). All cells were incubated in a humidified 5% CO2, 37℃ incubator. Human Doppel (hPRND) antibodies (A12, B2, B3, G5, H9) were obtained from KbioHealth (Osong, South Korea)

2.2 In vivo animal experiment

All in vivo experiments using live animals were carried out in compliance with the relevant laws and institutional guidelines of Seoul National University. Female Balb/c nude mice (6-weeks old, Orient Bio Inc. Korea) were injected subcutaneously with 1.0 x 10^7 HCT116 cells into the right side of the back after anesthetizing with Avertin. The human Doppel antibody (B2) was administered intravenously every 3 days at 10 mg/kg. The body

weight and tumor size of the mouse were measured once every three days. At 21 days after the start of the drug administration, the experiment was terminated, and the tumors were harvested, weighed and photographed.

2.3 Spheroid sprouting assay

HCTEC (Human colonic tumor endothelial cell) was used to form spheroids. HCTEC was cultured with ECGM-2 (C-22022, Promocell, Germany) supplemented with supplement mix (C-39226, Promocell, Germany) and 1% antibiotic antifungal agent (15240-062; Thermo Fisher Scientific). After HCTEC was fully grown, trypsinized and suspended in 1:3.3 mixture of ECGM and methocel (1.2% w/v of methylcellulose in ECGM). 1 spheroids (25 μl) include 1000 TECs and were created by a hanging drop method in a humidified 5% CO2, 37℃ incubator by overnight incubation. Next day, spheroids were collected and centrifuged at 150 g, 5 min, RT. Then, methocel solution containing 20% FBS (FP-0500-A; Atals), collagen I (Rat tail, 354236, Corning), 10% MEM 199 (10X, GIBCO; Invitrogen, CA) and 10% 0.2 N NaOH were added until the solution turns pink to adjust the pH to 7.4. 0.7 mL of the mixed solution containing 50 HCTEC spheroids were added into individual wells of a 24-well plate. Then polymerized for 30 min in a humidified 5% CO2, 37℃ incubator. After 30 min, 0.3 ml of ECGM containing hVEGF-A ($VEGF_{165}$; 100 ng/ml) was treated to induce sprouting. For the treatment group, all five Doppel antibodies (A12, B2, B3, G5, H9) were added in 0.3 ml of hVEGF-A

containing ECGM at 10 μg/ml, each. The plates were incubated overnight in a humidified 5% CO_2, 37℃ incubator. Spheroids were observed by Microscope (Eclipse TE2000-S; Nikon, Tokyo, Japan)

2.4 Western blot

Western blot was designed to investigate the effect of Doppel on VEGFR-2 and FGFR-1. Using the human Doppel antibody (B2), the effects of B2 on the phosphorylation of VEGFR-2 and FGFR-1 were observed.

When the cell is 80~90% proliferated in the dish, replace the media with fasting media, EBM Basal medium phenol red-free without supplement (CC-3129; Lonza). After fasting for 24 hours, wash the cell dish several times with PBS and add 1.0 ml of fresh fasting media. Human Doppel antibody (B2) is added at 10 μg/ml and incubated for 30 min in 37℃ cell incubator. Then add the target growth factor (VEGF-A or bFGF) to the cell at appropriate concentration and incubation time for activation of each receptor (70 ng/ml, 5 min for VEGF-A, and 50 ng/ml, 10 min for bFGF). Recombinant human VEGF-A (100-20) and recombinant human FGF-basic (100-18B) were purchased from PEPROTECH (Rocky Hill, NJ, USA). The cells were lysed on ice using NP-40 Cell lysis Buffer (FNN0021; Invitrogen) with protease inhibitor (P3100; GenDEPOT) and phosphatase inhibitor (P3200; GenDEPOT). The lysate was collected in a 1.0 ml tube and vortexed in a refrigerator for 30 min. Then, it was centrifuged at 14000 rpm, 4℃ for 10 min. The

supernatant was transferred to a new 1.0 ml tube and the protein amount using BCA protein assay was quantified. After quantification of the protein, the concentration of each sample was calculated to include the same amount of protein, and the loading buffer and DDW was added to denature the sample. Then each sample was loaded onto 7.5% polyacrylamide gel. The gel electrophoresis was started for 40 min at 200 V or 2 hours at 110 V. After the gel electrophoresis, proteins were transferred on the PVDF-membrane (IPVH00010; Millipore) using the transfer machine (Biorad Trans-Blot Turbo) at 70 V for 3 hours or 30 V for overnight in cold water bath. After transfer, the transferred membrane was blocked with 5% BSA solution for 1 hour at RT on the shaker. After blocking, membrane was treated with primary target antibodies (Doppel, Phosphorylated VEGFR-2, VEGFR-2, Phosphorylated FGFR-1, FGFR-1, GAPDH) to 5% BSA (1:2000) and incubated overnight at 4℃ on the shaker. The buffer was discarded from membrane and the membrane was washed with TBST buffer for 1 hour. Appropriate secondary antibodies was added for each of antibodies at 1:5000 ratio and incubated for 1 hour at RT on the shaker. The membrane washed with TBST several times. The luminol agent (DG-WP100; EZ-western Lumi Pico) was prepared and 1.0 ml luminol agent was added on each membrane and the image by LAS 4000 machine was obtained.

Phospho-FGF Receptor (Tyr654) antibody (ab59194; Abcam) was used to detect the phosphorylation of Tyr654 in FGFR-1. FGF Receptor antibody (ab824; Abcam) was used to detect the FGFR-1. Phospho-VEGFR-2 (Tyr1175) antibody (2478; Cell Signaling Technologies) was used to detect the

phosphorylation of Tyr1175 in VEGFR-2. Phospho-VEGFR-2 (Tyr1214) antibody (AF1766; R&D Systems) was used to detect the phosphorylation of Tyr1214 in VEGFR-2. VEGFR-2 antibody (2479; Cell Signaling Technologies) was used to detect the VEGFR-2. GAPDH antibody (5174; Cell Signaling Technologies) was used to detect the GAPDH. Human Doppel antibody (15113-T48; Sino biological) was used to detect the Doppel.

2.5 Proximity ligation assay (PLA)

HCTEC and HUVEC were grown in the glass bottom microwell dish (P35GCOL-0-10-C; MatTek Corporation) at 10^4cell/dish. After two days of seeding, cells were washed with PBS and fixed in formalin for 20 min at RT. Then, in situ PLA was performed using Duolink in situ Detection Reagents red (DUO92008; Sigma-Aldrich) according to the manufacturer's instructions.

For the Doppel and FGFR-1 PLA, rabbit anti-human Doppel antibody (15113-T48; Sino biological; rabbit) and mouse anti-FGFR-1 (ab824; Abcam) antibody were used. For the IgG control, Rabbit IgG Control (LF-SA8070U; AbFRONTIER), Mouse IgG Control (LF-SA8071U; AbFRONTIER) were used. For the PLA probe, a probe corresponding to the host of each primary antibody was used. Duolink In Situ PLA Probe Anti-Rabbit PLUS (DUO92002; Sigma-Aldrich) is used for Doppel antibody, Duolink In Situ PLA Probe Anti-Mouse MINUS (DUO92004; Sigma-Aldrich) is used for FGFR-1 antibody. After

completing the PLA step, cells were incubated for 30 min with FITC labeled phalloidin (P2141; Sigma-Aldrich) for the staining of cytoskeleton. Then cells were washed with PBS for 20 min and were mounted with Duolink In Situ Mounting Medium with DAPI (DUO82040; Sigma-Aldrich) for nuclear staining. CLSM II Confocal Laser Scanning Microscope (LSM710; Carl Zeiss) was used for the observation.

2.6 Immunofluorescence staining

HCTECs and HUVECs were grown in the glass bottom microwell dish (P35GCOL-0-10-C; MatTek Corporation) at 10^4cell/dish. After two days of seeding, cells were washed with PBS and fixed in formalin for 20 min at RT. Then blocked with blocking solution (DUO82007; Sigma-Aldrich) for 30 min in 37℃ incubator. After blocking, cells were incubated with primary antibodies in antibody diluent (DUO82008; Sigma-Aldrich) at 1:40 ratio for overnight in 37℃ incubator. Rabbit anti-human Doppel antibody (15113-T48; Sino biological; rabbit), mouse anti-FGFR-1 (ab824; Abcam) antibody, and goat anti-VEGFR-2 (AF644; R&D Systems) antibody were used. Next day, cells were washed with PBS and incubated with corresponding secondary fluorescent antibodies in antibody diluent at 1:40 ration for 1 hour at RT. Cy3 donkey anti-mouse (135395; Jackon Immuno Research) was used for the FGFR-1, Alexa Fluor 488 goat anti-rabbit (A11008; Invtrogen) was used for the Doppel in FGFR-1 & Doppel double staining. Alexa Fluor 555 donkey anti-rabbit (A-31572; Thermofisher) was used for

Doppel, Alexa Fluor 488 donkey anti-goat (A-11055; Thermofisher) was used for VEGFR-2 in VEGFR-2 & Doppel double staining. Then cells were washed with PBS for 1 hour and were mounted with Duolink In Situ Mounting Medium with DAPI (DUO82040; Sigma-Aldrich) for nuclear staining. CLSM II Confocal Laser Scanning Microscope (LSM710; Carl Zeiss) was used for the observation.

2.7 ITC (Isothermal titration calorimetry)

Human Doppel protein (obtained from KbioHealth; Osong, South Korea) and VEGFR-2 recombinant protein (10012-H08H; Sino Biological) were used for ITC. MicroCal iTC200 (GE Healthcare Life Sciences; USA) was used for calorimetry. Human VEGFR-2 recombinant protein at a concentration of 5 μM in a volume of 320 μl of PBS was added to 'Cell' part of the machine and human Doppel protein at a concentration of 0.3 mM in a volume of 110 μl of PBS was added to 'Syringe' part of the machine. Prior to ITC measurements, the concentration of each protein was measured by Nanodrop 2000 (Thermoscientific; USA). The ITC experiment parameters are as follows. The total measurement time = 1 hour, total number of injections = 20, Cell temperature= 25℃, Reference power=5, Initial delay= 60 sec, Stirring Speed= 750 rpm.

2.8 Human Phospho-Kinase microarray

Human Phospho-Kinase microarray was performed by Human phospho kinase array Kit (ARY003B; R&D) following the manufacturer's directions.

HCTEC was cultured with ECGM-2 (C-22022, Promocell, Germany) supplemented with supplement mix (C-39226, Promocell, Germany) and 1% antibiotic antifungal agent (15240-062; Thermo Fisher Scientific). After HCTEC was fully grown, the cells were washed with PBS and the media was replaced with fasting media, EBM Basal medium phenol red-free without supplement (CC-3129; Lonza). After fasting for 24 hours, the cells were washed several times with PBS and 1.0 ml of fresh fasting media was added.

For the treatment group, Human Doppel antibody (B2) is added at 10 μg/ml and incubated for 30 min in 37℃ cell incubator. And hVEGF-A ($VEGF_{165}$; 100-20; PEPROTECH) at 70 ng/ml was added and incubated 5 min in a humidified 5% CO_2, 37℃ in the incubator. The cells were lysed on ice using Lysis Buffer-6 with protease inhibitor and phosphatase inhibitor. Then, it was centrifuged at 14000 g, 4℃ for 5 min. The supernatant was transferred to a new 1.0 ml tube and the protein amount was quantified by using BCA protein assay. After quantification of the protein, each sample was calculated to include the 400 μg of protein.

Here is the summary of the array procedure of ARY003B. First, each well of an 8-well multi dish of Human phospho kinase array Kit is blocked with 1.0 ml of Array

Buffer-1 for 1 hour at RT. The Array Buffer-1 was discarded and 1.0 ml of diluted cell lysate (dilution with Array Buffer-1 to final volume 1.0 ml for each group) was added to both Part A and B membrane. The membrane was incubated overnight at 4° C on a rocking platform shaker. Next day, the membrane was washed with 1X Wash Buffer 3 times; 10 min per wash. After washing, 1.0 mL of diluted Detection Antibody Cocktail A was added on the Part A membranes and 1.0 mL of diluted Detection Antibody Cocktail B was added on the Part B membranes. Each membranes was incubated for 2 hours at room temperature on a rocking platform. After incubation, the membrane was washed with 1X Wash Buffer 3 times; 10 min per wash. Then 1.0 mL of diluted Streptavidin- HRP was added into each well and it was incubated for 30 min at room temperature on a rocking platform. The membrane was washed with 1X Wash Buffer 3 times; 10 min per wash. The Chemi Reagent Mix was prepared by mixing Chemi Reagent 1 and 2 in equal volumes and 1.0 ml of Chemi Reagent Mix was added on each membrane. The membrane was incubated for 1 min and the image was obtained by LAS 4000 machine.

3. Results and Discussion

3.1 Determination of target cancer cell line for mechanism study

3.1.1 Confirmation of Doppel expression in target cancer endothelial cell.

Previously, Doppel expression screening in various mouse tumor endothelial cell is performed in our lab and found out that colon cancer endothelial cell is most Doppel expressing cancer type [4]. Therefore, in this study, Human colonic tumor endothelial cell (HC-6203; Cell biologics; HCTEC) was designated as a target cell line for Doppel mechanism study. Prior to the start of the mechanism study, Doppel expression was confirmed by western blot in HCTEC **(Figure 6)**. Human Doppel antibody (15113-T48; Sino biological, 1:12500) is used to detect the human Doppel protein. It appears to have a molecular weight between 45 and 100 kDa depending on the degree of glycosylation (55, 70, 80 kda) and the thickest band appears at 70 kda. Therefore, western blot data of Doppel were shown at 70 kda. Normal endothelial cell, Human umbilical vein endothelial cell (HUVEC), was used as control comparing with HCTEC. The Doppel expression level was much higher in HCTEC compared to HUVEC **(Figure 7)**.

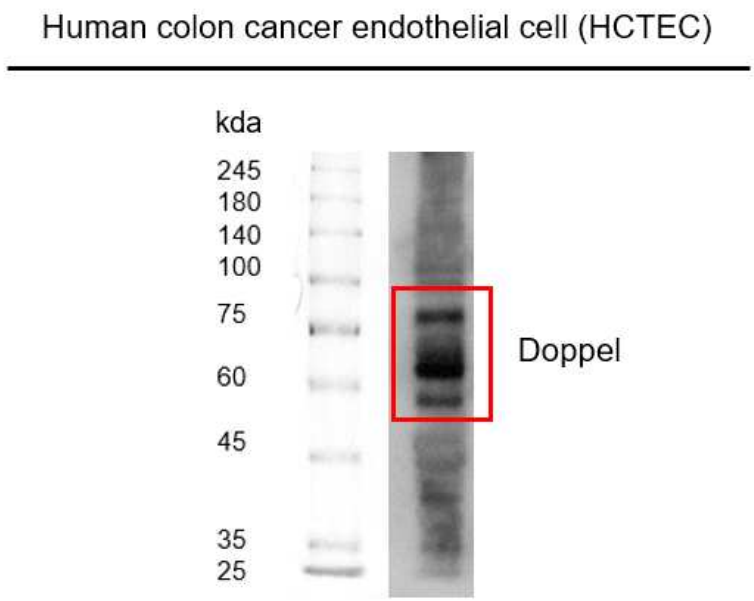


Figure 6. Western blot analysis of Doppel protein expression in Human colonic tumor endothelial cell (HCTEC)

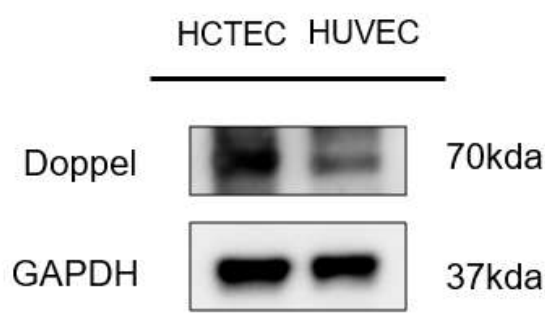


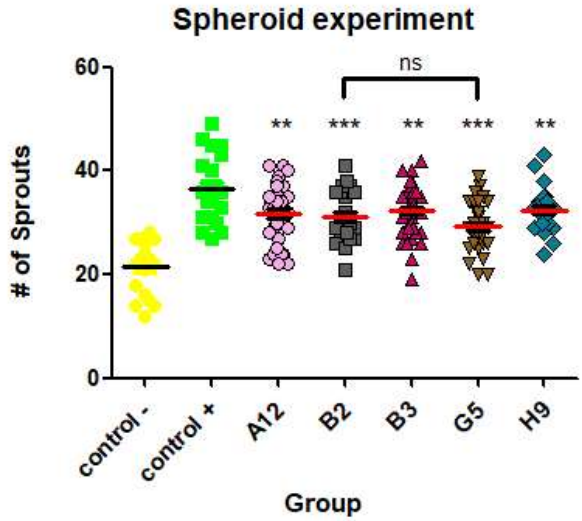
Figure 7. Western blot analysis of Doppel protein expression levels in HCTEC and HUVEC.

3.2 Effect of Doppel on VEGFR-2

3.2.1 Spheroid sprouting assay

The spheroid sprouting assay was performed to determine if the human Doppel antibodies had an anti-angiogenic effect associated with VEGFR-2. In addition, the most effective antibody was selected among five antibodies (A12, B2, B3, G5, H9). Human Doppel (hPRND) antibodies (A12, B2, B3, G5, H9) were obtained from KbioHealth (Osong, South Korea). These 5 antibodies were produced from human Fab antibody library service and selected among 87 antibodies by ELISA and SPR data using human Doppel protein. These 5 Doppel antibodies have cross-reactivity to mouse Doppel protein. Spheroids were made using HCTEC (Human colonic tumor endothelial cell). hVEGF-A ($VEGF_{165}$) was treated with 100 ng/ml to induce sprouting. All five Doppel antibodies were treated with 10 μg/ml and all exhibited an anti-angiogenic effect which inhibits sprouting. Among the five Doppel antibodies, B2 and G5 showed the greatest anti-angiogenic effect and the difference between them was not significant **(Figure 8).**

(a)



(b)

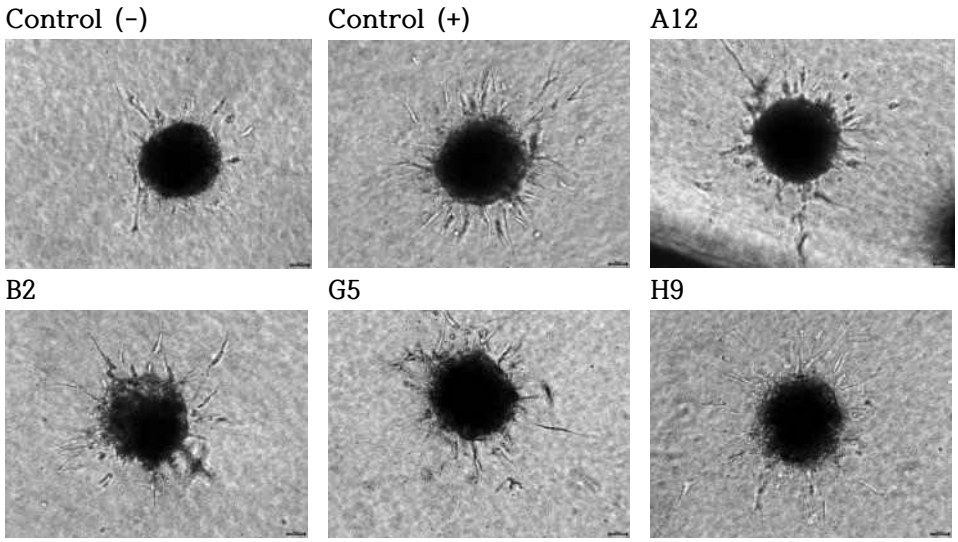


Figure 8. All five Doppel antibodies were treated with 10 ug/ml. hVEGF-A ($VEGF_{165}$) was treated with 100 ng/ml. (a) The number of sprouting in each group. (b) A microscopic image of spheroid in each group. (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ns, not significant; student t test)

3.2.2 Western blot (Tyr 1175, Tyr 1214)

The Western blot assay was also performed to determine if the human Doppel antibodies had an anti-angiogenic effect associated with VEGFR-2. Five human Doppel antibodies (A12, B2, B3, G5, H9; 10 μg/ml) were used as same as in the spheroid sprouting assay. Two tyrosine residues (Tyr1175 and Tyr1214) of VEGFR-2 were evaluated for the efficacy of Doppel antibodies. Tyr1175 is a primary auto-phosphorylation site of VEGFR-2 and Tyr1214 is a secondary auto-phosphorylation site of VEGFR-2. Phosphorylation of these two tyrosine residues activate downstream signal pathway related to angiogenesis.

The result shows that B2 and G5 human doppel antibodies decreased phosphorylation in Tyr1175, of which B2 showed the greatest efficacy **(Figure 9).** As previously mentioned, activation of Tyr1175 (phosphorylation) of VEGFR-2 affect cell proliferation and endothelial cell migration leading to promotion of angiogenesis, thus it can be concluded that Doppel antibodies inhibit angiogenesis. In the Western blot results, only the 230 kda band (fully glycosylated mature form) was shown for VEGFR-2 and P-VEGFR-2. Since the B2 antibody showed the greatest efficacy, B2 antibody was selected and used for remaining in vitro and in vivo experiments considering the results of spheroid sprouting assay and Western blot (Y1175).

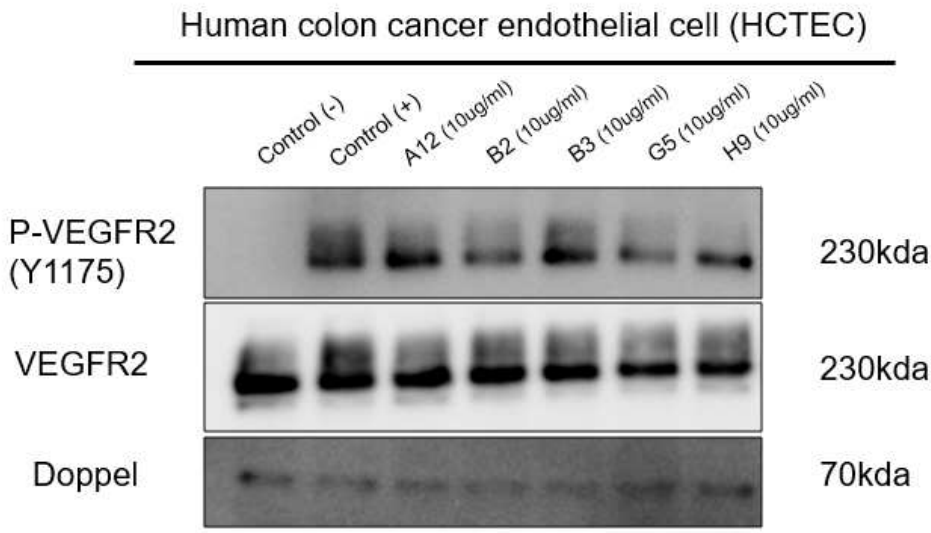


Figure 9. Western blot analysis of Tyr1175 of VEGFR-2 in Human colonic tumor endothelial cell (HCTEC).

B2 also decreased phosphorylation in Tyr1214 **(Figure 10).** The downstream of Tyr 1214 is linked to the P38MAPK pathway. Several studies have shown that the P38MAPK pathway is associated with resistance to conventional chemotherapeutic agents such as cisplatin, irinotecan and 5-fluorouracil (5-FU) in colorectal cancer [21, 22]. Moreover, P38MAPK is involved in the survival of cancer cells, and inhibition of P38MAPK increases the sensitivity of 5-FU of colon cancer cells [21]. Therefore, this experimental result suggests the possibility of using Doppel antibody with conventional anticancer drugs like 5-FU and the possibility of overcoming anticancer drug resistance problem which is a chronic problem of colorectal cancer.

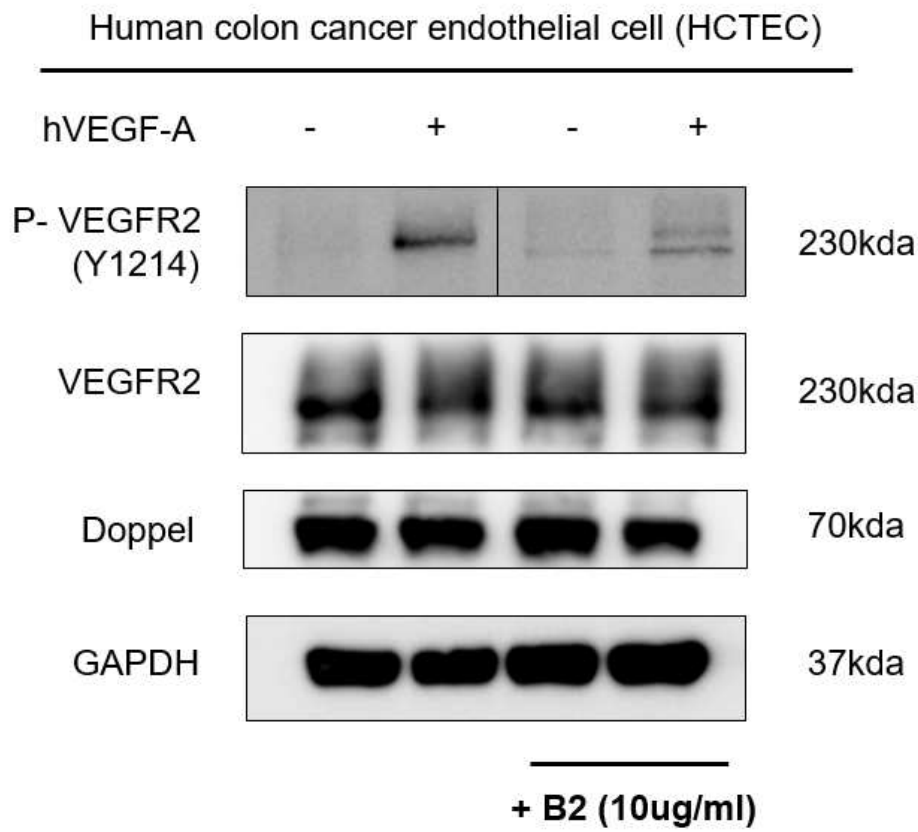


Figure 10. Western blot analysis of Tyr1214 of VEGFR-2 in Human colonic tumor endothelial cell (HCTEC).

3.2.3 Immunofluorescence staining

Since the phosphorylation of VEGFR-2 was decreased with Doppel antibody treatment, VEGFR-2 and Doppel would be physically located close to each other. Immunofluorescence staining (IF) was performed to confirm if the two proteins were expressed at similar positions on the cell membrane. Blue (DAPI) is nucleus, Green (Alexa Fluor 488) is VEGFR-2 and Red (Alexa Fluor 555) is Doppel. **(Figure 11).** Since the staining pattern of VEGFR-2 and Doppel is similar, it can be assumed that the two proteins are expressed at similar positions. However, immunofluorescence staining can not detect the exact relation between two proteins. Therefore, ITC was performed additionally to know more clearly.

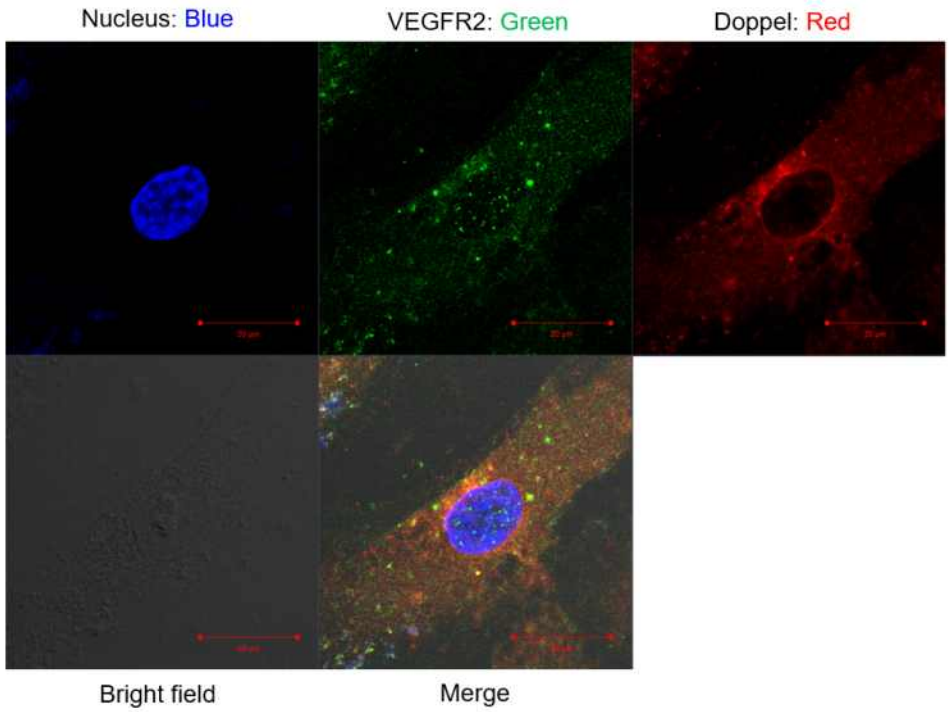


Figure 11. Immunofluorescence staining of Doppel and VEGFR-2 in HCTEC.

3.2.4 ITC (Isothermal titration calorimetry)

To determine if VEGFR-2 and Doppel are attached, the binding of Doppel protein with VEGFR-2 was measured using isothermal titration calorimetry (ITC). ITC is an experimental technique to judge whether two proteins are directly attached or not through the thermodynamic reaction of two proteins. Human VEGFR-2 recombinant protein (5 μM) was added to 'Cell' part of the machine and human Doppel protein (0.3 mM) was added to 'Syringe' part of the machine. Then, the protein of 'Syringe' part was titrated into the 'Cell' part for reaction. **(Figure 12)** As a result, thermodynamic graph did not show saturated sigmoid shape even though the concentration of the two proteins was differ 60 times **(Figure 13).** In summary, the two proteins are not attached. Considering IF and ITC data together, VEGFR-2 and Doppel appear to be expressed at similar positions on the membrane, but not attached directly. Additional PLA (proximity ligation assay) experiment should be performed to ensure that the two proteins are close within 10 nm range.

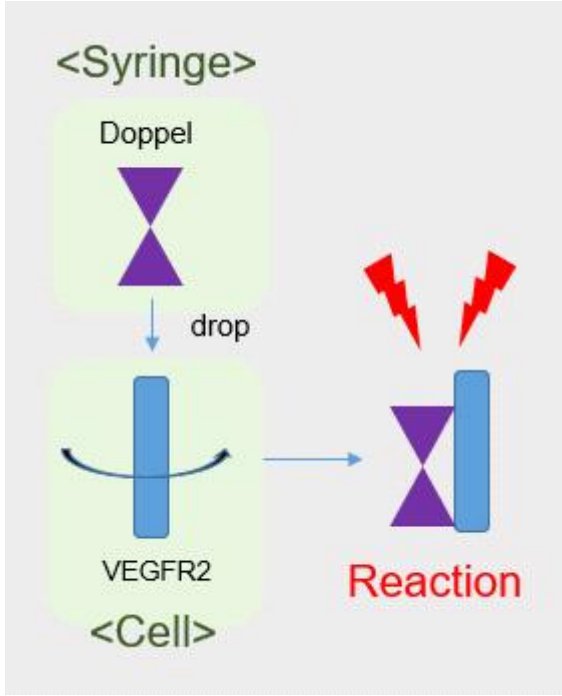


Figure 12. Scheme of ITC using Doppel and VEGFR-2.

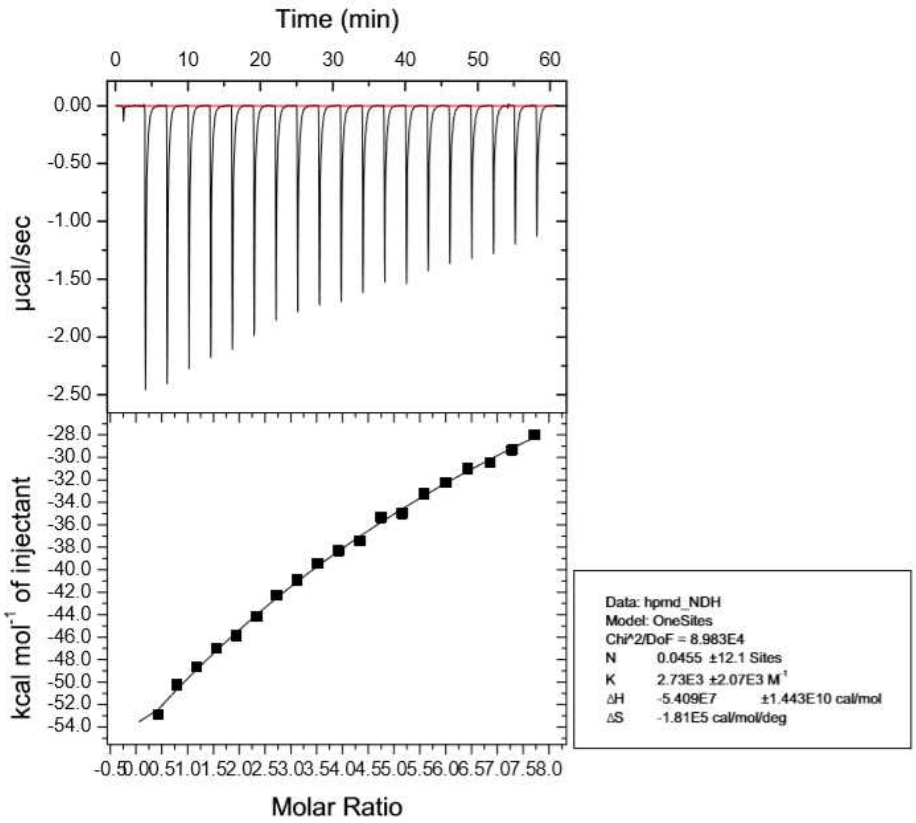


Figure 13. Thermodynamic analysis of Doppel binding to VEGFR-2.

3.2.5 Human Phospho-Kinase microarray assay

Human Phospho-Kinase microarray assay was performed using HCTEC to further investigate that Doppel antibody affects other kinases related to VEGF-A other than Y1175 & Y1214 of VEGFR-2. hVEGF-A ($VEGF_{165}$) was treated with 70 ng/ml and Human Doppel antibody (B2) was treated with 10 μg/ml. As a result, phosphorylation of β-catenin and STAT 5a was decreased in the B2 treatment group compared to Control. In the case of β-catenin, the difference was obvious, and STAT 5a was weaker than that, but inhibition was confirmed **(Figure 14).**

1) β-catenin (beta-catenin)

β-catenin (Catenin beta-1; CTNNB1) is a cadherin-associated protein involved in the regulation of cell adhesion and gene transcription. It acts as a transcriptional co-activator in the canonical Wnt signal transduction pathway that regulates gene transcription. The Wnt signal transduction pathway is also associated with carcinogenesis, and β-catenin is mutated or overexpressed in a variety of cancers (colon, lung, breast, ovary, etc.). In cancer, β-catenin is increased by hypoxia and activation of the Wnt / β-catenin pathway is maintained by the inactivation of p53 [23, 24, 25]. In particular, there is a close relationship between β-catenin signaling and regulation of VEGF-A expression in colorectal cancer. β-catenin increases the production of VEGF-A and initiates angiogenesis in colorectal cancer [25].

Therefore, microarray results suggest that treatment with

Doppel antibody inhibits the phosphorylation of the β-catenin pathway, which may result in anticancer effects and may inhibit angiogenesis in association with VEGF-A. Therefore, the decrease in phosphorylation of VEGFR-2 in (Figure 8) and (Figure 9) may be considered to be an indirect effect of β-catenin, other than the direct effect of Doppel on VEGFR-2.

2) STAT5a

Signal transducer and activator of transcription 5A (STAT5a) is a member of the STAT family and is present in the cytoplasm. STAT5a is activated (phosphorylated) by interleukin-2 (IL-2), IL-3, IL-4, IL-7, erythropoietin, thrombopoietin, and other growth factors. After activation of STAT5a, it translocates into the nucleus which affects the cell proliferation, migration, differentiation, angiogenesis, inflammation, apoptosis, and immune evasion. In addition, it is overexpressed in cancers such as colon, breast and prostate cancer [26, 27, 28]. Especially, previous studies on colorectal cancer have shown that inhibition of STAT5a promotes cell death of colorectal cancer cells by increasing the sensitivity to chemotherapeutic agents such as cisplatin or 5-fluorouracil [28]. Therefore, the combination of Doppel antibody with a chemical anticancer agent will increase the anticancer effect. In this experiment, the phosphorylation of STATa was observed to be decreased even though VEGF-A was treated. Since VEGF-A is not a major ligand for STAT5a [29], further studies with Western blot using IL-2 or 3 family cytokines, the major cytokine of STAT5a, can make the result more clearly.

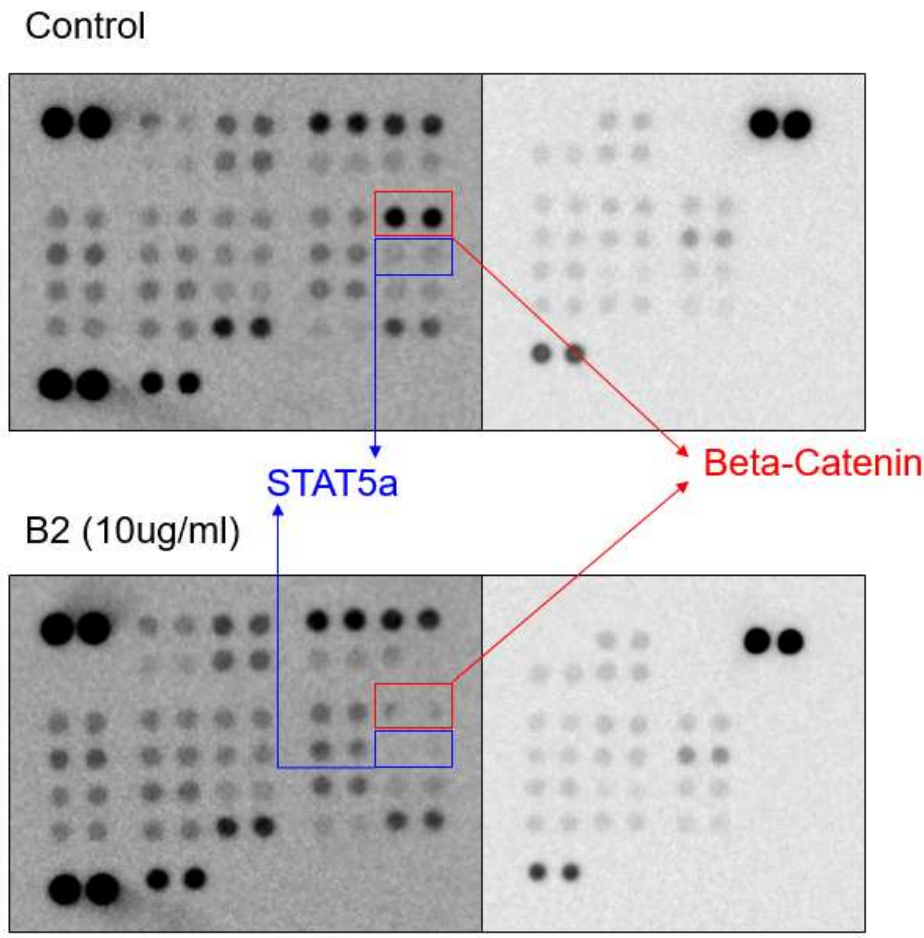


Figure 14. Human phospho-kinase microarray of HCTEC

3.3 Effect of Doppel on FGFR-1

3.3.1 Western blot (Tyr654)

The Western blot assay was performed to determine if the human Doppel antibodies has an anti-angiogenic effect associated with FGFR-1, another important RTK related to angiogenesis. 'B2' human Doppel antibody was used at concentration of 10 μg/ml and decreased phosphorylation in Tyr654 of FGFR-1 **(Figure 15).** FGFR-1 bands were observed at 120 and 145 kda, but only the fully glycosylated mature form of the top band (145 kda) was considered in the results [16]. By the result, it can be concluded that Doppel is involved in both phosphoryation of VEGFR-2 and FGFR-1 which are related to angiogenesis. Previous studies have shown that VEGF-independent angiogenesis of the tumors occurs in patients with anti-angiogenic drug targeting VEGF or VEGFR, leading to cancer relapse and upregulation of FGF ligands such as bFGF [14]. In addition, amplification of FGFR-1 and activated (phosphorylated) FGFR-1 are found in colorectal cancer (CRC) [19,32,33]. 24 of the 454 major CRCs exhibited FGFR-1 amplification and 18 CRCs showed membrane activated P-FGFR-1 [19]. In this situation, anti-FGF therapy effectively inhibit tumor growth and angiogenesis [14, 31]. Especially, RTK inhibitors that simultaneously target VEGFR and FGFR, such as brivanib, have emerged for overcoming this phenomenon [30], but they are still not specific to tumors. Therefore, the use of Doppel antibody which is tumor specific and simultaneously inhibit VEGFR-2 & FGFR-1 may be more effective and less

toxic in colorectal cancer patients with resistance to conventional anti-angiogenic drug.

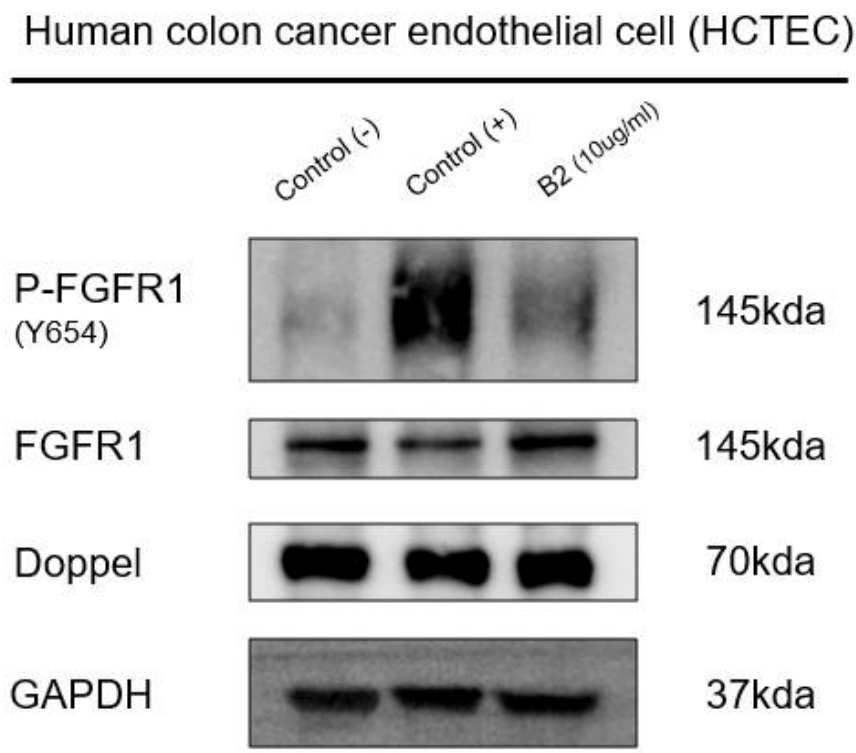


Figure 15. Western blot analysis of Tyr654 of FGFR-1 in Human colonic tumor endothelial cell (HCTEC).

3.3.2 Immunofluorescence staining

Since the phosphorylation of FGFR-1 was decreased with Doppel antibody treatment, FGFR-1 and Doppel would be physically located close to each other. Immunofluorescence staining (IF) was performed to confirm if the two proteins were expressed at similar positions on the cell membrane. HUVECs were used as controls for HCTEC. Blue(DAPI) is nulceus, Green (Alexa Fluor 488) is Doppel and Red (Cy3) is FGFR-1 **(Figure 16).** Similar to the Western blot results (Figure 6), the amount of Doppel expression of HUVEC is lower than that of HCTEC. Since the staining pattern of FGFR-1 and Doppel is similar, it can be assumed that the two proteins are expressed at similar positions. However, immunofluorescence staining can not detect the exact range between two proteins. Therefore, PLA was performed additionally to know more clearly.

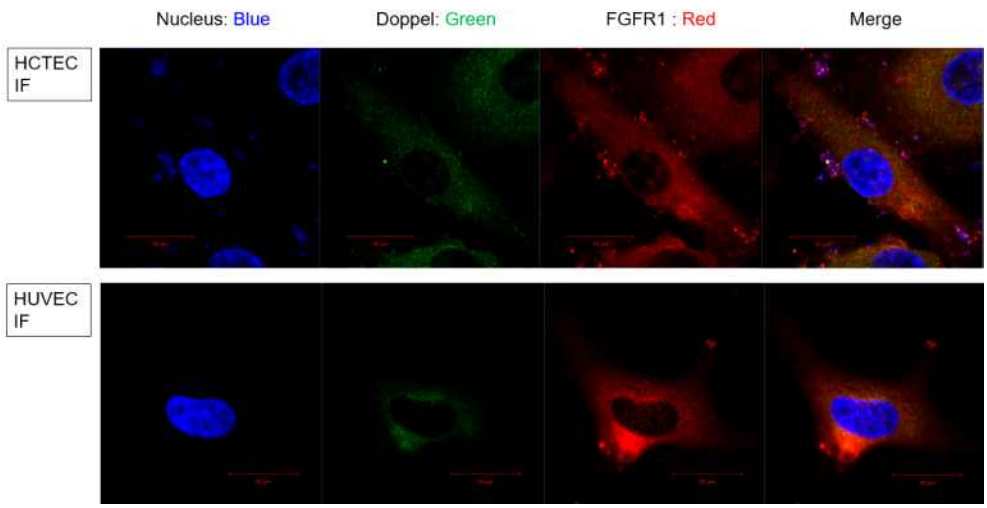


Figure 16. Immunofluorescence staining of Doppel and FGFR-1 in HCTEC and HUVEC.

3.3.3 PLA (Proximity Ligation Assay)

For the confirmation and accurate interpretation of IF result, PLA (Proximity Ligation Assay) was performed. HUVECs with low Doppel expression levels were used as controls for HCTEC. In PLA, a red dot is observed when the two proteins (Doppel and FGFR-1) are within the 10 nm range. As a result, a red dot was observed in the HCTEC. That is, Doppel and FGFR-1 are located close to each other within the 10 nm range. In the case of HUVEC, much less PLA dot was observed **(Figure 17)**. For the reliability of the results, PLA using IgG were also tested **(Figure 18).** Further, if ITC is performed, it will give a clue whether Doppel and FGFR-1 are directly attached and more detailed mechanism can be studied.

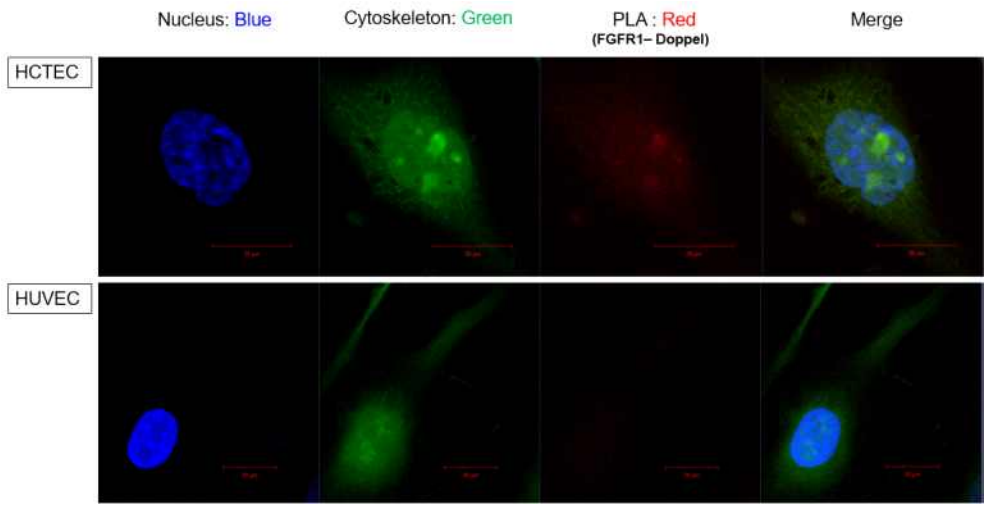


Figure 17. Doppel and FGFR-1 PLA in HCTEC and HUVEC

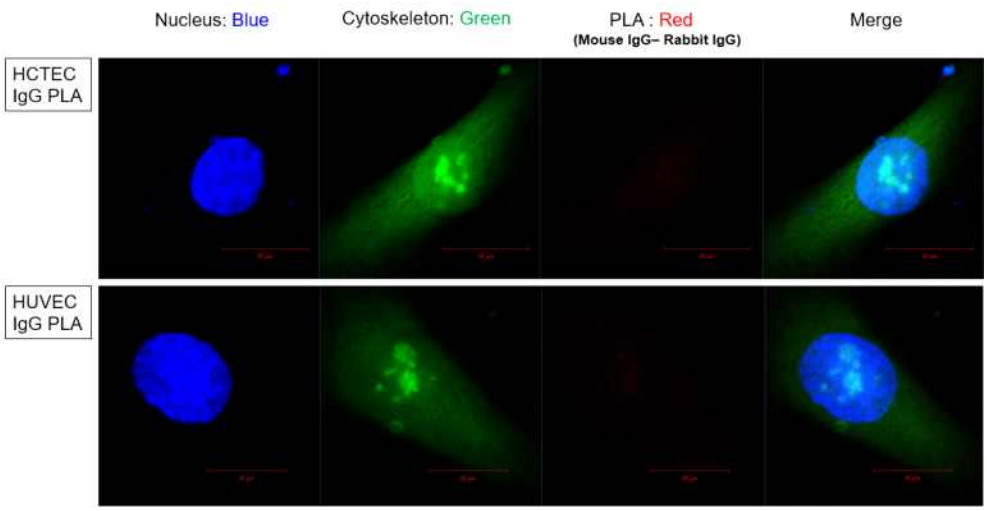


Figure 18. IgG PLA in HCTEC and HUVEC

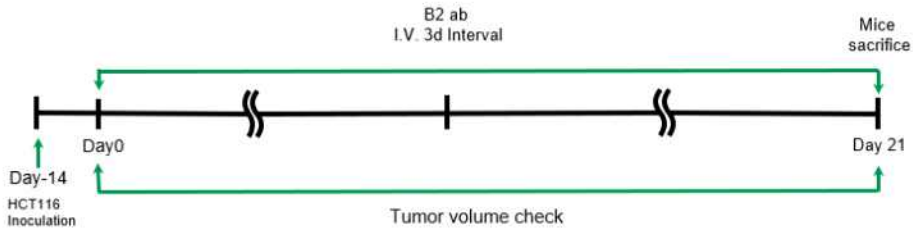
3.4 Tumor growth inhibition in *in vivo* mouse model

To evaluate the efficacy of the human Doppel antibody (B2) in vivo, a xenograft experiment using HCT116 human colon cancer cells was designed. Since in vitro experiments were performed using human colonic tumor endothelial cells (HCTEC), colon cancer cells (HCT116) were also selected for in vivo experiments. 1.0 x 10^7 HCT116 cells were injected subcutaneously into the right side of the back of Balb/c nude mice (Female, 6-weeks old). Control and B2 treatment group have 7 mice each respectively. Treatment began when the tumor had an average size of 50 mm^3. The human Doppel antibody (B2) was administered intravenously every 3 days at 10 mg/kg. At 21 days after the start of the drug administration, the experiment was terminated.

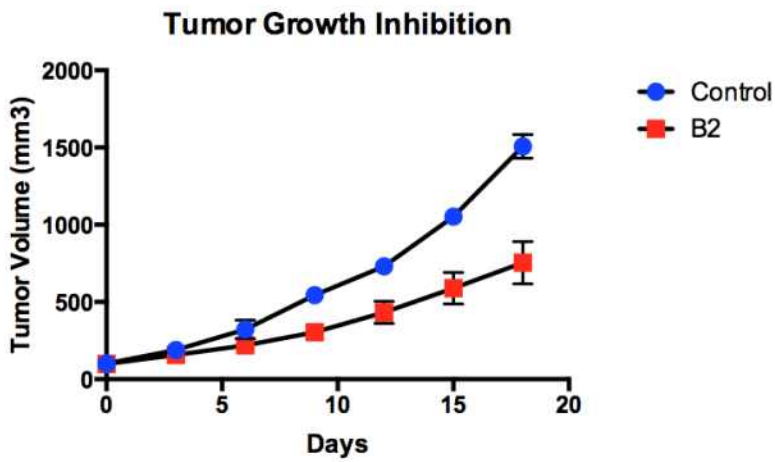
As a result, B2 group tumors were reduced by 50.3% compared to the control group. Therefore, it can be concluded that B2 has anticancer effect **(Figure 19).** This anticancer effect can be estimated by two reasons. First, human doppel antibody has cross-reactivity to mouse tumor endothelial cell therefore it shows anti-cancer effect. Second, human doppel antibody affect not only mouse tumor endothelial cell but also human colon cancer it self. Previously our lab found out that doppel are expressed in either tumor cell and tumor endothelial cell.

Furthermore, it is recommended to use Doppel antibody to chemo-resistance colorectal cancer cell line such as HT29. It will give confidence of Doppel mechanism which is supposed to increase the sensitivity of colorectal cancer cells to conventional chemotherapeutic agents.

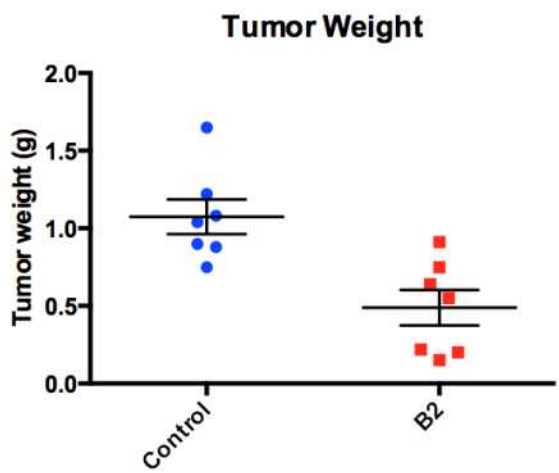
(a)


B2 ab
I.V. 3d Interval
Mice
sacrifice
Day0
Day 21
Day-14
HCT116
Inoculation
Tumor volume check

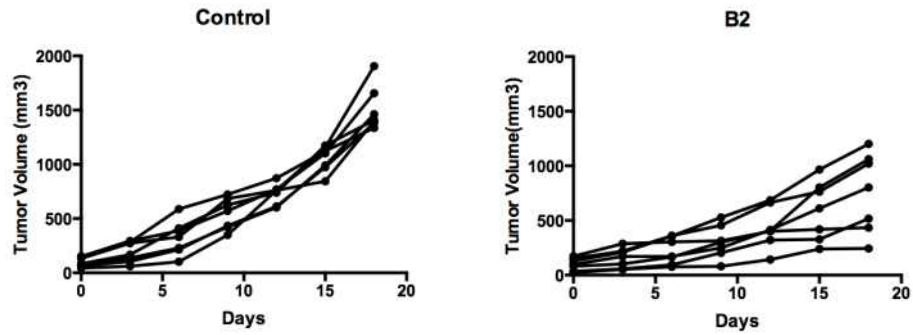
(b)


Tumor Growth Inhibition
Tumor Volume (mm3)
2000
1500
1000
500
0
0
5
10
15
20
Days
Control
B2

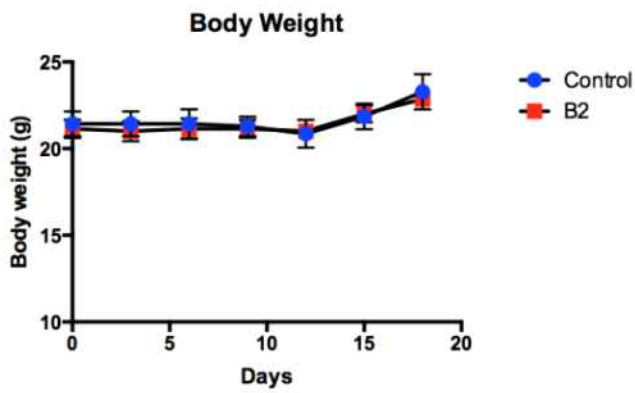
(c)


Tumor Weight
Tumor weight (g)
2.0
1.5
1.0
0.5
0.0
Control
B2

(d)



(e)



(f)

Figure 19. Human Doppel antibody (B2) in vivo experiment. (a) In vivo experiment schedule. (b) Tumor growth inhibition by B2 antibody. (c) Tumor weight. (d) Individual tumor grotwh graph of each group. (left: Control group, right: B2 antibody treatment group) (e) Body weight graph of each group. (f) Tumor image of each group.

4. Conclusion

This paper investigated the mechanism of 'Doppel' protein in colorectal cancer. In vivo mouse experiments using Human colon cancer (HCT116) and in vitro mechanism studies using Human Colonic Tumor Endothelial Cell (HCTEC) were performed by using the most efficacious antibody (B2) among various human Doppel antibodies. Mechanism studies were performed using various experimental methods such as Western blot, PLA, Immunofluorescence staining, ITC and Microarray, focusing on angiogenesis-related main receptors, VEGFR-2 and FGFR-1.

As a result, Doppel antibody inhibits angiogenesis by inhibiting phosphorylation of VEGFR-2 and FGFR-1. In the case of FGFR-1, Doppel and FGFR-1 are located within 10 nm. Therefore Doppel may affect to FGFR-1. If ITC is performed, it will give a clue whether Doppel and FGFR-1 are directly attached and more detailed mechanism can be studied. If they are not directly attached, the mechanism may be accessed as a co-receptor concept. Although many co-receptors are not directly attached, they may form complexes under specific conditions and interact with each other [34,35]. In the case of VEGFR-2, VEGFR-2 and Doppel are not attached as shown by the ITC result. However they may be close to each other because they have a similar expressing pattern on membrane as the IF result. Additional PLA data is needed to confirm the results. Microarray result shows that Doppel is also associated with β-catenin which is related to increased production of VEGF-A in colorectal cancer [25]. Therefore, Doppel may indirectly affect VEGFR-2. In addition, Doppel antibody reduced

the phosphorylation of Y1214 of VEGFR-2 and STAT5a. Activation of Y1214 is linked to activaion of P38MAPK. P38MAPK and STAT5a are related to chemo-resistance of colorectal cancer to conventional chemotherapy such as 5-FU and cisplatin [28].

Taken together, 1) Doppel is specific to tumor. 2) Doppel promotes angiogenesis in association with VEGF-A / VEGFR-2, bFGF / FGFR-1, and β-catenin. 3) Doppel reduces the chemotherapeutic sensitivity (apoptosis) of CRC cells related to the Y1214 -P38MAPK pathway and STAT5a **(Figure 20)**. Due to complex mechanism, it needs to be studied in various directions. The results obtained in this paper also need to be reconfirmed by other experiments. If more detailed and diverse experiments are available to support the conclusions of the paper, Doppel antibody can be used as novel therapeutic approach to CRC patients which has advantages such as high efficacy, low toxicity, low possibility of cancer relapse, and possibility of combination therapy with conventional chemotherapeutic agents.

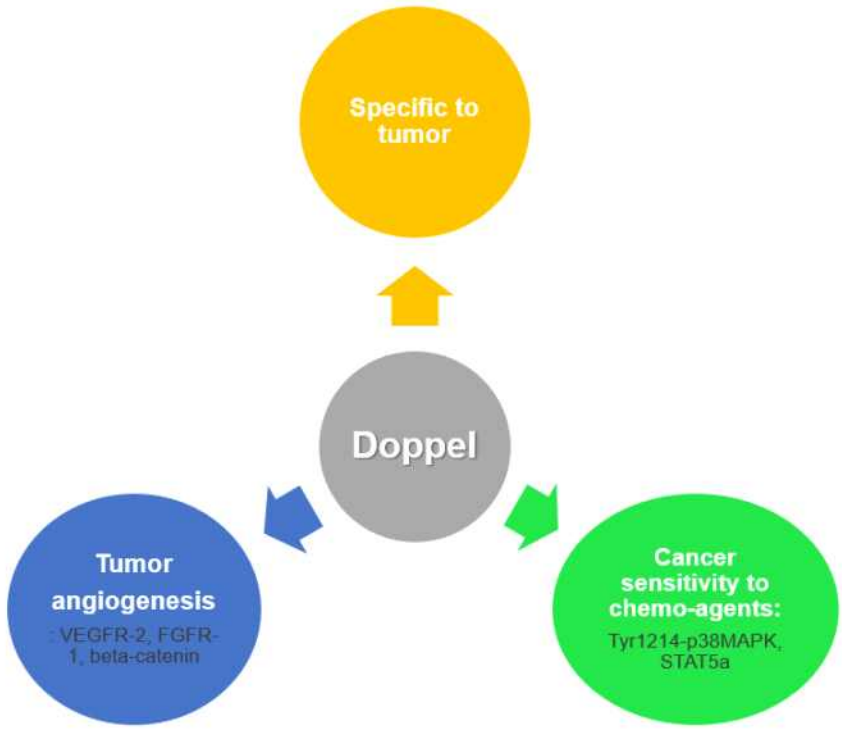


Figure 20. Effect of Doppel on human colorectal cancer.

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국문초록

혈관 형성(angiogenesis)은 정상조직의 성장 및 발달에서뿐만 아니라 암이 성장하고 전이하는데 필수적인 생물학적 과정이다. 이러한 혈관 형성을 억제하여 항암효과를 발휘하는 항암제의 개발이 활발하게 이루어졌으나, 그 타깃이 정상조직과 암에서 공통적으로 발현되는 것이기 때문에 혈관 형성이 구별 없이 저해된다는 한계점이 있었다.

본 연구에서는 정상 혈관 내피세포 (normal endothelial cell)에 비해 암 혈관 내피세포 (tumor endothelial cell)에서 훨씬 더 많이 발현되는 '도펠 (Doppel)' 단백질의 작용 메커니즘을 규명하였다.

실험 결과, 도펠 항체를 처리하였을 때 혈관 형성에 관련된 가장 주요한 수용체인 VEGFR-2 (혈관 내피 성장 인자 수용체-2) 그리고 FGFR-1 (섬유아세포 성장 인자 수용체-1)의 인산화가 감소하였으며, 도펠과 FGFR-1은 암의 내피세포 상에서 10 nm 범위 안에 가까이 있었다. 또한, 도펠 항체는 대장암에서 VEGF-A의 발현량을 조절하는 베타 카테닌 (β-catenin)과 화학 항암제에 대한 감수성과 연관된 STAT 5a 에도 영향을 주었다. 대장암 모델 동물 실험에서는 도펠 항체가 50% 이상의 항암효과를 보였다.

실험 결과를 종합해 보았을 때 도펠은 대장암에서 암의 혈관 형성 및 기존 화학 항암제에 대한 감수성과 관련되어 있다. 이 연구는 대장암에서 도펠의 역할을 처음으로 규명하였기 때문에 가치가 있다. 앞으로 더 자세한 메커니즘을 연구하면 도펠 항체를 기존의 화학항암제 및 혈관형성 억제 항암에 저항성을 지닌 대장암 환자에게 부작용이나 암의 재발은 줄이고 효능은 극대화하는 방안으로 사용할 수 있을 것이다.

주요어 : 도펠 단백질, 인간 대장암, 혈관 형성, 혈관 내피 성장 인자 수용체-2, 섬유아세포 성장 인자 수용체-1, 항암제 내성, 항암 항체 치료제

학 번 : 2016-27629