

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**SCREENING OF A SYNTHETIC HUMAN SCFV LIBRARY
AGAINST VASCULAR ENDOTHELIAL GROWTH FACTOR
RECEPTOR-2 (VEGFR-2) FOR IDENTIFICATION OF
POTENTIAL THERAPEUTIC CANDIDATES**

YUSUF EROL

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY**

ADVISOR: PROF. DR. İŞİL KURNAZ

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Co-ADVISOR: DR. OYA ARI UYAR

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

VASKÜLER ENDOTELYAL BÜYÜME FAKTÖRÜ
RESEPTÖRÜ-2'YE (VEGFR-2) KARŞI POTANSİYEL
TERAPÖTİK ADAYLARIN BELİRLENMESİ İÇİN
SENTETİK İNSAN SCFV KÜTÜPHANESİNİN TARANMASI

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YÜKSEK LİSANS TEZİ
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EKİM 2025



MASTER of SCIENCE JURY APPROVAL FORM

A thesis submitted by Yusuf EROL defended on 01/10/2025 before the jury formed with the 30/06/2025 date and 2025/34 numbered decision of the GTU Graduate Administration Board, has been accepted as a MASTER of SCIENCE thesis in the Department of Biotechnology.

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Dedicated To My Beloved Family

ABSTRACT

Angiogenesis, the development of new blood vessels from pre-existing ones, is an essential process in both physiological and pathological conditions. In cancer, tumor cells promote angiogenesis to ensure the delivery of oxygen and nutrients required for their growth and survival. Vascular endothelial growth factor (VEGF) and its receptors (VEGFRs), particularly VEGFR-2, are central regulators of tumor angiogenesis and represent important therapeutic targets. Although several anti-VEGF/VEGFR therapeutics, such as monoclonal antibodies and small-molecule inhibitors, have been clinically approved, their overall efficacy remains limited. With the growing interest in anti-angiogenic therapy and its combination with immunotherapy in cancer treatment, there is still a need for improved anti-VEGF/VEGFR drugs. In this study, VEGFR-2, the principal signaling receptor of the VEGF family, was targeted for the development of single-chain variable fragment (scFv) antibodies capable of inhibiting VEGF-VEGFR-2 interaction. For that purpose, a novel synthetic human scFv library was screened against VEGFR-2 using phage display technology. Four cycles of selection and enrichment, known as biopanning, were carried out using solution-based incubation of phage-scFv clones with VEGFR-2. Following the final round, 60 randomly selected clones were screened by phage ELISA to identify high-affinity binders to VEGFR-2. The selected scFv binders were then subjected to Sanger sequencing, and unique scFv antibodies with diverse CDR profiles were identified. These unique scFv antibodies were later successfully expressed in soluble form in *Escherichia coli* expression system and purified using protein L affinity chromatography. Binding specificity and affinity of soluble scFv antibodies to VEGFR-2 were confirmed via ELISA. Functional evaluation of their anti-angiogenic potential was performed using *in vitro* endothelial cell viability and wound healing assays. While the scFv antibodies showed limited inhibition of VEGF-driven cell survival and proliferation, one scFv notably suppressed endothelial cell migration. Further optimization of expression and stability profiles may enhance their anti-angiogenic effects. In conclusion, this study successfully identified and characterized novel anti-VEGFR-2 scFv antibody fragments derived from a synthetic human scFv antibody library.

Keywords: Anti-VEGF/VEGFR Antibodies, scFv Antibody, Synthetic Human scFv Library, Cancer Therapy, Phage Display

ÖZET

Anjiyogenez, mevcut damar yapılarından yeni kan damarlarının oluşumu, hem fizyolojik hem de patolojik koşullarda önemli bir süreçtir. Kanserde, tümör hücreleri büyümek ve hayatta kalmak için gerekli oksijen ve besin maddelerinin taşınmasını sağlamak amacıyla anjiyogenezi teşvik eder. Vasküler endotelial büyüme faktörü (VEGF) ve reseptörleri (VEGFR'leri), özellikle VEGFR-2, tümör anjiyogenezinin temel düzenleyicileridir ve bu nedenle önemli terapötik hedefler olarak kabul edilmektedirler. Monoklonal antikorlar ve küçük molekülü inhibitörler gibi çeşitli anti-VEGF/VEGFR ilaçları klinik olarak onaylanmış olsa da, bu ajanların genel etkinliği sınırlı kalmaktadır. Anjiyogenez karşıtı tedavilere ve bu tedavilerin immünoterapi ile kombinasyonlarına artan ilgi, daha etkili anti-VEGF/VEGFR ilaçlarına olan ihtiyacı sürdürmektedir. Bu çalışmada, VEGF ailesinin temel sinyal iletim reseptörü olan VEGFR-2, VEGF-VEGFR-2 etkileşimini engelleyebilecek tek zincirli değişken fragment (scFv) antikorlarının geliştirilmesi amacıyla hedeflenmiştir. Bu amaç doğrultusunda, sentetik olarak tasarlanmış yeni bir insan scFv kütüphanesi, faj gösterim teknolojisi kullanılarak VEGFR-2'ye karşı taranmıştır. Biopanning olarak bilinen seçim ve zenginleştirme işlemi, dört döngü boyunca phage-scFv klonlarının VEGFR-2 ile çözeltide inkübasyonu yoluyla gerçekleştirilmiştir. Son döngünün ardından, rastgele seçilen 60 klon faj ELISA ile taranmış ve yüksek afiniteye sahip bağlayıcılar belirlenmiştir. Seçilen scFv bağlayıcılar daha sonra Sanger sekanslama yöntemiyle dizilenmiş ve çeşitli CDR profillerine sahip özgün scFv antikorları tanımlanmıştır. Bu özgün scFv antikorları, *Escherichia coli* ekspresyon sistemi kullanılarak çözünür formda başarıyla üretilmiş ve protein L afinite kromatografisi ile saflaştırılmıştır. Çözünür scFv antikorlarının bağlanma özgüllüğü ve afinitesi ELISA ile doğrulanmıştır. Bu antikorların anti-anjiyojenik potansiyeli, *in vitro* endotelial hücre canlılığı ve yara iyileşmesi deneyleri ile fonksiyonel olarak değerlendirilmiştir. ScFv antikorları VEGF ile uyarılan hücre canlılığı ve proliferasyonunu sınırlı düzeyde inhibe etmiş olsa da, bunlardan birinin endotelial hücre göçünü anlamlı biçimde baskıladığı gözlemlenmiştir. Ekspresyon düzeyi ve stabilite profillerinin daha da optimize edilmesi, bu antikorların anti-anjiyojenik etkilerini artırabilir. Sonuç olarak, bu çalışma, sentetik insan scFv antikor kütüphanesinden türetilmiş yeni anti-VEGFR-2 scFv antikor fragmanlarını başarıyla tanımlamış ve karakterize etmiştir.

Anahtar Kelimeler: Anti-VEGF/VEGFR Antikorları, scFv Antikoru, İnsan Sentetik scFv Antikor Kütüphanesi, Kanser Terapisi, Faj Gösterimi

ACKNOWLEDGEMENTS

This study would not have been possible without the priceless help, guidance, and support of many wonderful people who believed in me and stood by me throughout this research journey. Firstly, I would like to express my deepest gratitude to my supervisor, Prof. Dr. Işıl KURNAZ, for her invaluable guidance, continuous support, and insightful suggestions throughout my master's research. Her expertise and unwavering encouragement have been instrumental in shaping this study and in helping me to develop a researcher's mindset.

I also extend my deepest and sincere thanks to my co-supervisor, Dr. Oya ARI UYAR, for her kind assistance and guidance during all stages of this work. I am deeply grateful for her continuous support, patience, technical guidance, and inspiring encouragement, which made this study possible. It has truly been a wonderful, enjoyable, and educational journey to work with her, and I am greatly indebted to her in ways that words cannot fully express.

I would also like to sincerely thank the members of my thesis jury, Prof. Dr. Işıl KURNAZ, Prof. Dr. Mehmet CANSEV, and Assist. Prof. Dr. Hüseyin ÇİMEN, for their valuable time, constructive feedback, and insightful suggestions, which greatly contributed to the completion of this study.

I would also like to extend my special thanks to all the members of the Medical Biotechnology Research Group of TÜBİTAK Marmara Research Center, for their help and support during this study. I am especially grateful to Dr. Hasan Ümit ÖZTÜRK, Dr. Beytullah ÖZGÜR, Dr. Melis DENİZCİ ÖNCÜ, and Dr. Bertan Koray BALCIOĞLU for their technical assistance, inspiring discussions, and valuable suggestions, which significantly improved this study. Thanks to their guidance, I have expanded my understanding of both theoretical and experimental aspects of this work, and I am sincerely grateful to them. I would also like to express my special thanks to my colleagues and lab mates, Kübra BİLGİÇ, Furkan ŞAHİN, Mustafa EMREM, and Sümeyye ULUBAY, for their constant technical and personal support throughout this research.

I would also like to thank all the members of AxanLAB and ÇimenLAB, Gebze Technical University. Special thanks to Dr. Ekin SÖNMEZ, Pınar MUTLU, Selen Özge ALMAMIŞ, and Sümeyye ONAT for their great support, technical assistance, and valuable feedback during the cell culture experiments.

I also extend my sincere thanks to Dr. Serhad ONAT from Balıkesir University for kindly providing the HUVECs.

I would also like to express my sincere gratitude to my dear friends Ahmet DİNÇ, Bünyamin BULKURCUOĞLU, and Enes Furkan SAKAR for their genuine friendship, which has supported and motivated me throughout this journey.

Finally, I would like to extend my heartfelt gratitude to my family for their endless love, patience, and unwavering support, which have been my greatest source of strength throughout every stage of my life.

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LIST OF SYMBOLS AND ABBREVIATIONS

AP-1	: Activator Protein-1
AMD	: Age-related Macular Degeneration
aa	: Amino acid
bp	: Base pair
BCA	: Bicinchoninic Acid
BSA	: Bovine Serum Albumin
cfu	: Colony-forming unit
CDR	: Complementary-Determining Region
4C1N	: 4-Chloro-1-Naphthol
CTD	: C-terminal Domain
mm ³	: Cubic millimeter
Dll4	: Delta-like 4
DNA	: Deoxyribonucleic Acid
DME	: Diabetic Macular Edema
DR	: Diabetic Retinopathy
DPBS	: Dulbecco's Phosphate-Buffered Saline
DMEM	: Dulbecco's Modified Eagle Medium
EPC	: Endothelial Progenitor Cells
ELISA	: Enzyme-linked Immunosorbent Assay
EGFR	: Epidermal Growth Factor Receptor
EDTA	: Ethylenediaminetetraacetic Acid
ECD	: Extracellular Domain
ERK	: Extracellular Signal-regulated Kinase
FBS	: Fetal Bovine Serum
FGF	: Fibroblast Growth Factor
Flt-1:	: Fms-like Tyrosine Kinase-1
Flt-4	: Fms-like tyrosine kinase-4
Fab	: Fragment Antigen-binding
Fc	: Fragment Crystallizable
g	: Gram
HC	: Heavy Chain
HSPGs	: Heparan Sulfate Proteoglycans
HRP	: Horseradish Peroxidase
HUVEC	: Human Umbilical Vein Endothelial Cell
HRE	: Hypoxia Response Element
HIF	: Hypoxia-inducible Factor
Ig	: Immunoglobulin
IgD	: Immunoglobulin-like Domain
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
JMD	: Juxtamembrane Domain
KDR	: Kinase Insert Domain Receptor

kDa	: Kilodalton
LC	: Light Chain
LB	: Lysogeny Broth
mTOR	: Mammalian Target of Rapamycin
mRNA	: Messenger Ribonucleic Acid
µg	: Microgram
µL	: Microliter
mL	: Milliliter
mm	: Millimeter
MEK	: Mitogen-activated protein kinase kinase
ng	: Nanogram
nm	: Nanometer
NRP-1/2	: Neuropilin-1/2
NGS	: Next-generation Sequencing
NSCLC	: Non-small Cell Lung Cancer
OD ₆₀₀	: Optical Density at 600 nm
PBS	: Phosphate Buffered Saline
PI3K	: Phosphoinositide 3-Kinase
EPCs	: Placental Growth Factor
PDGF	: Platelet-derived Growth Factor
PEG	: Polyethylene Glycol
PCR	: Polymerase Chain Reaction
PD-L1	: Programmed Cell Death Ligand Protein-1
PD-1	: Programmed Cell Death Protein-1
RAF	: Rapidly Accelerated Fibrosarcoma
RAS	: Rat Sarcoma
RTK	: Receptor Tyrosine Kinase
RCC	: Renal Cell Carcinoma
STAT3	: Signal Transducer and Activator of Transcription 3
scFv	: Single-chain variable fragment
ssDNA	: Single-stranded Deoxyribonucleic Acid
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sp1	: Specificity Protein 1
SOC	: Super Optimal Medium with Catabolic Repressor
TMB	: Tetramethylbenzidine
TMD	: Transmembrane Domain
TME	: Tumor Microenvironment
TKD	: Tyrosine Kinase Domain
TKI	: Tyrosine Kinase Inhibitor
FDA	: US Food and Drug Administration
VH	: Variable Heavy
VL	: Variable Light
VEGF	: Vascular Endothelial Growth Factor
VEGFR	: Vascular Endothelial Growth Factor Receptor

VPF	: Vascular Permeability Factor
PIGF	: Placental Growth Factor
wAMD	: Wet Are-related Macular Degeneration
2xYT	: 2x Yeast Extract Tryptone
YENB	: Yeast Extract Nitrogen Base



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1. INTRODUCTION

1.1. Blood Vessel Formation

Blood vascular system is fundamental to all cells in the body throughout embryonic and postnatal development. By carrying nutrients, oxygen, and blood cells to tissues and organs, and removing waste metabolites, blood vasculature helps maintain body homeostasis [1]. Endothelial cells form the primary cellular layer of the blood vasculature. Beyond their structural role, they directly regulate many functions, such as vascular permeability, blood flow, coagulation, inflammation, and immune responses, in response to microenvironment stimuli [2]. Throughout the life of higher organisms, blood vessel formation is mediated by two fundamental mechanisms: vasculogenesis and angiogenesis.

1.1.1. Vasculogenesis

In the early phases of embryonic development, the primitive blood vasculature is formed from embryonic endothelial progenitor cells (EPCs) through a mechanism termed vasculogenesis [3]. During vasculogenesis, EPCs derived from hemangioblasts within blood islands differentiate into simple tube-like vascular structure (**Figure 1.1**) [4]. Vasculogenesis is genetically predetermined and driven mainly by potent angiogenic factors, such as vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGFR-2), and fibroblast growth factor (FGF) in the early days of embryogenesis [5], [6]. Additionally, early markers of EPCs, including VEGFR-2, characterize EPC and drive vasculogenesis along with their signaling pathways [7], [8].

Previously, vasculogenesis was considered a mechanism limited to embryonic development. With the isolation of circulating EPCs (cEPCs) in the peripheral blood of adults, however, this concept was in a debate [9]. Subsequent findings proposed that cEPCs contribute to physiological and pathological vasculogenesis in postnatal development [1], [10], [11]. Nevertheless, to date, there is still no consensus about

whether postnatal vasculogenesis relies on the incorporation of cEPCs into the vascularization or by secreting proangiogenic factors like VEGF [2].

1.1.2. Angiogenesis

Following the formation of primary capillaries through vasculogenesis, the vascular network undergoes further expansion and remodeling via angiogenesis. Unlike vasculogenesis, angiogenesis involves the formation of new vessels from pre-existing vasculature (**Figure 1.1**) [12]. Angiogenesis is essential in establishing a mature and functionally organized vascular system during both growth and adult physiology. In embryonic development, it is particularly active and mediates the formation of a major part of vasculature. This is because the continuously growing embryo requires adequate blood flow to supply nutrients and oxygen, which in turn drive angiogenesis. In contrast, angiogenesis is quiescent in adults and limited to wound healing, female menstrual cycles, and pregnancy [13].

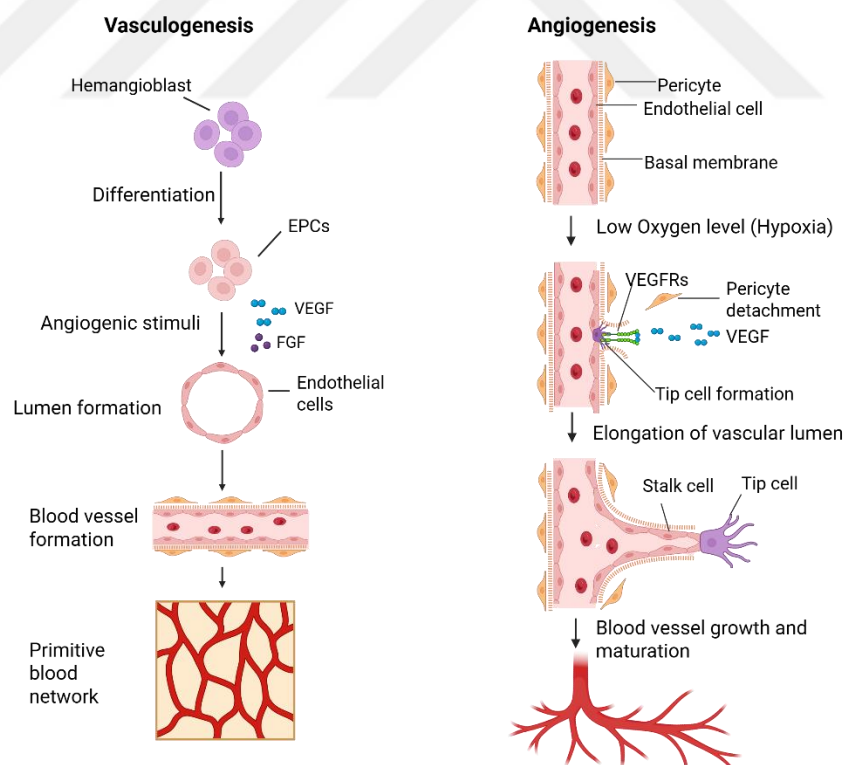


Figure 1.1: Schematic overview of vasculogenesis and angiogenesis. Created in BioRender.com [14].

Several modes of angiogenesis controlled by various signaling factors such as hypoxia, the presence of low-level oxygen, and VEGF-VEGFR signaling pathway take place in new blood vessel formation. These include sprouting angiogenesis, intussusceptive angiogenesis, and coalescent angiogenesis. Sprouting angiogenesis is the most well-characterized and considered the classical mechanism of angiogenesis. Under hypoxic conditions, new blood vessel formation is necessary to satisfy the elevated demand for oxygen. This triggers the secretion of pro-angiogenic factors, primarily VEGF [15]. Through interaction with its receptors (VEGFRs) on endothelial cells, VEGF initiates downstream signaling cascades that activate and guide new vessel formation. In response, specialized endothelial cells known as tip cells proliferate toward VEGF signaling, making an outward extension of existing blood vessel. Following tip cells, adjacent stalk cells undergo proliferation and migration to extend the sprout and form vascular lumen (**Figure 1.1**) [15]. Once new sprouts are formed, they connect to existing vessels via anastomosis [16]. The spatial organization of new sprouts and tip-stalk cell determination are regulated by gradient VEGF and VEGFR concentration, and Notch-Delta signaling [17]. Following VEGF-induced tip cell extension, tip cells secrete abundant delta-like 4 (Dll4), a ligand of Notch receptor. Dll4 activates Notch receptor on the adjacent cell through cell-to-cell contact and suppress tip cell determination in those cells [18]. This feedback mechanism directs neighboring cells to adopt the stalk cell fate, thus maintaining an organized sprout formation.

A fine equilibrium between pro-angiogenic and anti-angiogenic signaling factors maintains physiological angiogenesis. Pro-angiogenic factors, such as platelet-derived growth factor (PDGF), angiopoietins, FGF, and VEGF, induce endothelial cell activation, proliferation, and vascularization [19]. Conversely, anti-angiogenic molecules, including thrombospondins, endostatin, and angiostatin, inhibit excessive or aberrant vessel formation, thereby maintaining vascular homeostasis [20]. However, in pathophysiological states such as cancer, retinopathies, atherosclerosis, arthritis, obesity, and chronic inflammation, this balance is disrupted [1]. With the over-expression of pro-angiogenic factors, disorganized excessive blood vasculature occurs in pathological angiogenesis. Given that angiogenesis is central to both physiological processes and many pathological states, its mechanisms have been extensively studied over the last five decades. Research has firmly substantiated that

VEGF-VEGFR axis is the primary mediator of angiogenesis, thus attracting enormous interest in both physiological development and disease treatment [4], [21].

1.2. VEGF-VEGFR Family

The VEGF-VEGFR family constitutes the principal angiogenic molecules involved in both vasculogenesis and angiogenesis under normal physiology and pathological states. In mammals, the VEGF-VEGFR family comprises five ligands, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) and three receptors, VEGFR-1, VEGFR-2, and VEGFR-3 (**Figure 1.2**). In addition, non-mammalian VEGF homologs, such as VEGF-E encoded by parapoxvirus Orf virus and VEGF-F isolated from snake venom, have also been identified [22]. VEGF is a member of the VEGF/PDGF superfamily, distinguished by a highly conserved cystine knot motif [23]. This motif contains cysteine amino acids that form intrachain and interchain disulfide bonds, which are essential for the dimeric conformation and biological activity of these molecules. Consequently, VEGFs are structurally dimeric proteins, mainly as homodimers and sometimes as heterodimers [24].

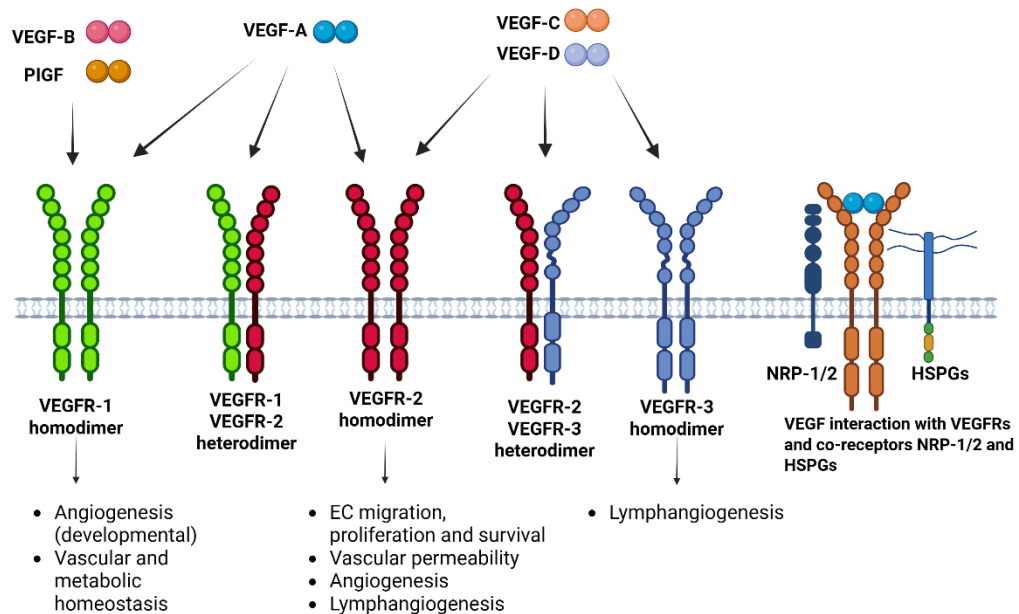


Figure 1.2: VEGF-VEGFR family. Illustration was adapted from [21] and re-created with modifications in BioRender.com.

Among the VEGF ligands, VEGF-A is the principal mediator for blood vessel formation and remodeling, primarily through its interaction with VEGFR-2 [25].

VEGF-C and VEGF-D bind to VEGFR-3 and play critical roles in lymphangiogenesis, the lymphatic vessel development [26]. VEGF-B and PlGF interact with VEGFR-1 and are generally considered non-essential for physiological angiogenesis. Nevertheless, VEGF-B is involved in cardioprotective and neuroprotective functions, as well as in regulating lipid uptake by endothelial cells [28]-[30]. PlGF contributes to the development and maturation of the placental vasculature, while also contributing to pathological angiogenesis under certain conditions [30]. Given the significance of VEGF-A and VEGFR-2, the present study focused on this signaling axis.

1.2.1. VEGF-A

VEGF-A is the most potent and extensively investigated angiogenic member of the VEGF family, owing to its critical roles in physiological and pathological angiogenesis. It is often referred to simply as VEGF. Initially, it was described in 1983 as a mediator of high permeability in tumor vasculature and, hence, named vascular permeability factor (VPF) [31]. Few years later in 1989, a growth factor with growth-stimulating effects on endothelial cells was identified and designated vascular endothelial growth factor (VEGF) [32]. Further studies on the isolation and sequencing of VPF and VEGF revealed that their identity as a single protein [33], [34]. Subsequent studies identified additional components of the VEGF family.

Human VEGFA gene is positioned on chromosome 6p12 and comprises eight exons interrupted by seven introns. Alternative splicing of VEGF-A mRNA generates multiple splice variants, each characterized by distinct amino acid lengths, bioavailability, binding affinity for VEGFRs and co-receptors, and angiogenic potential [35], [36]. While the majority of VEGF-A isoforms are pro-angiogenic, some exhibit reduced angiogenic activity or even inhibit angiogenesis and are thus considered as anti-angiogenic isoforms [37]. Pro-angiogenic isoforms are designated as VEGF-A_{xxx}a, whereas anti-angiogenic isoforms are VEGF-A_{xxx}b, where “xxx” indicates the specific number of amino acids. The most abundant and widely studied isoforms are VEGF-A_{111a}, VEGF-A_{121a}, VEGF-A_{145a}, VEGF-A_{145b}, VEGF-A_{165a}, VEGF-A_{165b}, VEGF-A_{189a}, and VEGF-A_{206a}. Among these, VEGF-A_{165a} is the predominant pro-angiogenic isoform expressed under both physiological and pathological states [38]. Notably, the expression profiles of these isoforms vary among different cancer types and are linked with the tumor progression [37], [39], [40]. For

instance, VEGF-A_{111a}, VEGF-A_{121a}, VEGF-A_{165a} are found to be upregulated in non-small cell lung cancer (NSCLC), prostate cancer, and renal cell carcinoma (RCC), respectively [39], [41], [42]. As understanding of VEGF splice variants in cancer deepens, improved therapies are now explored with a focus on VEGF-A isoforms.

1.2.2. VEGFRs

VEGFs mediate their angiogenic signaling through three main canonical receptors VEGFR-1, VEGFR-2, and VEGFR-3, as well as co-receptors neuropilin-1/2 (NRP-1 and NRP-2) and heparan sulfate proteoglycans (HSPGs) (**Figure 1.2**) [43]. They fall within the receptor tyrosine kinase (RTK) superfamily, a major class of transmembrane receptors regulating diverse processes including cellular growth, proliferation, differentiation, survival, and metabolism [44]. VEGFRs share an extensive level of structural similarity which presents an extracellular domain of seven immunoglobulin-like (Ig-like) repeats domain, a transmembrane domain, and an intracellular tyrosine kinase domain [43]. VEGFs interact with the extracellular Ig-like domains of VEGFRs, inducing receptor homo- or hetero-dimerization and activation of the receptor tyrosine kinase. Subsequent autophosphorylation of specific tyrosine residues within the intracellular domain triggers various downstream signaling pathways that control key processes, including endothelial cell proliferation, migration, survival, and vascular permeability [43].

Despite their structural homology, VEGFRs exhibit significant differences in ligand-binding affinity, selectivity, and downstream signaling responses. VEGFR-1 contributes to blood vessel formation, particularly in embryogenesis; VEGFR-3 participates in lymphangiogenesis, while VEGFR-2 serves as the major mediator of angiogenic signaling in the blood vascular system and also participates in lymphangiogenesis [21].

VEGFR-1 (also known as Fms-like tyrosine kinase-1, Flt-1), is mainly present on vascular endothelial cells and also found on monocytes, macrophages, neuronal cells, and certain tumor-associated cells [45], [46]. It exists as two splice variants: a full-length membrane-bound receptor and a short soluble isoform. VEGFR-1 engages with VEGF-A, VEGF-B, and PlGF with a very high affinity. Despite the strong binding, VEGFR-1 exhibits significantly lower angiogenic signaling activity compared to VEGFR-2.

Functionally, VEGFR-1 plays a significant role in vascular maturation during embryogenesis. Mouse embryos lacking both alleles of *Vegfr1* gene exhibit abnormal, disorganized blood vessel formation, resulting in embryonic lethality around days 8-9 [47]. Interestingly, embryos with deleted tyrosine kinase activity of VEGFR-1, meaning no angiogenic signal transduction via VEGFR-1, are able to develop normal vasculature and survive [48]. These findings indicate that VEGFR-1 functions primarily as a ligand-trapping receptor, thereby limiting VEGF availability to VEGFR-2. It is now widely accepted that VEGFR-1 acts both as a negative regulator of physiological angiogenesis during development, and as a positive regulator in pathological angiogenesis [46].

VEGFR-2, also referred to as kinase insert domain receptor (KDR), is expressed on both blood and lymphatic endothelial cells. It interacts with its primary ligand VEGF-A, as well as VEGF-C and VEGF-D. Through VEGF-A binding, VEGFR-2 transduced the bulk of VEGF-driven angiogenic signaling in vasculogenesis and angiogenesis [43]. Additionally, by responding to processed VEGF-C and VEGF-D, it contributes to lymphangiogenesis. The vital role of VEGFR-2 in vascular development is evidenced by *Vegfr2*-deficient mice model, which exhibits embryonic lethality at days 8-9 due to impaired hematopoietic and endothelial cell development. [49]. In pathological conditions, it also serves as the main responder of VEGF. Aberrant VEGFR-2 signaling is implicated in various diseases, including cancer, age-related macular degeneration (AMD), rheumatoid arthritis, and diabetic retinopathy (DR) [50]. This makes the VEGF-VEGFR2 interaction a central focus of anti-angiogenic therapies.

Human VEGFR-2 gene is positioned at chromosome locus 4q11-12, encoding a full-length protein of 1356 amino acids (aa). VEGFR-2, in its mature form, is a 230-kDa transmembrane glycoprotein. Structurally, it comprises multiple domains: an extracellular domain (ECD, 1–764 aa), a transmembrane domain (TMD, 765–789 aa), and intracellular domain (790-1356 aa) (**Figure 1.3**). Intracellular domain includes a juxtamembrane domain (JMD, 790–833), and a tyrosine kinase domain (TKD, 834–1162) and a flexible C-terminal domain (CTD, 1163–1356) [51]. The ECD domain consists of an N-terminal signal peptide (1-19 aa) and seven Ig-like subdomains, IgD 1-7, which are essential for ligand binding and receptor dimerization. IgD1 regulates receptor-ligand binding, whereas IgD2 and IgD3 mediate strong binding to VEGF and

VEGF-induced receptor dimerization [52]. IgD2, 4, 5, and 6 are involved in regulating the ligand binding rate and stability [53]. After VEGF-binding, membrane-proximal IgD7 mediates and stabilizes the receptor dimerization (**Figure 1.3**) [54].

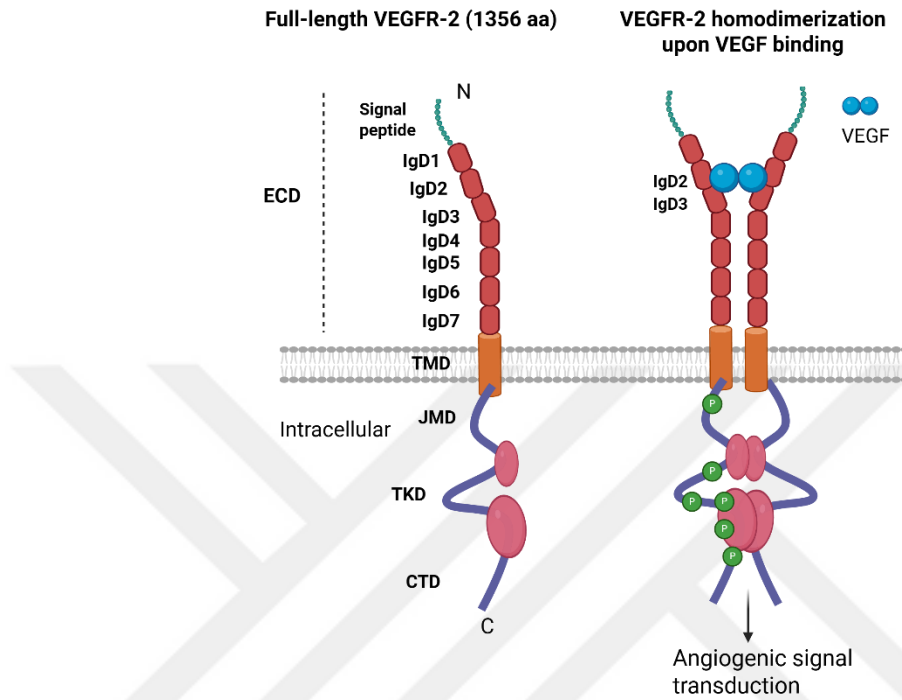


Figure 1.3: VEGFR-2 structure and homodimerization upon VEGF binding. Created in BioRender.com.

VEGFR-3 (also known as Fms-like tyrosine kinase-4, Flt-4) is present in diverse cell types, including capillary, venous and lymphatic endothelial cells, neuronal progenitors, macrophages, and osteoblasts. It is engaged by VEGF-C and VEGF-D and contributes to lymphangiogenesis during both embryonic and adult development. In early embryogenesis, it is found on both blood and lymphatic endothelial cells, highlighting its significance in vascular development [55]. *Vegfr3* gene inactivation in mice leads to death at embryonic day 9.5, caused by defective vascular organization and subsequent cardiovascular failure [56]. Moreover, excessive upregulation of VEGFR-3 and VEGF-C is associated with pathological lymphangiogenesis and angiogenesis in breast and gastric cancers [57], [58].

1.3. VEGF-VEGFR-2 Signaling in Pathological Angiogenesis

Pathological angiogenesis refers to the formation of structurally and functionally abnormal blood vessels, which are often excessive, disorganized, and highly permeable. This process is a hallmark of various diseases, including cancer, DR, AMD, atherosclerosis, and chronic inflammatory disorders such as rheumatoid arthritis [1]. Among them, cancer was arguably the first disease that was shown to promote the formation of excessive vasculature [59]. In 1971, Judah Folkman, a pioneer in angiogenesis research, postulated that the tumor growth beyond 1-2 mm³ depends on the development of new blood vessels [60]. He introduced the concept of anti-angiogenesis and hypothesized that inhibiting the formation of tumor-associated vasculature could offer a novel approach in cancer therapy. Following Folkman's hypothesis, research over decades has established angiogenesis as a fundamental hallmark of cancer [61]. Central to this process, the VEGF-VEGFR signaling axis drives tumor-induced angiogenesis, particularly through the VEGF-A-VEGFR-2 binding (**Figure 1.4**) [62].

Tumors are characterized by rapid and uncontrolled proliferation, which requires significantly higher amounts of oxygen and nutrients compared to normal tissues [63]. Once a tumor outgrows a size of 1-2 mm³, sufficient oxygen and nutrient supply become critical. To meet this demand, tumors and tumor-associated cells within tumor microenvironment (TME) overexpress VEGF and VEGFR-2 under hypoxic conditions, thereby promoting new vessel formation [64]. Several mechanisms upregulate VEGF expression to drive tumor angiogenesis. These include hypoxia, oncogenic mutations, and inflammatory signaling.

Hypoxia is a well-established regulator of VEGF expression. Under low oxygen conditions, cells and tissues primarily respond through the activation hypoxia-inducible factor (HIF) transcription factors [65]. HIF-1 binds to a well conserved region in the 5' promoter of the VEGFA gene, known as hypoxia response element (HRE), leading to overexpression of VEGF and promoting angiogenesis [66]. Additionally, hypoxia-independent induction of HIF-1 and subsequent VEGF-A overexpression has been observed in several cancers, such as breast, neuroblastoma, RCC and NSCLC [38], [67]. Beyond HIF-1, several other transcription factors are known to contribute to VEGF overexpression. These include Sp1, AP-1, STAT3, RAS

and RAF, which can be activated by various oncogenic signals and inflammatory pathways, further enhancing VEGF-mediated angiogenesis in tumors [38], [68].

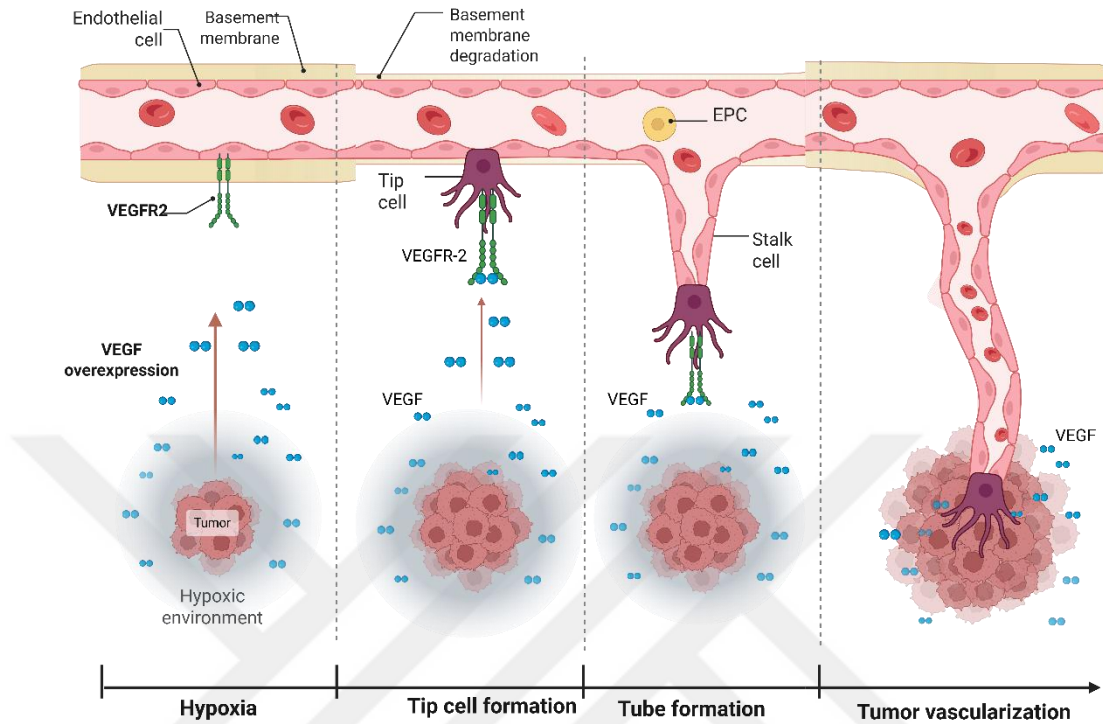


Figure 1.4: Schematic representation of VEGF-driven tumor angiogenesis. Created in BioRender.com.

VEGF expression is upregulated in a wide range of tumors, such as glioblastoma multiforme, juvenile hemangioma, melanoma, lung, breast, head and neck, ovarian, gastric, colorectal, and renal carcinomas. Similarly, VEGFR-2 is also overexpressed in various malignancies, including breast and colorectal cancers, melanoma, thyroid carcinoma, ovarian cancer, NSCLC, urothelial carcinoma, and acute myeloid leukemia [69].

Excessive VEGF-VEGFR2 signaling drives multiple processes that support tumor survival, progression, and metastasis. One of key effects is the promotion of abnormal angiogenesis in tumor. This results in the formation of dense, disorganized, and leaky vasculature that ensures a continuous delivery of oxygen and nutrients to tumor, thereby promoting its survival. VEGF signaling, primarily thorough VEGFR-2, stimulates endothelial cell proliferation and migration, processes essential for tumor angiogenesis. Additionally, VEGF can directly influence tumor cells through VEGFRs expressed on their surface, thereby enhancing tumor aggressiveness and metastatic potential [38]. Moreover, VEGF increases microvascular permeability, facilitating

cancer cells entry into the bloodstream and promoting metastasis [70]. It also contributes to the establishment of a supportive endothelial vascular plexus within the TME by recruiting cEPC from the bone marrow [71].

Importantly, VEGF is produced by tumor cells and stromal cells, including tumor-associated endothelial cells, cancer-associated fibroblasts, and various immune cells. Beyond its function in tumor angiogenesis, VEGF acts as an immunosuppressive cytokine, impairing the functionality of multiple immune cells within the TME [38]. These actions include the suppression of T cell development, induction of T cell exhaustion, and the inhibition of dendritic cell maturation [72]-[74]. Specifically, VEGF enhances VEGFR-2 upregulation on immunosuppressive regulatory T cells and M2 macrophages [75]. Furthermore, VEGF-VEGFR-2 signaling facilitates the recruitment of immunosuppressive cell populations into the TME [76], [77]. The formation of abnormal vasculature within the TME in response to high level of VEGF also impairs the infiltration of cytotoxic T cells [78]. Collectively, VEGF-VEGFR signaling promotes an immunosuppressive, pro-tumorigenic microenvironment that supports tumor immune evasion and progression.

1.4. Targeting VEGF-VEGFR-2 in Cancer

Since the inception of the anti-angiogenic cancer therapy approach by Judah Folkman in 1970s, the factors and pathways regulating angiogenesis have been intensively studied [60]. Decades of research on angiogenesis under both physiological and pathological states have highlighted the VEGF–VEGFR axis as a key therapeutic target. Consequently, numerous anti-angiogenic therapeutics targeting the VEGF-VEGFR axis have since been engineered and approved to treat various cancers and ocular diseases (**Figure 1.5**).

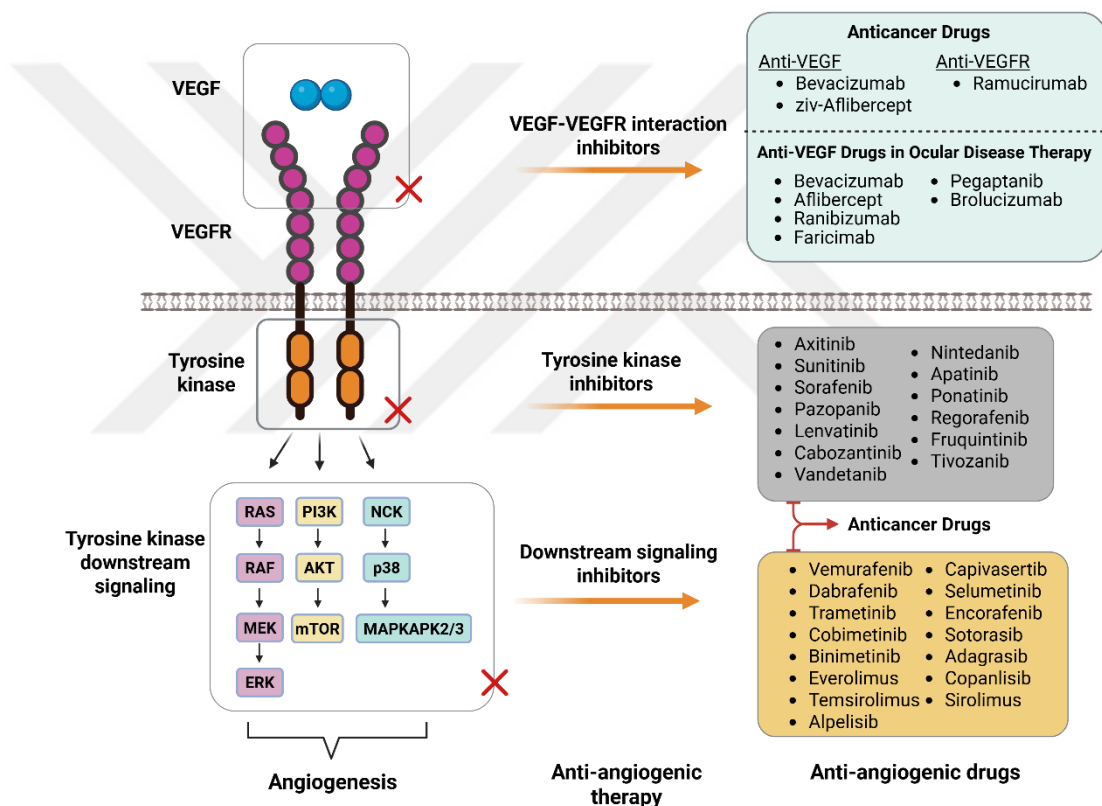


Figure 1.5: FDA-approved anti-angiogenic drugs targeting VEGF-VEGFR pathway. Illustration was adapted from [79], re-created in BioRender.com, and updated to include recently approved drugs.

The therapeutic strategy is conceptually straightforward: to prevent the interaction between VEGF and VEGFRs, to inhibit the tyrosine kinase activity or to block the downstream signaling pathways. Such therapeutics can be generally categorized into two groups: (i) large recombinant protein macromolecules including antibodies and fusion proteins that neutralize VEGF or block VEGFRs, and (ii) small-molecule tyrosine kinase inhibitors (TKIs) interfering with intracellular signaling of VEGFRs.

On account of their large molecular size, antibodies and fusion proteins are unable to penetrate the cell membrane. Consequently, they exert their anti-angiogenic effects extracellularly by binding either to VEGF ligands or to VEGFRs, thus, preventing the VEGF-VEGFR interaction [80]. In contrast, small molecules can cross cell membrane and inhibit the intracellular signaling of the VEGF-VEGFR system [79].

Bevacizumab (Avastin; Genentech/Roche) is the first major breakthrough in targeting the VEGF–VEGFR signaling axis. The first experimental result came in 1993, showing monoclonal antibodies targeting VEGF inhibited tumor growth *in vivo* [81]. This study was instrumental the development of humanized VEGF-specific monoclonal antibody bevacizumab capable of binding to all VEGF-A isoforms. Bevacizumab subsequently emerged as the first anti-angiogenic therapeutic approved by the US Food and Drug Administration (FDA) in 2004 for the first-line treatment of metastatic colorectal cancer with the combination of chemotherapy [82]. To date, bevacizumab has received approval for eight types of cancer, including colorectal cancer (2004), NSCLC (2006), metastatic breast cancer (2008), glioblastoma (2009), RCC (2009), cervical cancer (2014), ovarian cancer (2018). Clinical trials of bevacizumab in combination of chemotherapeutic agents showed enhanced overall survival, progression-free survival, and response rates [80]. In addition, bevacizumab is also used as off-label treatment for neovascular AMD and diabetic macular edema (DME).

Targeting VEGFRs have also showed improvement in cancer therapy. Ziv-Aflibercept (Zaltrap, Regeneron, Sanofi) is a recombinant decoy receptor fusion protein consisting of one domain from VEGFR-1 (IgD2) and one from VEGFR-2 (IgD3), fused to the Fc domain of human IgG1 [80]. It targets VEGF-A, VEGF-B and PlGF and blocks their interaction with endogenous VEGFRs. It was approved in 2012 for metastatic colorectal cancer in combination of chemotherapy [83]. A related formulation, aflibercept (Eylea, Regeneron, Bayer), is approved for ocular diseases, including wet AMD (wAMD), DME and DR.

Ramucirumab (Cyramza; Eli Lilly & Company) is a recombinant human monoclonal antibody that targets VEGFR-2 and prevents its interaction with VEGF. Unlike bevacizumab, which targets VEGF, ramucirumab directly blocks receptor VEGFR-2 itself, offering an alternative approach to VEGF inhibition. It was approved as a single agent or in combination of chemotherapy for the treatment of stomach, gastric,

colorectal, and hepatocellular cancers [84]. In a recent clinical trial, ramucirumab plus erlotinib, an EGFR TKI, has notably improved the progression-free survival compared with placebo plus erlotinib, in the treatment of EGFR-mutated metastatic NSCLC [85]. This has led to an approval with combination of erlotinib as a first-line treatment for EGFR-mutated metastatic NSCLC in 2020.

Alongside macromolecular biologics, numerous small-molecule TKIs have also been engineered and clinically approved for treating diverse cancers. These drugs inhibit either tyrosine kinase activity of VEGFRs or downstream signaling pathways [79]. For example, axitinib (Inlyta, Pfizer), sorafenib (Nexavar, Bayer), and sunitinib (Sutent, Pfizer) are approved for the treatment of metastatic RCC and other cancers. VEGFR downstream signaling inhibitors target RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, and NCK/p38/MAPKAP2/3 pathways, which are critical for cell proliferation, survival, and angiogenesis [79]. A major disadvantage of small-molecule TKIs is that they simultaneously block multikinase receptors, which can lead to serious off-target effects [86].

Notably, the combination of anti-angiogenic therapy with immunotherapy in cancer treatment has recently gained considerable attention. Multiple clinical trials have investigated the safety and efficacy of this approach. Some of the trials have demonstrated improvements in progression-free survival and overall survival relative to monotherapy [87]-[89]. These findings have resulted in approval of anti-angiogenic and immunotherapy combinations for different cancers [90]. For instance, the combination of bevacizumab and atezolizumab, an anti-programmed cell death ligand protein-1 (PD-L1) monoclonal antibody, was approved as a first-line therapy for hepatocellular carcinoma [87]. Likewise, axitinib, a VEGFR TKI, was approved in combination of pembrolizumab, an anti-programmed cell death protein-1 (PD-1) antibody, for the first-line treatment of advanced RCC [88].

1.5. Antibody Phage Display

1.5.1. Antibodies

Over the past decades, advances in biotechnology have contributed to the generation of numerous biopharmaceuticals targeting diverse conditions, including autoimmune diseases, cancer, and infectious diseases. These biopharmaceuticals include monoclonal and bispecific antibodies, antibody fragments, recombinant proteins, vaccines, hormones, growth factors, peptides, and cell-based therapies. Among these, antibodies represent the largest and best-selling class of biotechnological products [91].

Antibodies are immunoglobulin proteins secreted by B lymphocytes upon encountering foreign antigens including toxins, pathogens, and abnormal cells. Each antibody recognizes and binds to a unique region on the antigen, called an epitope, with high specificity and affinity. Structurally, they consist of two identical heavy chains (HC) and two identical light chains (LC), bound by disulfide bonds into a characteristic Y-shaped structure (**Figure 1.6**). Each antibody is composed of two main functional regions: fragment antigen-binding (Fab) region and fragment crystallizable (Fc) region. The Fab region contains the variable domains of the heavy (VH) and light (VL) chains, which jointly constitute the antigen-binding site responsible for epitope recognition. The Fc region, made up of the constant domains of the heavy chains, regulate effector functions through interactions with immune cells and the activation immune pathways [92]. Upon binding, antibodies can directly neutralize the antigen or initiate immune responses including complement-mediated lysis of infected cells and pathogens, antibody-dependent cellular cytotoxicity, and phagocytosis [93].

Owing to their high specificity, affinity, and functional versatility, antibodies remain essential tools in both biomedical research and clinical therapy. To expand their applications and optimize their properties, various recombinant antibody formats have been engineered for specific therapeutic and diagnostic purposes. These formats include full-length monoclonal antibodies, bispecific antibodies, Fab fragments, single-chain variable fragments (scFv), nanobodies (single-domain antibodies), diabodies, among others. Each format offers unique advantages in terms of size, tissue

penetration, stability, and target specificity, making them suitable for diverse biomedical applications.

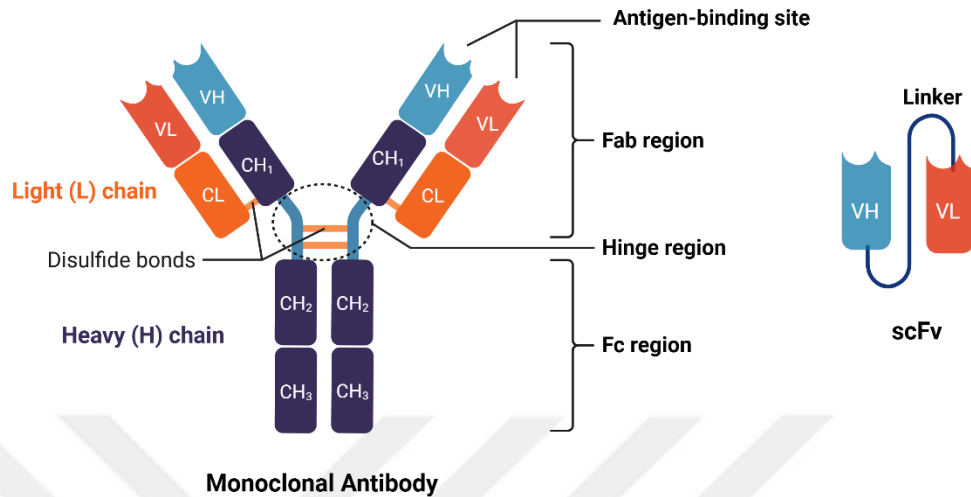


Figure 1.6: Structure of monoclonal antibody and scFv. Created in BioRender.com.

1.5.2. Single-chain Variable Fragment (scFv)

ScFv is an engineered recombinant antibody fragment consisting of a variable heavy chain and a variable light chain, linked by a flexible polypeptide linker (**Figure 1.6**). With a molecular weight of 25–30 kDa, scFvs are typically arranged in either a VL-linker-VH or VH-linker-VL orientation, depending on structural stability and functional requirements [94]. This design preserves the antigen-binding capability of the parent antibody yet significantly reducing the molecular size. One of the major advantages of scFvs is their compact design and the ease of genetic manipulation. Their simple structure allows rapid and versatile engineering for a wide range of applications, including diagnostics, targeted drug delivery, radiotherapy, and clinical therapy [95]. They are usually monovalent but can be easily engineered into multivalent or bispecific formats (e.g., diabodies, tandem scFvs). These features make scFvs highly attractive candidates for next-generation antibody-based technologies. ScFv offers several advantages over full-length antibody. For instance, due to its small size, scFv has greater rate of penetration into tissue and solid tumors compared to intact antibody [96]. In addition, scFvs are rapidly cleared from blood, which is beneficial for diagnostic imaging and targeted therapy. This property makes them particularly attractive for targeting specific markers on tumor cells, neutralizing tumor-associated

proteins and effectively delivering drugs and toxins. Moreover, the absence of Fc region reduces their immunogenicity, making them more suitable for repeated or long-term administration. Another major advantage is the easy, cost-effective, and large-scale production in bacterial expression systems including *Escherichia coli* [97]. Finally, they are easily developed from *in vitro* display systems such as phage display and yeast display.

To date, only a few scFvs have received approval from the FDA. These include brotuzumab (Beovu, Novartis), a scFv monoclonal antibody binding to VEGF and approved for treatment of wAMD and DME, and blinatumomab (Blincyto, Amgen), a bispecific antibody fragment consisting of two scFvs (diabody) and approved for the treatment of B-cell acute lymphoblastic leukemia [98]. Nevertheless, numerous scFv-based therapeutic strategies, including scFv-protein and scFv-drug fusions, and bispecific antibodies, are now in various clinical trials for the treatment of a wide range of cancers [99].

1.5.3. Phage Display

Phage display is a powerful *in vitro* antibody selection technology broadly employed to discover peptides and antibodies specific to diverse antigens. It is based on the genetic insertion of DNA sequences encoding peptides or antibody fragments into the genome of filamentous bacteriophages, also called phage, viruses that infect bacteria [100]. By genetically fusing the inserted protein to a bacteriophage surface protein, the fused protein can be successfully displayed on the phage surface. This technology maintains the link between genotype and phenotype and enables the development and selection of vast peptide and antibody libraries against specific targets.

Phage display was originally developed in 1985 by George P. Smith. He demonstrated that fusing a peptide-encoding DNA sequence into gene III of the filamentous M13 bacteriophage enabled surface presentation of the peptide without compromising virion infectivity [101]. Later, Gregory Winter and colleagues successfully extended this approach for screening antibody and antibody fragments against a specific antigen [102]. Since then, phage display has become a widely utilized platform for the generation of numerous peptides and antibodies exhibiting high specificity and affinity toward their target antigens. In recognition of their pioneering contributions, George

P. Smith and Sir Gregory P. Winter were awarded the Nobel Prize in Chemistry in 2018 for “the phage display of peptides and antibodies” [103].

Several *E. coli* bacteriophages have been employed in the phage display systems, including filamentous phages f1, fd, and M13, as well as lytic phages like T4 and lambda. Among these, M13 is the most widely used phage. The M13 bacteriophage has a circular single-stranded DNA (ssDNA) genome of 6407 base pairs (bp), comprising of nine genes that encode 11 distinct proteins [104]. Some of these proteins serve as coat proteins encapsulating the genome, while the others are required for phage replication and assembly (Figure 1.7).

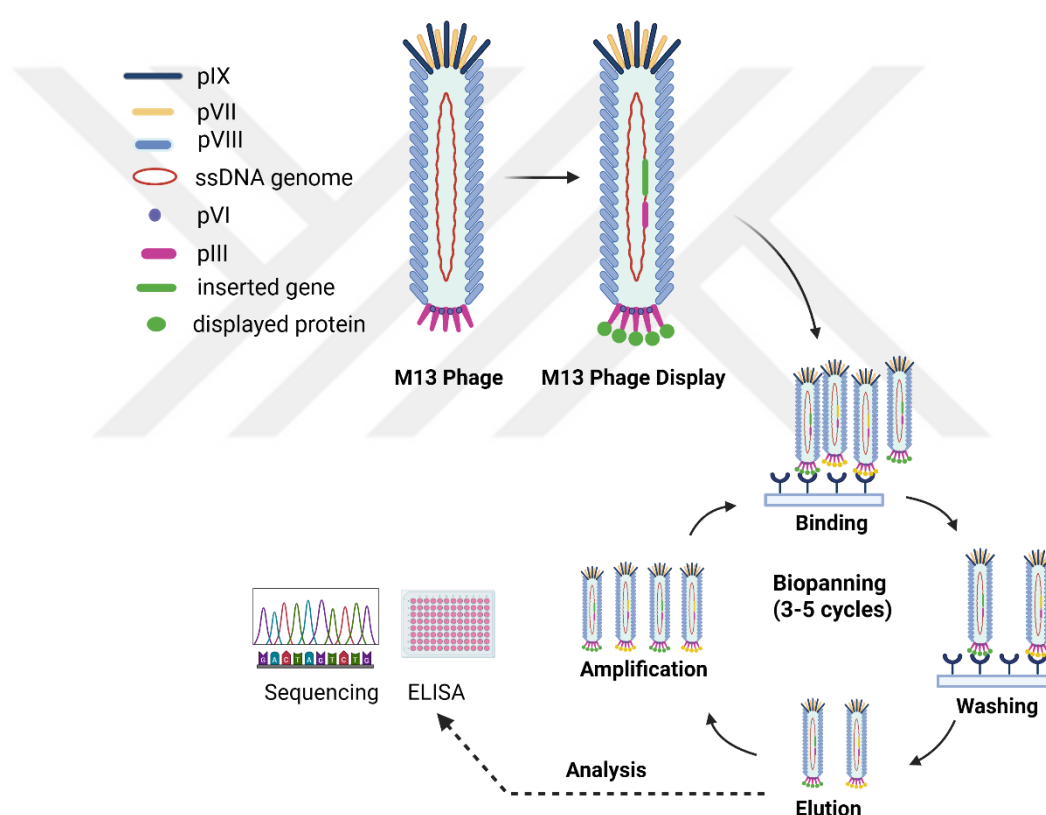


Figure 1.7: M13 phage display. Created in BioRender.com.

For phage display, the DNA of interest, typically encoding an antibody fragment or peptide, is mainly linked to the gene encoding the minor coat protein pIII (3–5 copies per phage). Although less common, the major coat protein pVIII (approximately 2,700 copies per phage) is also utilized in some studies [105]. These antibody-pIII fusions can be generated either by direct insertion into the phage genome or, more commonly, via phagemid. The phagemid is a plasmid-based vector that bears the antibody-pIII fusion gene, an origin of replication, and antibiotic selection gene [106]. Phagemids

can also be used in the standard cloning and expression of antibody-pIII fusions. To generate recombinant phage particles displaying the antibody, *E. coli* cells are first transformed with phagemid vector and then superinfected with a helper phage. Helper phages provide the necessary phage proteins for the phage assembly but contain a defective version of the targeted coat protein gene (e.g., pIII or pVIII). This dual system enables the preferential incorporation of phagemid-encoded antibody-pIII fusion proteins into the assembling phage particles. Finally, the antibody is displayed on the surface of the M13 bacteriophage as a fusion with pIII, while its encoding gene is simultaneously packaged in the same phage virion [107].

After the generation of antibody-phage particles, they are subjected to a process known as biopanning or panning (**Figure 1.7**). Biopanning is a selection and affinity enrichment procedure used to isolate antibody-phage clones that exhibit high affinity and specificity toward a target antigen [108]. In this process, the phage-antibody fusions are incubated with the target antigen, typically immobilized onto a solid surface like microtiter plate or presented in solution. This incubation allows specific binders to interact with the antigen. Unbound or weakly bound phages are washed away through rigorous washing, whereas strongly bound phages are subsequently recovered. The recovered virions are then amplified in *E. coli* and used in subsequent biopanning round. This cycle of binding, washing, elution, and amplification is generally repeated three to five times to progressively enrich the antigen-specific, high-affinity antibody-phage clones. At the end of the biopanning process, individual antibody-phage clones exhibiting high affinity against the antigen are identified then characterized using phage enzyme-linked immunosorbent assay (phage ELISA) and DNA sequencing.

1.5.4. Antibody Library

A key component of successful phage display screening against an antigen is the antibody library. Due to limitations of phage display and *E. coli* expression systems, the library should consist of small genes encoding small proteins. Full-length antibodies are not efficiently screened or expressed in these systems, although several examples of full-length IgG display have been reported [109]. Instead, antibody fragments including scFv, Fab, nanobodies and diabodies are common formats used in antibody phage display.

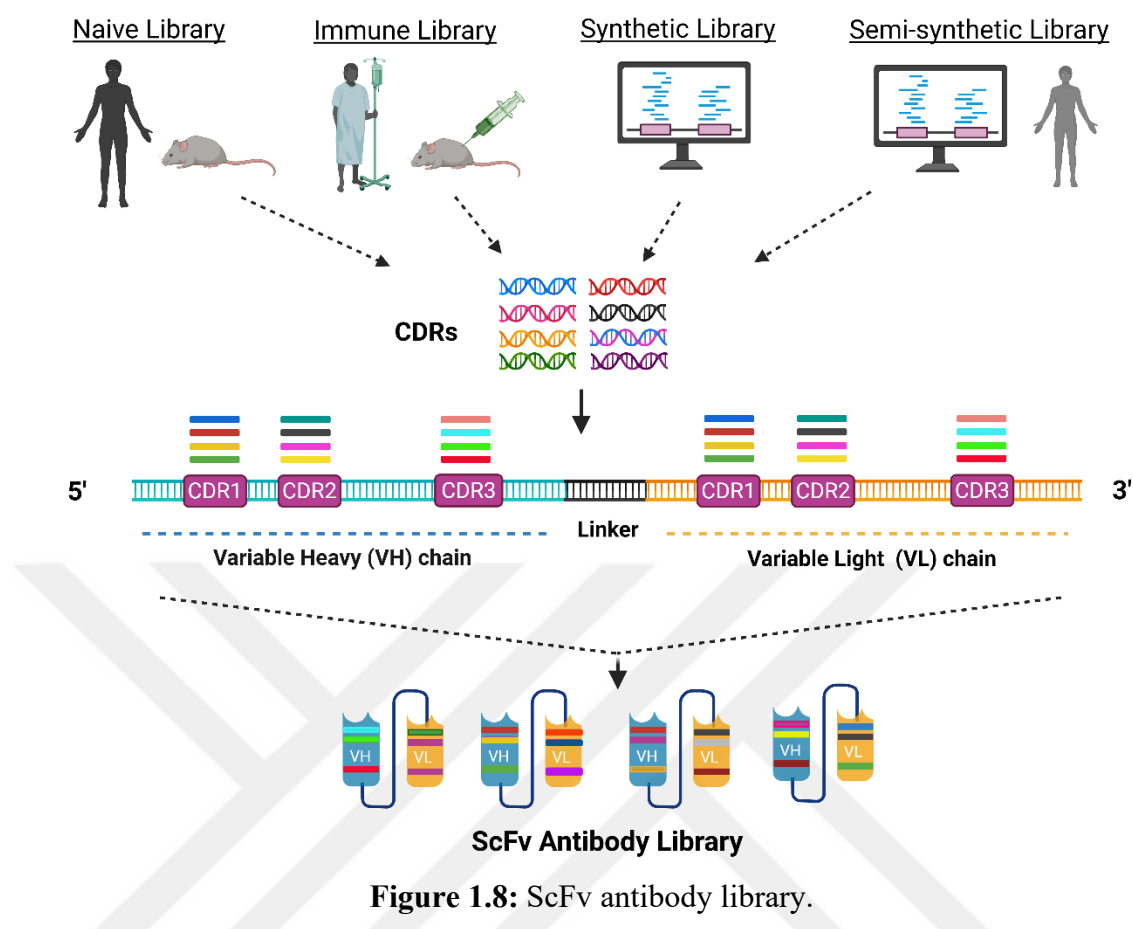


Figure 1.8: ScFv antibody library.

An effective antibody library should be well-constructed to ensure the functional display of antibody fragments and should possess high diversity to maximize the chances of isolating high-affinity, target-specific binders. The diversity is primarily introduced into the complementarity-determining regions (CDRs) of antibody frameworks, typically ranging from 10^8 to 10^{11} unique clones (**Figure 1.8**) [110]. The antibody library is generally generated in four approaches: naïve library, immune library, semi-synthetic library, and synthetic library. Naïve antibody libraries are developed from antibody variable gene repertoire of non-immunized healthy donors, such as humans and mouse. Since they are not naturally affinity-maturated against the target, they tend to have less affinity and specificity for the target but often exhibit a broad CDR diversity. In contrast, immune libraries are derived from variable antibody genes obtained from donors immunized with the target antigen or individuals with a relevant disease. It is valuable for the isolation of high-affinity and specific antibodies toward antigen used for immunization. However, the use of immune antibody library is limited against different antigens because of the diversity is biased toward a specific

target. In addition, the diversity of antibody libraries can be further enhanced with the synthetic or semi-synthetic approaches. While the semi-synthetic library is designed by the integration of natural and synthetic antibody genes, synthetic library is based on the generating artificial CDR sequences on common antibody frameworks [111]. These strategies are employed to increase not only the diversity but also the solubility, stability, and functional performance of antibody libraries.

To date, a vast number of phage display-derived antibodies have received FDA approval for treating a wide range of diseases, including cancer, autoimmune disorders, and infectious diseases. Notably, adalimumab (Humira, AbbVie) is the first phage display-derived human monoclonal antibody approved for the treatment of rheumatoid arthritis and other inflammatory bowel diseases in 2002. Additional examples include raxibacumab (ABthrax, HGS/GSK), atezolizumab (Tencentriq, Roche/Genentech), avelumab (Bavencio, Merck/Pfizer), among others. Focusing on the VEGF-VEGFR axis, anti-VEGFA ranibizumab (Lucentis; Genentech/Roche) and faricimab (Vabysmo, Roche) were also developed from the phage display technology. Importantly, anti-VEGFR-2 monoclonal antibody ramucirumab (Cyramza; Eli Lilly & Co.) was developed from a naïve Fab phage display library [112].

2. PURPOSE OF THE STUDY

The primary aim of this study was to develop and characterize novel scFv antibodies with high specificity and affinity against VEGFR-2, the key regulator of VEGF-driven tumor angiogenesis. For this purpose, a previously constructed synthetic human scFv library was screened for the first time against an antigen, VEGFR-2, using phage display technology. To specifically target the VEGF–VEGFR-2 interaction, the extracellular IgD2–D3 domains of VEGFR-2, known for their VEGF-binding activity, were used as bait in the biopanning process. Following the selection of scFv antibodies, the study aimed to evaluate their diversity, sequence characteristics, and enrichment through functional screening. Identified scFv antibodies were expressed and purified using *E. coli* expression system, and their binding to VEGFR-2 was assessed by ELISA. Finally, the biological activity of the purified anti-VEGFR-2 scFv antibodies was evaluated using endothelial cell viability and wound healing assays to determine their potential anti-angiogenic effects. Through these objectives, the study aimed to identify functionally active, high-affinity anti-VEGFR-2 scFv antibodies with the potential to contribute to the development of improved anti-angiogenic therapeutics.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Bacterial Strains and Helper Phages

The bacterial strains and helper phages used throughout this study are provided in **Table 3.1**.

Table 3.1: Bacterial strains and helper phages used in this study.

<u>Name</u>	<u>Provider/ Cat No.</u>	<u>Genotype</u>	<u>Application</u>
<i>E. coli</i> TG1	Lucigen, 60502	[F' traD36 proA+B+ lacIqZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrB hsdSM)5(rK- mK-)	Phage Propagation
<i>E. coli</i> ER2738	New England Biolabs, E4104	[F' proA ⁺ B ⁺ lacI ^q Δ(lacZ)M15 zzf::Tn10(Tet ^R)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5]	Phage Propagation
<i>E. coli</i> BL21(DE3)	New England Biolabs, C2527I	[fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::lacI::PlacUV5::T7 gene1) i21 Δnin5]	scFv expression
<i>E. coli</i> HB2151	Amersham Biosciences	K12 (lac-pro), ara, nalr, thi/F'[proAB, lacIq, lacZ M15]	scFv expression
M13KO7 Helper Phage	New England Biolabs, N0315S	-	Phage Propagation
CM13d3 Helper Phage	Antibody Design Labs, PH040L	-	Phage Propagation

3.1.2. Synthetic Human scFv Library, Phagemid Vector, and Primers

The synthetic human scFv antibody library used in this study was previously designed by Dr. Bertan Koray Balcıoğlu and Dr. Beytullah Özgür from Medical Biotechnology Research Group at TÜBİTAK Marmara Research Center, Kocaeli, Türkiye (unpublished data). This library consists of fully human scFvs engineered for broad antigen recognition by introducing diversity in the CDRs of both VH and VL chains.

Prior to this study, the scFv library was cloned into pCANTAB 5E phagemid vector for phage display screening by GenScript. The pCANTAB 5E phagemid carries an ampicillin resistance gene, a lac promoter, a signal peptide, an E-tag affinity epitope, and gene encoding the M13 pIII coat protein for surface display (**Figure 3.1**). The scFv library was inserted at the N-terminus of pIII protein, with the signal peptide at the N-terminal end of the scFv and E-tag affinity epitope at the C-terminal end. This vector was used both for the selection of scFv binders against VEGFR-2 in phage display screening and for the expression of recombinant scFv antibodies.

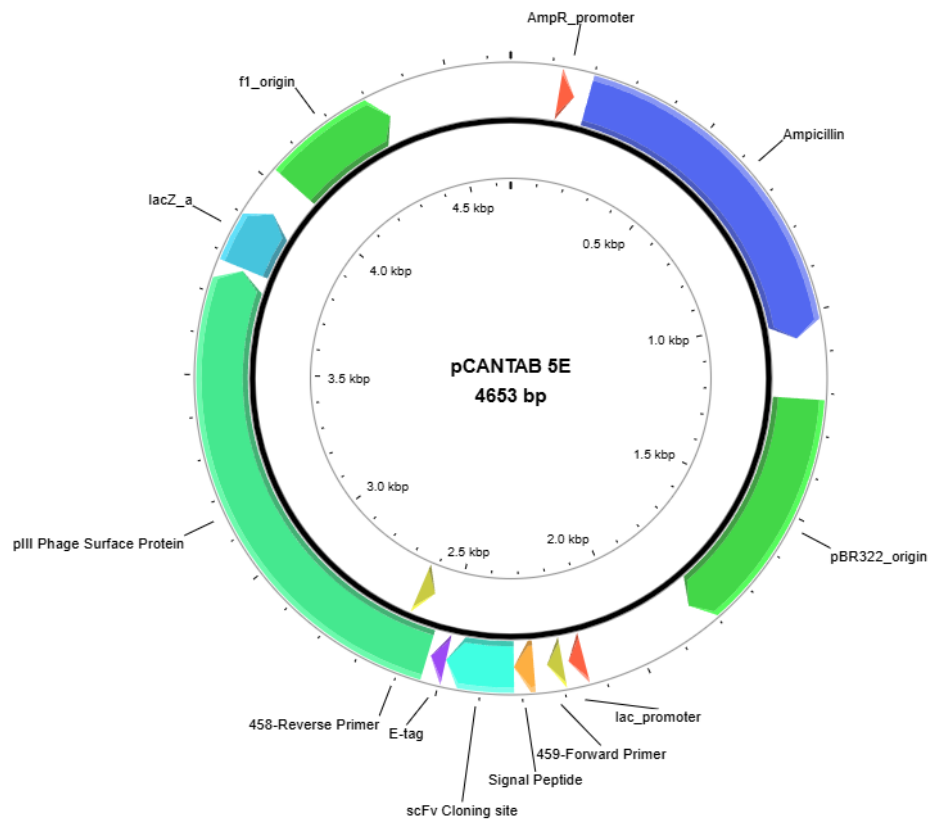


Figure 3.1: Map of pCANTAB 5E phagemid vector. Created in PlasMapper [113].

For polymerase chain reaction (PCR) application and sequencing, primers 459 (forward) and 458 (reverse) were used (**Table 3.2**). The binding sites of these primers are shown in **Figure 3.1**.

Table 3.2. Primers 458 and 459.

Primer	Sequence
459 Forward	5'-TATGACCATGATTACGCCAAG-3'
458 Reverse	5'- TTTTGTCTGCTTTCCAGACGTT-3'

3.1.3. Recombinant Proteins and Antibodies

Recombinant proteins and antibodies used in this study are provided in **Table 3.3**.

Table 3.3: Recombinant proteins and antibodies used in this study.

<u>Name</u>	<u>Provider</u>	<u>Cat No</u>
Recombinant Human VEGF-A (VEGF165) Protein	Sino Biological	1066-HNAB
Recombinant Human VEGFR2/KDR Protein (Domain 2&3, hFc Tag)	Sino Biological	10012-H02H2
Recombinant Human VEGFR2/KDR Protein (ECD, His Tag)	Sino Biological	10012-H08H
Anti-M13 Major Coat Protein Mouse Monoclonal Antibody	Santa Cruz Biotechnology	sc-53004
Anti-Mouse IgG-Peroxidase Antibody	Sigma-Aldrich	A9044
Anti-E tag Horseradish Peroxidase Polyclonal Antibody	Abcam	ab19400
Pierce™ Protein A Magnetic Beads	Thermo Scientific	88846

3.1.4. Kits, Enzymes, and Markers

All kits, enzymes and markers used throughout this study are provided in **Table 3.4**.

Table 3.4: List of kits, enzymes, and markers used in this study.

<u>Name</u>	<u>Provider</u>	<u>Catalog No</u>
NucleoSpin® Plasmid Miniprep Kit	Macherey-Nagel	740588.50
NucleoSpin® Gel and PCR Clean-up Kit	Macherey-Nagel,	740609.50
ZR DNA Sequencing Clean-Up Kit	Zymo Research	D4050
GenomeLab DTCS Quick Start Kit	Beckman Coulter	608118 AH
Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit	Thermo Scientific	23227
<i>Bst</i> NI Restriction Enzyme	New England Biolabs	R0168S
MyTaq™ DNA Polymerase	Bioline	BIO-21105
DNase I Enzyme	Thermo Scientific	PI90083
Lysozyme	Thermo Scientific	89833
PageRuler™ Plus Prestained Protein Ladder	Thermo Scientific	26619
GeneRuler 1 kb DNA Ladder	Thermo Scientific	SM0311
MassRuler Low Range DNA Ladder	Thermo Scientific	SM0383

3.1.5. Chemicals

Chemicals and reagents used throughout this study are provided in **Table 3.5**.

Table 3.5: List of chemicals and reagents used in this study.

<u>Name</u>	<u>Provider</u>	<u>Catalog No</u>
Acetic Acid	Sigma-Aldrich	A6283
30% Acrylamide/Bis Solution	Bio-Rad	1610158
Agarose	Sigma-Aldrich	A9539
Ammonium Persulfate	Bio-Rad	1610700
Ampicillin	Sigma-Aldrich	A9518
Bacto Agar	BD Biosciences	214010

Table 3.5: Continued.

Bacto Tryptone (Peptone)	Thermo Fisher Scientific	211705
Bovine Serum Albumin	Roche	3117332001
B-PER™ Bacterial Protein Extraction Reagent	Thermo Scientific	90079
Calcium Chloride	Sigma-Aldrich	C4901
Carbonate-Bicarbonate Buffer	Sigma-Aldrich	C3041
4-Chloro-1-Naphthol (4C1N)	Sigma	C6788
Coomassie Brilliant blue R 250	Sigma-Aldrich	1125530025
Ethanol	Sigma-Aldrich	1009831011
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich	E5134
Glucose D (+)	Sigma-Aldrich	G8270
Glycerol	Sigma-Aldrich	G7757
Glycine	Sigma-Aldrich	G7126
Hydrogen Peroxide	Kimpa	-
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	I5502
Kanamycin	Sigma-Aldrich	K1377
Magnesium Chloride	Sigma-Aldrich	M8266
2-Mercaptoethanol	Sigma-Aldrich	M3148
Methanol	Sigma-Aldrich	1060122500
Nonfat-Dried Milk	Sigma-Aldrich	M7409
Phosphate Buffered Saline	Sigma-Aldrich	P4417
Polyethylene Glycol (PEG) 6000	Merck	25322-68-3
SigmaFast™ Protease Inhibitor Cocktail	Sigma	S8830
SOC Medium	Thermo Fisher Scientific	15544034
Sodium Acetate	Millipore	7610
Sodium Chloride	Sigma-Aldrich	S9888
Sodium citrate	Sigma-Aldrich	C8532
Sodium Dodecyl Sulfate	Sigma-Aldrich	L3771
Sodium Phosphate	Sigma-Aldrich	30435
Sucrose	Sigma-Aldrich	S0389
Sulfuric Acid	Sigma-Aldrich	339741
SYBR™ Safe DNA Gel Stain	Invitrogen	S33102
Tetracycline	Sigma-Aldrich	T7660
Tween 20	Sigma	P1379
Tetramethylbenzidine (TMB)	Sigma-Aldrich	860336
Yeast Extract	Millipore	1037530500

3.1.6. Buffers and Media

The buffers and media used throughout this study, along with their composition, are provided in **Table 3.6**.

Table 3.6: Buffers and media used in this study.

<u>Name</u>	<u>Composition</u>	<u>Application</u>
Binding Buffer	20 mM sodium phosphate, 150 mM NaCl, pH 7.2	Protein purification
Biopanning Elution Buffer	0.2 M glycine pH 2.0	Biopanning
Coating Buffer	0.1 M carbonate-bicarbonate (0.07 M Sodium bicarbonate, 0.03 M sodium carbonate), pH 9.6	Coating in ELISA
4C1N Solution	1 tablet of 4C1N in 10 mL Methanol	Western blot
Destaining Buffer	10% Methanol, 7% Acetic acid	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Elution Buffer	0.1 M sodium citrate, pH 2.8	Protein purification
LB Agar	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar (per liter)	Bacterial growth
LB Medium	10 g tryptone, 5 g yeast extract, 10 g NaCl (per liter)	Bacterial growth
Lysis Buffer	4 mL B-PER™ buffer supplemented with 2 µM MgCl ₂ , 8 µL lysozyme, 8µL DNase I and 400 uL SigmaFast™ EDTA-free protease inhibitor cocktail (per 1 gram cell pellet)	Protein extraction
PEG/NaCl Buffer	20% PEG 6000, 2.5 M NaCl	Phage isolation
1x SDS Running Buffer	25 mM Tris, 192 mM Glycine, 0.1% SDS (w/v)	SDS-PAGE

Table 3.6: Continued

Staining Buffer	0.1% Coomassie Blue (w/v), 45% Methanol, 10% Acetic acid	SDS-PAGE
Stop Solution	2 μ L of 3M Sodium Acetate pH 5.2, 2 μ L of 100 mM Na ₂ - EDTA pH 8.0 and 1 μ L of 20 mg/mL of glycogen	Sequencing
Transfer Buffer	25 mM Tris, 192 mM Glycine, 20% Methanol (v/v)	Western blot
Triethanolamine Buffer	0.128 M NaCl, 0.28% triethan- olamine (v/v)	Western blot
1x Tris-Acetic acid-EDTA (TAE) Buffer	40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA pH 8.0	Agarose gel
Tris/EDTA/Sucrose (TES) Buffer	200 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, 8 μ L of DNase I, 400 uL Sig- maFast™ EDTA-free protease inhibitor cocktail (4 mL per 1 gram cell pellet)	Protein extrac- tion
4C1N Substrate Buffer	2 mL of 4C1N solution, 10 mL triethanolamine buffer, 150 uL hydrogen peroxide	Western blot
YENB Broth	7.5 gr yeast extract, 8 gr nutri- ent broth (per liter)	Bacterial growth
2xYT Agar	16 g tryptone, 10 g yeast ex- tract, 5 g NaCl, 15 g agar (per liter)	Bacterial growth
2xYT Broth	16 g tryptone, 10 g yeast ex- tract, 5 g NaCl (per liter)	Bacterial growth

3.1.7. Cell Culture Reagents and Consumables

Cell culture reagents and consumables used in this study are provided in **Table 3.7**.

Table 3.7: Cell culture reagents and consumables used in this study.

<u>Name</u>	<u>Provider</u>	<u>Catalog No</u>
alamarBlue Cell Viability Reagent	Thermo Scientific	DAL1025
Human Umbilical Vein Endothelial Cells (HUVECs)	Kind gift from Dr. Serhad ONAT (Balıkesir University, Türkiye)	-
1% Antibiotic-Antimycotic solution	Vicent	450-115 EL
96-well Flat Bottom Cell Culture Plate	Isolab	-
Dulbecco's Phosphate-buffered Saline (DPBS)	Gibco	14190144
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	41966-029
Fetal Bovine Serum (FBS)	Gibco	10438026
Trypsin-EDTA	Gibco	25200056

3.1.8. Laboratory Equipment

Laboratory equipment used throughout this study is provided in **Table 3.8**.

Table 3.8: Laboratory equipment used in this study.

<u>Name</u>	<u>Provider</u>	<u>Cat No</u>
AKTA Pure™ 25 Chromatography System	Cytiva	-
Autoclave	Systec	DX-150
Azure c600 Gel Imaging System	Azure Biosystems	-
CEQ™ 8000 Genetic Analysis System	Beckman Coulter	
Class II Biological Safety Cabinet	Faster	BH2004
Class II Biological Safety Cabinet	Esco	AC2-4E8
Eppendorf ThermoMixer® C	Eppendorf	2231001127
Gene Pulser® II Electroporation System	Bio-Rad	165-2105
GloMax® Explorer Multimode Microplate Reader	Promega	GM3500

Table 3.8: Continued.

HiTrap™ Protein L Affinity Column	Cytiva	29048665
Horizontal Electrophoresis System	Bio-Rad	1704405
Incubator	Nuve	EN500
Inverted Light Microscope	Soptop	ICX41
NanoDrop™ One/One ^C Microvolume UV-Vis Spectrophotometer	Thermo Scientific	ND-ONE-W
Nunc™ 96-Well DeepWell™	Thermo Scientific	260251
NUNC™ Maxisorp™ 96-well plate	Thermo Scientific	439454
pH meter	Mettler Toledo	30046240
Precision Balance	Sartorius	CPA32025
Protein Electrophoresis Equipment	Bio-Rad	1658033
Protein LoBind® Tubes	Eppendorf	22431081
Refrigerated Incubator	New Brunswick Scientific	Innova 4230
Refrigerated Centrifuge	Beckman Coulter	Allegra X-30
Refrigerated Microcentrifuge	Eppendorf	5415R
Refrigerated Ultracentrifuge	Beckman Coulter	Avanti J-26 XP1
Rotator	Lab Companion	CRT 350
SnakeSkin™ Dialysis Tubing, 3.5 kDa	Thermo Scientific	88242
Sonifier	Branson	SSE-1
Thermocycler	Biometra	T3000
Trans-Blot Turbo Transfer System	BioRad	17001915
Ultra-low temperature freezer -80 C	Haier Biomedical	DW-86L T/ST
UV-Vis Spectrophotometer	Beckman Coulter	DU730
Varioskan™ Flash Spectral Scanning Multimode Reader	Thermo Scientific	5250040
Vivaspin® 500 centrifugal concentrator, 10 kDa	Sartorius	VS0102
Vivaspin® 500 centrifugal concentrator, 5 kDa	Sartorius	VS0112

3.2. Methods

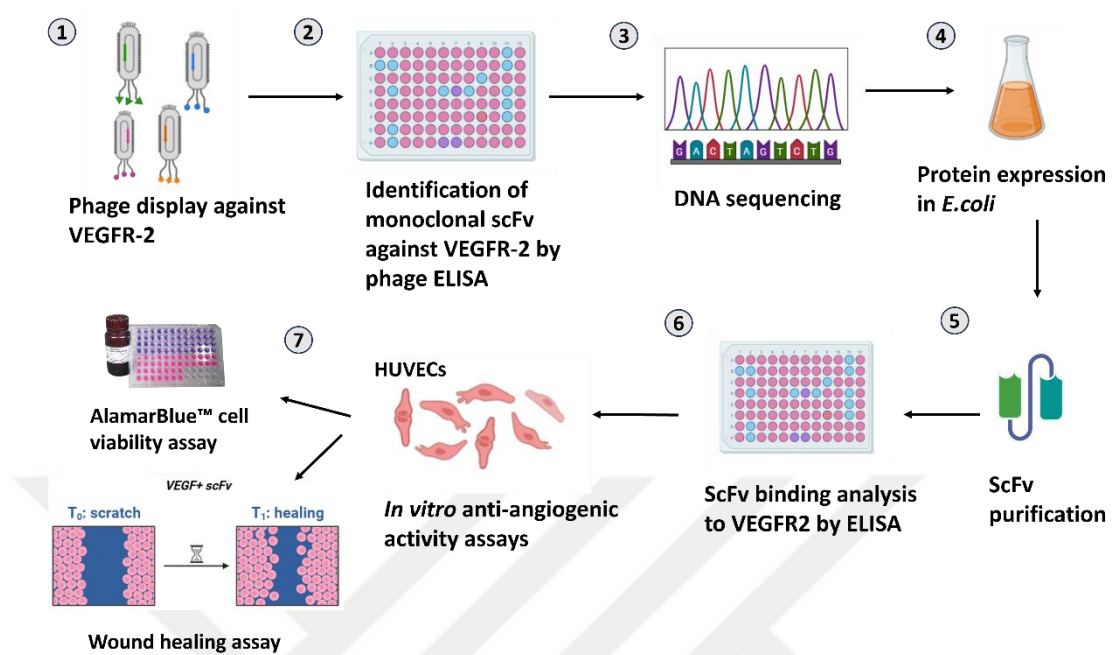


Figure 3.2: The experimental workflow of the study. Created in BioRender.com

3.2.1. Phage Display Screening Against VEGFR-2

Phage display screening of the synthetic human scFv library against VEGFR-2 (extracellular domains 2–3) was performed as the initial step in the experimental workflow of this study (**Figure 3.2**). Phage virions displaying the scFv library were first produced in *E. coli* through superinfection with helper phages, followed by an isolation step. For this purpose, the phagemid plasmid (bearing the scFv library) was transformed into *E. coli* TG1 cells. Then, the first phage-scFv propagation was carried out in TG1 cells, followed by PEG/NaCl precipitation to isolate phage-scFv virions. From this point onward, all subsequent phage propagations were conducted in *E. coli* ER2738 cells.

The produced phage-scFv virions were subjected to the first biopanning cycle. The output phages of first biopanning cycle were propagated in ER2738 cells to obtain sufficient phage-scFv quantities for the subsequent cycle. In total, four phage propagation steps were carried out, each corresponding to one of the four rounds of solution-phase biopanning. After each biopanning cycle, the integrity and diversity of scFv library were controlled by colony PCR and restriction digestion. Following the

final (fourth) round of biopanning, monoclonal phage ELISA was conducted to assess the binding affinity and specificity of individual phage-displayed scFv antibodies against VEGFR-2.

3.2.1.1. Electrocompetent Cell Preparation and Phagemid scFv Library Electroporation

Phagemid scFv library was transformed into electrocompetent *E. coli* TG1 cells. *E. coli* TG1 is an amber suppressor possessing F pili, which facilitates infection of M13 filamentous phage (**Figure 3.3**). Due to these characteristics, TG1 is commonly used in phage display library screening, allowing both efficient infection by helper phage and read-through of amber stop codons in phagemid constructs. Electroporation was chosen over the heat shock transformation due to its higher transformation efficiency with lower quantity of plasmid DNA [114]. Electrocompetent cell preparation and electroporation were conducted according to the manufacturer's instructions (Lucigen), with minor modifications.

Initially, electrocompetent TG1 cells were prepared as follows: glycerol stock of *E. coli* TG1 (Lucigen), stored at -80°C , was streaked onto LB agar plates and incubated at 37°C for 16–18 hours. The following day, a single TG1 colony was inoculated into 5 mL YENB broth and grown for 16–18 hours at 37°C with shaking at 220 rpm. The overnight culture was then diluted 1:100 in 250 mL fresh YENB broth and incubated under the same conditions until the optical density at 600 nm (OD_{600}) of culture reached 0.4–0.5. Once the desired OD_{600} was reached, the culture was chilled on ice for 20 minutes. From this step onward, all materials were pre-cooled to maintain low temperature. Cells were harvested by centrifugation at $4,000 \times g$ at 4°C for 15 minutes. Supernatant was carefully discarded, and the cells were gently resuspended in ice-cold 125 mL 10% glycerol solution. The wash step with 10% glycerol was repeated once more under identical centrifugation conditions. Finally, the cells were gently resuspended in 2 mL of 10% glycerol, aliquoted into 40 μL aliquots, and stored at -80°C . Several aliquots were reserved for immediate electroporation-based transformation of the phagemid scFv library.

For the electroporation, 40 μL electrocompetent TG1 cells were thawed on ice, and well-mixed with 5 ng of phagemid scFv plasmid library in a chilled 0.5 mL microcentrifuge tube. The mixture was then transferred to a chilled electroporation cuvette (with a cuvette gap of 1.0 mm). The electroporation cuvette was placed in the

electroporator device (Gene Pulser II, Bio-Rad) and pulsed at manufacturers recommended conditions (voltage of 1,800 V; capacitance of 10 μ F and impedance 600 Ohms for a time of 3.5 to 4.5 milliseconds). Immediately, 900 μ L SOC recovery medium was added, followed by incubation at 37 °C for 1 hour with agitation. All of transformation mixture was spread on LB agar plates containing ampicillin (100 μ g/mL) and incubated at 37 °C for 16 hours. As a positive control, a plasmid bearing an ampicillin resistance gene was transformed in parallel. Competent cells without plasmid served as a negative control to confirm the effectiveness of antibiotic selection. The following day, all colonies were scraped from the agar plates into 5 mL LB medium containing ampicillin. The culture was incubated at 37 °C for 16 hours with shaking at 250 rpm. Glycerol stocks of overnight culture were prepared and stored at -80 °C. Remaining culture was used for phage-scFv production.

3.2.1.2. Phage-scFv Library Propagation in *E. coli*

Phage-scFv propagation was performed prior to each biopanning round, resulting in a total of four amplification steps. The initial propagation was performed using *E. coli* TG1 cells, while the subsequent three amplifications were conducted using *E. coli* ER2738 strain (**Figure 3.3**). ER2738 is an amber suppressor strain suitable for M13 phage propagation due to its rapid growth and high phage yield. Notably, *E. coli* ER2738 strain has tetracycline antibiotic resistance.

Phage propagation was performed according to CM13d3 pIII-defective helper phage supplier's manual (Antibody Design Labs), with minor changes [115]. CM13d3 is a helper phage with a wild-type pIII phenotype, allowing efficient bacterial infection, yet it lacks a functional gIII gene on its genome [116]. This design drives the exclusive display of pIII-fusion proteins encoded by the phagemid on the phage surface. The use of such pIII-defective helper phages has been reported to enhance the display efficiency of scFv fragments by up to 100-fold or more, thereby improving the sensitivity and success rate of phage display selection [117]. CM13d3 helper phage also confers kanamycin resistance, which is used for selection following the superinfection.

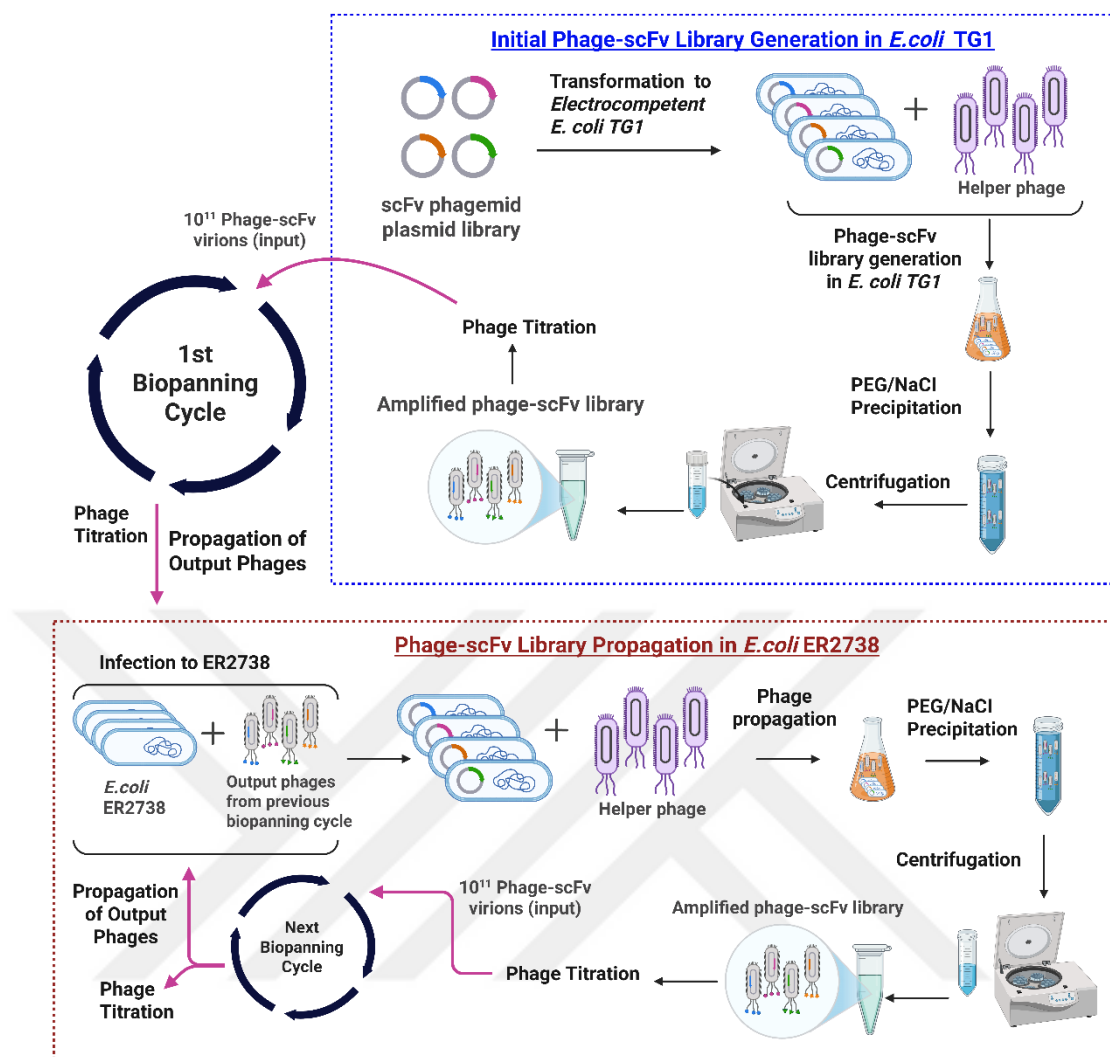


Figure 3.3: Propagation and isolation of the phage-displayed scFv library in *E. coli*. Created in BioRender.com.

An overnight culture of the appropriate host strain containing phagemid plasmid was diluted 1:100 into 50 mL of 2×YT medium supplemented with ampicillin (100 µg/mL) and incubated at 37 °C with shaking at 250 rpm. When OD₆₀₀ of culture reached 0.5, CM13d3 helper phage was introduced to the culture at a ratio of 1 µL per 1 mL of bacterial suspension for superinfection. The mixture was incubated at 37 °C for 1 hour with shaking at 250 rpm.

Following superinfection, the culture volume was adjusted to 100 mL with fresh 2×YT medium containing both ampicillin (100 µg/mL) and kanamycin (50 µg/mL) to maintain selection pressure for both the phagemid and the helper phage. The temperature was then reduced to 30 °C, and the culture was incubated for 16 hours with agitation at 250 rpm to facilitate phage particle assembly and secretion.

The output phage-scFv virions obtained from each biopanning cycle were amplified in *E. coli* ER2738 strain (**Figure 3.3**). Briefly, 100 μ L of eluted phage-scFv virions were incubated with 1 mL of ER2738 culture ($OD_{600} = 0.5$) at 37 °C for 30 minutes without shaking, followed by an additional 30 minutes with agitation at 250 rpm. The infected culture was then transferred into 20 mL of fresh 2 $\times$ YT medium supplemented with ampicillin (100 μ g/mL), tetracycline (20 μ g/mL), and 1% glucose, and incubated at 37 °C until the OD_{600} reached 0.5. Subsequently, CM13d3 helper phage was introduced at a ratio of 1 μ L per 1 mL of bacterial culture for superinfection. The culture was incubated for 1 hour at 37 °C with shaking at 250 rpm. Cells were harvested by centrifugation at 4,000 $\times$ g for 10 minutes at room temperature, and the pellet was resuspended in 100 mL of fresh 2 $\times$ YT medium containing ampicillin (100 μ g/mL) and kanamycin (50 μ g/mL). The culture was then incubated at 30 °C for 16 hours with shaking at 250 rpm to facilitate phage particle assembly and secretion.

3.2.1.3. Isolation of Phage-scFv Library Propagated in *E. coli*

After each phage propagation step, phage-scFv virions were isolated using the PEG/NaCl precipitation method (**Figure 3.3**) [118]. First, bacterial cells from the overnight-grown culture producing phage-scFv virions were removed by two consecutive centrifugation steps at 8,000 $\times$ g at room temperature. The resulting phage-containing supernatant was filtered through a 0.45 μ m filter to eliminate any remaining bacterial debris. The clarified supernatant was mixed with 1/5 volume of ice-cold PEG/NaCl buffer (20% PEG 6000, 2.5 M NaCl) and incubated on ice for 2 hours with occasional gentle inversion to promote phage precipitation. Phage particles were then pelleted by centrifugation at 10,000 $\times$ g for 1 hour at 4 °C. The supernatant was discarded, and the phage pellet was resuspended in 20 mL of sterile distilled water. To further purify the phage particles, the suspension underwent another round of centrifugation for 10 minutes at 10,000 $\times$ g. The collected supernatant was again mixed with 1/5 volume of ice-cold PEG/NaCl buffer and incubated on ice for 30 minutes. A final centrifugation step was performed at 10,000 $\times$ g for 40 minutes at 4 °C. The phage pellet was then resuspended in 1 mL sterile 1 $\times$ PBS. The purified phage-scFv virions were titrated to determine phage concentration and subsequently stored at 4 °C until further use.

3.2.1.4. Biopanning Selection Against VEGFR-2

Antibody selection and screening of the synthetic human scFv library against VEGFR-2 were performed using a modified version of the solution-phase biopanning protocol described by Heine *et al.* [119]. Four rounds of biopanning were conducted to enrich scFv antibodies specific for VEGFR-2 (**Figure 3.4**). In each panning round, approximately 10^{11} phage scFv-displayed virions were incubated with recombinant human VEGFR-2 protein (Extracellular domains 2 and 3, hFc-tagged, Sino Biological) in 1% BSA-PBST (PBS containing 0.05% Tween-20 and 1% bovine serum albumin) to minimize nonspecific binding. Pierce™ Protein A Magnetic Beads (Thermo Scientific) were used in each round to pull down the VEGFR-2:phage-scFv complexes by binding the hFc tag of the antigen, following the manufacturer's immunoprecipitation manual with minor modifications.

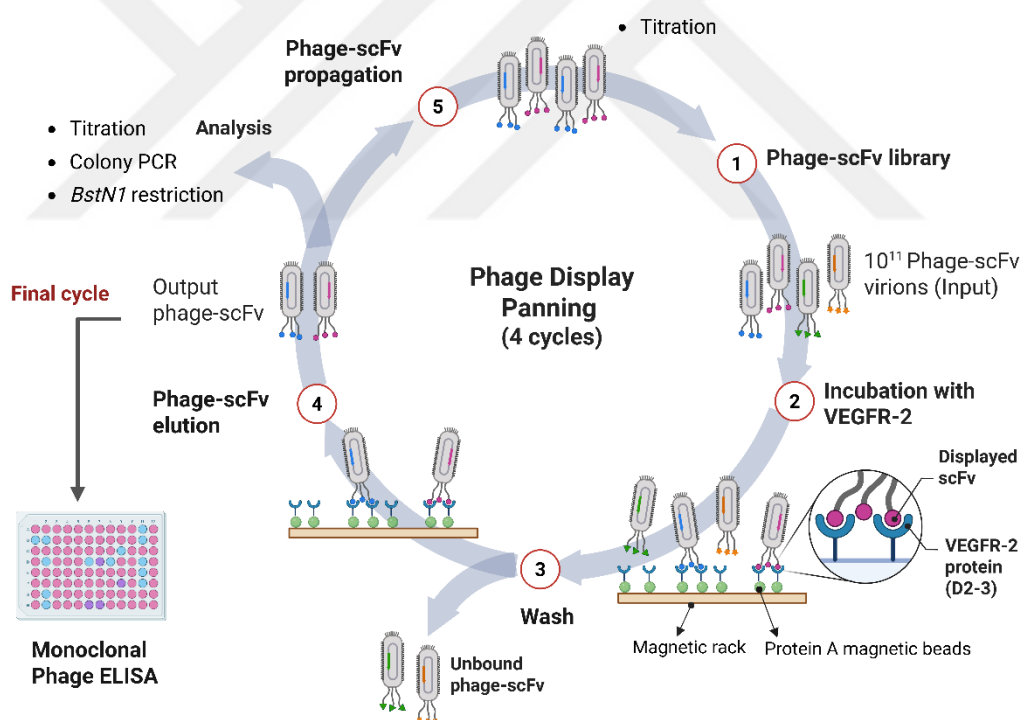


Figure 3.4: Phage display biopanning cycle. Created in BioRender.com.

Firstly, the required volume of Protein A magnetic beads was transferred into 1.5 mL protein low-binding tubes (Protein LoBind, Eppendorf) and placed on a magnetic stand for 1 minute to remove the storage buffer. Beads were washed twice with 500 μ L PBS, and the supernatant was discarded. A pre-incubation step was carried out to eliminate

non-specific binders that may result from incorrect antibody folding or background binding to Protein A or magnetic beads. For this, washed magnetic beads were incubated with 10^{11} phage scFv-displayed virions for 1 hour at room temperature under overhead rotation. After incubation, beads were magnetically separated for 5 minutes, and the supernatant, now containing the cleared phage-scFv virions, was transferred to a new protein LoBind tube for subsequent antigen incubation.

Recombinant VEGFR-2 protein was added to the scFv phage solution, which was pre-incubated with magnetic beads. For first round of biopanning, 7.5 μ g of VEGFR-2 was used. To increase the selection pressure and favor the isolation of higher-affinity binders, the amount of VEGFR-2 was sequentially reduced in subsequent rounds: 5 μ g in the second, 2.5 μ g in the third, and 1 μ g in the fourth round. For the first biopanning cycle, the solution was incubated for 120 minutes at room temperature with overhead shaking. The incubation time was also decreased to 90 minutes in second biopanning, 60 minutes in third and fourth biopanning cycles. After incubation, an equal volume of pre-washed Protein A magnetic beads was added to the mixture and further incubated for 60 minutes with rotation at room temperature to capture VEGFR-2:scFv-phage complexes. Following binding, the solution was placed on magnetic stand for 1 minute and the supernatant was removed. Magnetic beads were then washed with 300 μ L PBST to remove non-specific and unbound phage-scFvs to VEGFR-2, with supernatants discarded after each wash. Washing stringency was increased progressively in each round: 10 washes with 0.05% PBST in the first round, 20 washes with 0.05% PBST in the second, and 30 washes with 0.1% PBST in both the third and fourth rounds.

After the final washing, magnetic beads were incubated with elution buffer (0.2 M glycine pH 2.0) to elute VEGFR-2 specific phage-scFv virions, at room temperature for 10 minutes. The recovered phages were immediately neutralized with 15 μ L of 1 M Tris pH 9.1. The beads were separated by placing the tube on the magnetic stand, and the supernatant containing eluted VEGFR-2-specific scFv-phages were collected. The eluted phages were titrated to determine the output of each biopanning cycle and stored at 4 °C for further amplification or analysis.

3.2.1.5. Phage Titration

After each phage propagation step and each biopanning round, the purified phage-scFv virions were titrated to determine their concentration by colony counting assay [119]. For this purpose, a single colony of *E. coli* ER2738, freshly streaked on an LB agar, was inoculated into 5 mL of 2×YT medium supplemented with tetracycline. The culture was kept at 37 °C and shaken at 250 rpm until the culture reached an OD₆₀₀ of 0.5. Serial 100-fold dilutions of purified phage stock were prepared in 1×PBS, ranging from 10⁻² to 10⁻¹⁰. For infection, 10 µL of each phage dilution was added to 100 µL of the ER2738 culture and kept at 37 °C for 30 minutes without agitation. Following the infection step, the infected culture was plated on 2×YT agar plates supplemented with ampicillin and 1% glucose. The plates were placed in a 37 °C incubator overnight (approximately 16 hours). The following day, the number of colonies on each plate was counted, and the phage titer was determined as colony-forming units (cfu) per mL using the formula below (3.1).

$$\text{Phage titer (cfu/mL)} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Plated volume (mL)}} \quad (3.1)$$

where “Number of colonies” is the average of colonies, the “Dilution factor” is the reciprocal of the dilution plated, and “Plated volume” is the volume of diluted phage used to infect bacteria and then added to the plate.

3.2.1.6. Colony PCR

After each biopanning cycle, the diversity and integrity of phage-scFv library were evaluated by colony PCR and restriction digestion using *Bst*NI enzyme [118]. For this purpose, DNA inserts from the scFv library were amplified by colony PCR using primers 458 and 459, which target the insert region of the pCANTAB 5E vector carrying the scFv library. Following the plating of phage dilutions for phage titration on LB agar plates, well-separated individual colonies were obtained. Nineteen of these colonies are randomly picked with sterile toothpick and suspended in 10 µL LB medium in a 0.5 mL microcentrifuge tube. After well resuspension, 2 µL from each bacterial suspension served as the template, and the PCR reaction mixture was prepared for each sample (Table 3.9).

PCR cycling was carried out under the following conditions: an initial denaturation at 95 °C for 5 minutes; 35 cycles of 95 °C for 15 seconds, 55 °C for 15 seconds, and 72 °C

for 10 seconds; with a final extension at 72 °C for 2 minutes. The colony PCR products were analyzed on a 1% agarose gel to verify the integrity of the scFv inserts.

Table 3.9: Colony PCR components.

Components	Volume (μL)
Template	2
MyTaq™ Reaction Buffer	5
MyTaq™ DNA Polymerase (2 units)	0.4
Primer 459 (forward, 5pmol)	0.5
Primer 458 (reverse, 5pmol)	0.5
dH ₂ O	16.6
Total	25

3.2.1.7. Restriction Digestion with *Bst*N1

*Bst*N1 is a restriction endonuclease that recognizes and cleaves DNA at the 5'-CCWGG-3' (W= A or T) site. Due to its high frequency of cutting within immunoglobulin variable regions, *Bst*N1 is widely used to assess the uniqueness of selected clones and to evaluate clonal diversity by generating distinct restriction fragment length patterns. 20 μL of insert bearing PCR products verified by colony PCR were incubated with 1 μL *Bst*N1 (New England Biolabs), 5 μL 10x NEB 3.1 RE buffer and 24 μL nuclease-free water. The reaction mixture was maintained at 60 °C for 5 hours. Digestion products were separated on a 2% agarose gel. The resulting agarose images were analyzed for distinct patterns between the selected clones.

3.2.1.8. Monoclonal Phage ELISA

Following the final cycle (fourth) of biopanning, monoclonal phage ELISA was performed to assess the binding affinity and specificity of individual phage-displayed scFv clones against VEGFR-2. This procedure involved two main steps: (i) production of monoclonal phage-scFv particles in small-scale bacterial cultures, and (ii) screening of their antigen-binding properties via ELISA [119].

For monoclonal phage production, 60 single bacterial colonies containing scFv inserts were randomly picked from 2×YT agar plates spread with the output of the fourth biopanning round. Each colony was used to inoculate 1 mL of 2×YT medium containing ampicillin and 1% glucose in 96-well DeepWell plates. Cultures were incubated at 37 °C with agitation until reaching an OD₆₀₀ of ~0.5. M13KO7 helper phage (New England Biolabs) was introduced at a multiplicity of infection corresponding to 1.0×10^8 cfu. Following 1 hour incubation, bacterial cells were collected by centrifugation at $2,700 \times g$ for 10 minutes. Cells were then resuspended with 1 mL fresh 2×YT medium containing ampicillin (100 µg/mL) and kanamycin (50 µg/mL). Finally, cultures were grown at 30 °C for 16-18 hours with agitation to allow for phage particle production and secretion into the supernatant. After the overnight incubation, cultures were centrifuged at $2,700 \times g$ for 10 minutes at 4 °C to pellet the bacterial cells. The clarified supernatants, containing monoclonal phage-scFv virions, were carefully isolated and stored at 4 °C for subsequent ELISA screening against VEGFR-2.

For ELISA-based screening, a 96-well microtiter plate (Nunc™ Maxisorp™, Thermo Scientific) was coated in triplicate with 100 ng of recombinant VEGFR-2 (Domain 2 and 3, hFc-tagged) diluted in 100 µL of coating buffer (0.1 M carbonate-bicarbonate, pH 9.6). As a negative control, 1% BSA was similarly coated in separate wells. The plate was sealed with parafilm and kept overnight at 4 °C. The following day, wells were washed three times with 300 µL of PBST (PBS with 0.1% Tween-20) to remove unbound protein. To reduce non-specific binding, wells were blocked with 300 µL of 3% (w/v) skim milk prepared in PBST, for 2 hours at room temperature. After blocking, wells were again washed three times with PBST. Next, 100 µL of clarified monoclonal phage-scFv supernatant (prepared from individual bacterial cultures) was added to both VEGFR-2-coated and BSA-coated wells. Plates were incubated for 2 hours at room temperature with gentle agitation. Following incubation, wells were washed six times with PBST to remove unbound phage-scFv particles. To determine phage-scFv binding to coated-antigens, wells were incubated with 100 µL of primary mouse anti-M13 monoclonal antibody (Santa Cruz Biotechnology, Cat. No. sc-53004), diluted 1:1,000 in 3% skim milk/PBST, for 1 hour at room temperature. Wells were then washed as previously described. Next, 100 µL of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich, Cat. No. A9044), diluted 1:10,000 in 3% skim milk/PBST, was incubated for 1 hour, as secondary antibody. After a 3-time

washing step, 100 μ L TMB substrate solution as HRP enzyme substrate was added to each well, and color development was allowed for up to 30 minutes at room temperature in the dark. The reaction was stopped by adding 50 μ L of 2 M H_2SO_4 to each well. Absorbance was read at 450 nm using a microplate reader (GloMax® Explorer Multimode Microplate Reader, Promega).

3.2.2. Sequence Determination of VEGFR-2 Specific Antibodies

3.2.2.1. Plasmid Isolation

Plasmid isolation was performed to obtain phagemid plasmid containing VEGFR-2-specific scFv gene inserts. The isolated plasmids were used for two purposes: (i) sequence determination of selected scFv antibodies showing positive binding to VEGFR-2, and (ii) transformation into *E. coli* BL21(DE3) and HB2151 strains for recombinant protein expression of soluble scFv antibodies. Plasmid DNA isolation was carried out using the NucleoSpin® Plasmid Miniprep Kit (Macherey-Nagel) according to the manufacturer's instructions with minor modifications [120]. This kit relies on alkaline lysis of bacterial cells, with subsequent binding of plasmid DNA to a silica membrane using chaotropic salts. *E. coli* strain ER2738 was employed as the host for plasmid isolation.

Freshly streaked, phagemid-harboring single bacterial colonies were inoculated into 5 mL LB medium supplemented with 100 μ g/mL ampicillin. The culture was incubated at 37 °C for 16 hours with shaking at 250 rpm. The next day, cells were collected by centrifugation at $11,000 \times g$ for 30 seconds. Supernatant was discarded and the cells were thoroughly resuspended with 250 μ L resuspension Buffer A1. After complete resuspension, 250 μ L of lysis Buffer A2 was introduced, and the mixture was gently inverted 6–8 times. The mixture was kept at room temperature for 5 minutes for complete lysis. Next, 300 μ L of neutralization Buffer A3 was added, and the mixture was gently mixed until blue samples turned colorless completely. Cell lysate was pelleted out by centrifugation at $11,000 \times g$ for 10 minutes at room temperature. The resulting clear supernatant was loaded onto a kit-provided column and inserted into a collection tube. The column was centrifuged at $11,000 \times g$ for 30 seconds, and the flow-through was removed. For washing, 600 μ L washing Buffer A4 was added to the column, followed by centrifugation at $11,000 \times g$ for 30 seconds. An additional dry-

spin was conducted for 1 minute at $11,000 \times g$ to discard residual wash buffer. For elution, 50 μ L of nuclease-free water was applied directly to the center of the column membrane and incubated at room temperature for 1 minute. Elution was completed by centrifugation at $11,000 \times g$ for 1 minute. The concentration and purity of the isolated plasmid DNA were measured with a NanoDrop spectrophotometer (Thermo Scientific). All plasmid stocks were kept at -20°C until required.

3.2.2.2. PCR Amplification and Purification of scFv Gene Insert

PCR amplification of VEGFR-2-specific scFv gene inserts was carried out for sequence determination, following the colony PCR protocol with minor modifications. Specifically, 20 ng of isolated phagemid DNA was used as the template instead of colonies picked from LB agar plates. All other PCR components and cycling conditions remained unchanged, see **Table 3.9**. The resulting PCR products were examined on a 1% agarose gel to verify the presence and expected size ($\sim 1,000$ bp) of the scFv inserts.

Gel-confirmed scFv bearing PCR products were excised and isolated using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer's protocol [121]. In short, excised gel fragments were incubated with NTI DNA binding buffer at 50°C until fully dissolved. The mixture was then passed through a DNA-binding column via centrifugation. After two wash steps, the DNA was eluted using nuclease-free water.

To ensure high-purity DNA suitable for sequencing, a secondary purification was performed using the ZR DNA Sequencing Clean-Up Kit (Zymo Research). The scFv DNA was mixed with binding buffer, loaded onto a clean-up column, washed twice, and eluted with nuclease-free water. The final purified products were re-examined on a 1% agarose gel to assess integrity and purity. The concentration and purity of well-purified PCR products were determined using a NanoDrop spectrophotometer. Isolated PCR products were kept at -20°C until required.

3.2.2.3. DNA sequencing of VEGFR-2-specific scFv Antibodies

To analyze the sequence and diversity of VEGFR-2-specific scFv clones, DNA sequencing was performed using GenomeLab DTCS Quick Start Kit (Beckman Coulter) on the GenomeLab GeXP Analysis System (Beckman Coulter), following the manufacturer's instructions [121]. Two sequencing reactions were prepared for each scFv antibody, one for the forward read and one for the reverse read, using primers 458 (reverse) and 459 (forward). Sequencing reactions were set up with 120 ng of highly purified PCR product as template DNA, along with other reagents as recommended by the manufacturer (Table 3.10). A thermal cycling program of 30 cycles was performed, consisting of denaturation at 96 °C for 20 s, annealing at 50 °C for 20 s, and extension at 60 °C for 4 min, followed by a final hold at 4 °C.

Table 3.10: Sequencing reaction using DTCS Quick Start Kit.

Components	Volume (μL)
dH ₂ O	10.4
DNA template (120 ng)	1.2
Primer (4 pmol)	0.4
DTCS Quick Start Master Mix	8
Total	20

Following PCR amplification, sequencing-reaction DNA products were purified by ethanol precipitation. Briefly, 5 μL of stop solution (2 μL of 3M Sodium Acetate pH 5.2, 2 μL of 100 mM Na₂-EDTA pH 8.0 and 1 μL of 20 mg/mL of glycogen) was added to each reaction tube. Immediately thereafter, 60 μL of ice-cold 95% ethanol was added, and the mixture was gently mixed. Samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was discarded, and DNA pellets were rinsed twice with 200 μL of chilled 70% ethanol, each followed by a 5-minute centrifugation under the same conditions. After the final wash, residual ethanol was removed, and the pellets were vacuum-dried for approximately 10 minutes. The dried DNA pellets were reconstituted in 40 μL of sample loading solution and transferred to sequencing plate. To prevent evaporation during sequencing, a drop of mineral oil was layered over each well. A corresponding buffer plate was prepared simultaneously to match the number of samples. Both plates were then loaded into the GenomeLab GeXP genetic analysis system, and the LFR-a method was used to perform the sequencing runs. Following

data acquisition, raw reads were processed using the GenomeLab GeXP Analysis software. Further sequence editing, alignment, and analysis were performed using BioEdit (<https://bioedit.software.informer.com/7.2/>), Chromas (<https://technelysium.com.au/wp/chromas/>), and ApE (<https://jorgensen.biology.utah.edu/wayned/apE/>) [122], [123].

3.2.3. Recombinant Soluble Protein Expression and Purification of scFv Antibodies

3.2.3.1. Chemically Competent Bacterial Cell Preparation and Transformation

Phagemid plasmids of phage ELISA-positive scFv antibodies were transformed into competent *E. coli* BL21(DE3) and HB2151 strains for recombinant protein expression, by heat shock transformation method [124]. For that, first, competent BL21(DE3) and HB2151 cells were prepared. Glycerol stocks of each strain stored at -80 °C were streaked onto LB agar medium plates and incubated at 37 °C for 16-18 hours. The following day, a single bacterial colony was inoculated into 5 mL LB medium and grown for 16-18 hours at 37 °C with shaking at 250 rpm. The overnight culture was diluted 1/100 into 100 mL fresh LB medium and grown until OD₆₀₀ of culture reached 0.4-0.5. Once the target optical density was reached, the culture was chilled in an ice bath for 10 minutes. After this step, all materials used were pre-cooled before use. Cells were harvested by centrifugation at $2,700 \times g$ at 4 °C for 10 minutes. Supernatant was carefully discarded, and the cells were gently resuspended in ice-cold 3.2 mL 100 mM CaCl₂. The cells were incubated on ice-water bath for 30 minutes and then harvested by centrifugation at same conditions. Another round of incubation with 1.6 mL of ice-cold 100 mM CaCl₂ was done for 20 minutes. Finally, glycerol stocks were prepared, aliquoted and frozen at -80 °C.

20 ng of each phagemid plasmid was used for the transformation of 200 µL competent bacterial strains by heat shock method. First, competent cells were thawed on ice, gently mixed with the phagemid DNA plasmid, and incubated on ice for 30 minutes. This was followed by a heat shock at 42 °C for 45 seconds in a water bath, then returned to ice for an additional 5 minutes. Subsequently, pre-heated 800 µL LB medium was added and cells were incubated at 37 °C for 1 hour with shaking at 250 rpm. 100 µL of each sample was plated on LB agar plates containing ampicillin (100 µg/mL) and

incubated at 37 °C for 16 hours. As a positive control, a plasmid bearing an ampicillin resistance gene was transformed in parallel. Competent cells without plasmid served as a negative control to confirm the effectiveness of antibiotic selection. The next day, individual bacterial colonies harboring the phagemid were selected for recombinant protein expression.

3.2.3.2. Recombinant Protein Expression of scFv Antibodies in *E. coli*

Recombinant protein expression of anti-VEGFR-2 scFv antibodies was optimized by varying induction temperatures and incubation times under IPTG stimulation in Erlenmeyer flasks [125]. Protein expression studies were conducted with *E. coli* BL21(DE3) cells, the expression profile of scFv clones were also controlled in *E. coli* HB2151 cells. For each scFv antibody, a freshly transformed single colony harboring the scFv-encoding phagemid was inoculated into 20 mL of 2×YT medium (supplemented with ampicillin and 1% glucose) and cultured overnight at 37 °C with shaking at 240 rpm. The next day, cultures were diluted 1:100 into fresh 2×YT medium containing ampicillin and 1% glucose and incubated until mid-log phase (OD₆₀₀ of ~0.6). Bacterial cells were then collected by centrifugation at 4,000 × g for 5 minutes and resuspended in fresh 2×YT medium containing ampicillin and 1 mM IPTG to induce protein expression. Time-zero samples were collected prior to induction as expression controls. Induction was performed under various temperatures (16 °C, 25 °C, and 30 °C) and incubation times (4, 16, 24, and 42 hours). Following induction, cells were harvested by centrifugation at 4,000 × g for 10 minutes at 4 °C. Resulting cell pellets were either directly analyzed for scFv expression using SDS-PAGE and Western blot or stored at –20 °C for later processing. After optimization of conditions (induction temperature and duration), protein expression was scaled up to 400 mL with 1 mM IPTG induction at 16 °C for 24 hours to maximize soluble protein yield for downstream purification.

3.2.3.3. Soluble Protein Extraction

Bacterial cell lysis for soluble scFv protein extraction was carried out using two different approaches: cytoplasmic proteins were extracted using B-PER™ Bacterial Protein Extraction Reagent (Thermo Scientific) following the manufacturer's

instructions, while periplasmic proteins were obtained through osmotic shock using TES buffer [125].

For cytoplasmic soluble protein extraction, bacterial cell pellets obtained from protein expression studies were resuspended at a ratio of 1 g of pellet per 4 mL B-PER™ lysis buffer (supplemented with 2 μ M MgCl₂, 8 μ L lysozyme, 8 μ L DNase I and 400 μ L SigmaFast™ protease inhibitor cocktail). The suspension was incubated at room temperature for 20 minutes with slow shaking to allow efficient lysis. After incubation, an aliquot was collected to represent the total protein fraction, which includes both soluble and insoluble protein fractions. The remaining lysate was centrifuged at 14,000 $\times$ g for 15 minutes at 4 °C. The resulting supernatants, containing soluble proteins, were carefully collected and stored on ice for downstream analysis. The pellet fraction was resuspended in an equal volume of B-PER to obtain the insoluble protein fraction. Total, soluble and insoluble protein fractions were examined by SDS-PAGE and Western blot.

For periplasmic soluble protein extraction, bacterial pellets were resuspended in TES buffer at a ratio of 1 g of pellet per 4 mL of buffer (200 mM Tris-HCl, pH 8.0; 500 mM sucrose; 1 mM EDTA), supplemented with 8 μ L of DNase I and 400 μ L SigmaFast™ protease inhibitor cocktail. Periplasmic protein extraction was carried out on ice for 30 minutes, followed by the same fractionation procedure described for cytoplasmic protein extraction. Periplasmic protein extraction fractions were also analyzed by SDS-PAGE and Western blot.

3.2.3.4. SDS-PAGE and Western Blot Analysis

Recombinant protein expression profile of each scFv antibody was analyzed by SDS-PAGE and Western blot [125]. Specifically, the solubility and expression levels of scFv clones were assessed using the soluble and insoluble protein fractions, following standard protocols with minor modifications. Each sample (30 μ L) was mixed with 4 $\times$ Laemmli sample buffer (Bio-Rad) (supplemented with 355 mM 2-mercaptoethanol for reducing conditions). The samples were denatured by heating at 95 °C for 5 minutes. Subsequently, 15 μ L of each denatured sample was loaded onto a 12% polyacrylamide gel. Electrophoresis was performed at a constant voltage of 120 V in SDS running buffer until distinct protein bands were resolved. Following electrophoresis, gels were stained with staining buffer (0.1% w/v in 40% methanol,

10% acetic acid) for 30 minutes and destained with the destaining buffer (40% methanol and 10% acetic acid).

For Western blotting, Trans-Blot Turbo Transfer System (Bio-Rad) was used to transfer proteins to nitrocellulose membranes. Nitrocellulose membranes were blocked with blocking buffer (1% BSA in PBS and 0.1% Tween-20) for 2 hours. Detection of scFv protein bands was performed using an HRP-conjugated anti-E tag polyclonal antibody (Abcam, Cat. No. ab19400), diluted 1:5,000 in 1% BSA (in 0.1% PBST). Membranes were incubated with the antibody for 1 hour at room temperature with gentle agitation. After antibody incubation, membranes were washed three times with PBST (5 minutes each) to remove unbound antibody. Colorimetric signal detection was carried out with 4C1N substrate solution. The substrate was freshly prepared and added to the membrane surface at room temperature in the dark until bands became visible. Once adequate signal intensity was achieved, the reaction was stopped by rinsing the membranes with distilled water.

3.2.3.5. Soluble scFv Antibody Purification

Soluble anti-VEGFR-2 scFv proteins were purified via affinity chromatography using the ÄKTA Pure™ 25 chromatography system (Cytiva) with 1 mL HiTrap™ Protein L affinity column (Cytiva, Cat. No. 29048665). All purification steps were conducted according to the manufacturer's recommended protocol [126]. Since the synthetic human scFv library was designed to include human kappa light chain subtypes 1 and 3, the majority of recombinant scFv proteins were expected to bind Protein L resin [127]. Thus, Protein L affinity chromatography was the primary method used for purification.

Soluble scFv protein extraction was performed by using either BPER lysis buffer or TES periplasmic extraction buffer, as previously described. For BPER protein extraction, the cell lysate was additionally subjected to a sonication step at 10% amplitude for 6 cycles (pulse on: 10 seconds, pulse off: 20 seconds) (Branson). The lysates were then centrifuged, and the resulting supernatants containing soluble proteins were used for affinity purification. For purification using HiTrap™ Protein L affinity column, soluble scFv protein extraction was diluted 1/1 with the binding buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.2). The mixture was filtered through a 0.45 µm syringe filter. The column was equilibrated with at least 5 column volumes

of binding buffer before sample loading. The sample was then loaded at flow rate of 0.5 mL/min. After loading, the column was washed with a minimum of 5 column volumes of binding buffer or until the absorbance returned to near baseline. Bound proteins were eluted using 5 column volumes of elution buffer (0.1 M sodium citrate, pH 2.8). Eluted fractions were immediately neutralized by adding 60 μ L of 1.0 M Tris-HCl (pH 8.0) per mL of eluate. Fractions were stored at -20°C for subsequent analysis, including SDS-PAGE, Western blot, BCA, and protein ELISA.

Following purification, the eluted protein fractions were dialyzed against PBS using SnakeSkin™ dialysis tubing with a 3.5 kDa molecular weight cut-off (MWCO) (Thermo Scientific, Cat. No. 88244). Dialysis was performed at 4°C , with the PBS buffer changed three times to ensure efficient buffer exchange. The proteins were concentrated using Vivaspin® 500 centrifugal concentrator tubes with 5 kDa and 10 kDa MWCO (Sartorius), following the manufacturer's instructions.

3.2.3.6. Protein Quantification by BCA

The concentration of soluble scFv proteins and total cell lysates used for ELISA assays was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's protocol [128]. Briefly, a standard curve was prepared using serial dilutions of BSA within the concentration range of 0–2,000 $\mu\text{g/mL}$. Working reagent was freshly made by combining Reagent A with Reagent B at a ratio of 50:1. For the assay, 25 μL of each protein sample or standard was first pipetted into a 96-well microplate, and then 200 μL of the prepared BCA working reagent was added. The plate was gently agitated and kept at 37°C for 30 minutes. After incubation, absorbance was read at 562 nm using a microplate reader. Protein concentrations of unknown samples were determined by plotting their absorbance values on the BSA standard curve.

3.2.4. Protein ELISA

To assess the binding specificity and affinity of recombinant scFv proteins against VEGFR-2, ELISA assay was performed using both crude bacterial cell lysates and purified protein fractions [118]. NUNC™ Maxisorp™ 96-well plates were coated in triplicate with a defined concentration of VEGFR-2 antigen (Extracellular domain 2

and 3) in carbonate-bicarbonate coating buffer (pH 9.6). In some assays, recombinant human VEGFR-2 Protein (Full extracellular domain 1-7, His-tagged, Sino Biological, Cat. No. 10012-H08H) was also coated. BSA was coated at an equal concentration in control wells as negative control. Plates were sealed with parafilm and stored overnight at 4 °C. Next day, wells were washed three times with 300 µL PBST (0.1% Tween-20) and blocked with 300 µL of 4% (w/v) skim milk in PBST for 2 hours at room temperature. After blocking, wells were washed again three times with PBST. Equal amount of either cell lysate or purified scFv protein fractions in 100 µL were added to both VEGFR-2-coated and BSA-coated wells and incubated for 2 hours at room temperature with gentle shaking. Next, wells were washed four times with PBST to remove unbound proteins. To detect bound scFv proteins, wells were incubated with 100 µL of HRP-conjugated anti-E tag antibody (1:2,500 dilution in 4% skim milk/PBST; Abcam, Cat. No. ab19400) for 1 hour at room temperature. After a 4-time washing step, 100 µL of TMB substrate solution was added to each well, and color development was allowed for up to 30 minutes at room temperature in dark. The reaction was stopped by adding 50 µL of 2 M H₂SO₄ to each well. Absorbance was measured at 450 nm using a microplate reader (GloMax® Explorer Multimode Microplate Reader, Promega).

3.2.5. *In Vitro* Anti-angiogenic Activity Assays

3.2.5.1. Cell Culture

HUVECs were a kind gift from Dr. Serhad ONAT (Balıkesir University, Türkiye). HUVECs were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) and 1% Antibiotic-Antimycotic solution (Vicent). Cells were maintained at 37 °C in a humidified 5% CO₂ incubator. Cell passage was performed twice a week, considering the population doubling time.

3.2.5.2. Cell Viability Assay

The effect of anti-VEGFR-2 scFv antibodies on endothelial cell proliferation was evaluated using fluorescence-based alamarBlue assay. HUVECs were seeded into 96-well tissue culture plates at a density of 5,000 cells per well and incubated for 24

hours at 37 °C in a humidified 5% CO₂ incubator. The following day, cells were serum-starved by replacing the culture medium with starvation medium (containing 0.5% FBS) and incubated for 16–18 hours. After starvation, cells were treated with various concentrations of purified anti-VEGFR-2 scFv antibodies in starvation medium supplemented with 100 ng/mL recombinant human VEGF-A (VEGF165; Sino Biological, Cat. No. 1066-HNAB). Control groups included: (i) starvation medium with 100 ng/mL VEGF-A, (ii) starvation medium alone, and (iii) complete medium alone (containing 10% FBS). All treatments were carried out in triplicate and incubated for 72 hours. Following treatment, the medium was replaced with serum-free medium containing 10% alamarBlue cell viability reagent (Thermo Scientific, Cat. No. DAL1025). After a 4-hour incubation at 37 °C, fluorescence intensity was measured at 560 nm excitation and 590 nm emission using a Varioskan™ Flash spectral scanning multimode reader (Thermo Scientific).

3.2.5.3. Wound Healing Assay

To assess the effect of anti-VEGFR-2 scFv proteins on endothelial cell migration, wound healing assay was conducted using HUVECs [130]. Cells were seeded into 96-well plates at a density of 40,000 cells per well and incubated for 24 hours at 37 °C in a humidified incubator with 5% CO₂. The next day, cells were serum-starved by replacing the culture medium with starvation medium (containing 0.5% FBS) and incubated for 16–18 hours. On the following day, a linear scratch was generated in the center of each well using a sterile 100 µL micropipette tip. Detached cells were removed by gently washing the wells with DPBS (Gibco). After washing, cells were treated with various concentrations of purified anti-VEGFR-2 scFv antibodies in starvation medium supplemented with 100 ng/mL recombinant human VEGF-A (VEGF165; Sino Biological, Cat. No. 1066-HNAB). Control groups included: (i) starvation medium with 100 ng/mL VEGF-A, (ii) starvation medium alone, and (iii) complete medium (containing 10% FBS) alone. Treatments were incubated for 24, 48, and 72 hours. Images of the scratch area were taken immediately after scratching (time zero) and again after 24, 48, and 72 hours using an inverted light microscope (Soptop) at 4× magnification. Wound closure was quantified by normalizing the wound area at each time point (24, 48, and 72 h) to the initial wound area at time zero using ImageJ (NIH) with wound healing size tool updated plugin [129], [130].

4. RESULTS

4.1. Screening a Synthetic Human scFv Library Against VEGFR-2

In this study, a previously designed synthetic human scFv library was screened against VEGFR-2 using phage display technology to develop anti-VEGFR-2 scFv antibodies. The scFv library, cloned into pCANTAB 5E phagemid vector, was transformed into *E. coli* TG1 cells for phage-scFv particle production. Phage virions displaying the scFv library were generated by superinfection with CM13d3 helper phages. The produced phage-scFv particles were isolated by PEG/NaCl precipitation, and their titers were determined by colony-counting titration.

Screening of the synthetic human scFv library against VEGFR-2 was carried out using a solution-phase biopanning approach. Four rounds of biopanning were conducted to select scFv antibodies with specificity for recombinant human VEGFR-2 protein (comprising of extracellular domains 2 and 3, hFc-tagged). In each biopanning round, approximately 10^{11} phage virions displaying scFv library were incubated with recombinant human VEGFR-2 protein. After incubation period, protein A magnetic beads were employed to isolate the VEGFR-2:phage-scFv complexes via binding the hFc tag on VEGFR-2. Extensive washing steps were applied to eliminate unbound and weakly bound phages. As the rounds progressed, the selection stringency was increased by lowering antigen concentration and incubation time, while performing additional washing steps, shown in **Table 4.1**.

After washing steps, VEGFR-2-bound phage-scFv particles were collected with a low-pH elution step. The output phage, number of eluted phages, of each biopanning round was titrated using colony-counting titration method, shown in **Table 4.1**. Output phages of each round were then propagated in *E. coli* ER2738 cells with the superinfection of CM13d3 helper phages for subsequent biopanning round following same isolation process.

Table 4.1: Phage display biopanning results against VEGFR-2.

Panning Round	VEGFR-2 (μ g)	Incubation (h)	Wash	Input phage (cfu)	Output Phage (cfu)	Output/Input (%)	Enrichment
First	7.5	2	10x PBS-T (0.05%)	10^{11}	6.0×10^8	0.60	1.00
Second	5	1.5	20x PBS-T (0.05%)	10^{11}	1.0×10^9	1.00	1.66
Third	2.5	1	30x PBS-T (0.1%)	10^{11}	4.3×10^9	4.3	7.17
Fourth	1	1	30x PBS-T (0.25%)	10^{11}	1.2×10^8	0.12	0.2

The output/input ratio serves as a measure of affinity selection efficiency and was determined by dividing the output phage titer by the input phage titer for each round. An increase in this ratio indicates successful enrichment of specific and high-affinity clones. The enrichment factor, also another indicator, was calculated by normalizing each round's output/input ratio to that of the first round.

The output phage titer was initially 6.0×10^8 cfu in the first round, corresponding to a 0.60% output/input ratio, shown in **Table 4.1**. The output phage titer increased to 1.0×10^9 cfu in the second round with a 1% output/input ratio and 1.66 enrichment factor despite less incubation time with reduced amount of VEGFR-2 and increased number of washings. This shows a positive selection of VEGFR-2 specific phage-scFv clones and successful amplification of these clones after the first round. The enrichment of VEGFR-2-specific phage-scFv clones also continued in the third round. The output was reached to 4.3×10^9 cfu with a 4.3% output/input and enrichment factor of 7.17, demonstrating efficient enrichment of high-affinity binders. Unexpectedly, the output phages dropped to 1.2×10^8 cfu with a lower output/input of 0.2% and enrichment factor of 0.2 in the fourth round.

4.2. Colony PCR and *Bst*NI Restriction Digestion Analysis of VEGFR-2-specific scFv Library

To evaluate the diversity and integrity of the scFv library across four rounds of biopanning, scFv DNA was amplified via colony PCR. After each panning round, 19 colonies randomly selected from the titration plates were used as template for colony PCR. Analysis of PCR products on a 1% agarose gel confirmed the presence of the

expected ~1000 bp scFv insert (**Figure 4.1A**, **Figure 4.2A**, **Figure 4.3A**, and **Figure 4.4A**). Positive clones harboring the expected ~1,000 bp scFv insert were subjected to *Bst*NI restriction digestion. The resulting digestion patterns, analyzed on a 2% agarose gel, demonstrated high diversity of scFv library (**Figure 4.1B**, **Figure 4.2B**, **Figure 4.3B**, and **Figure 4.4B**).

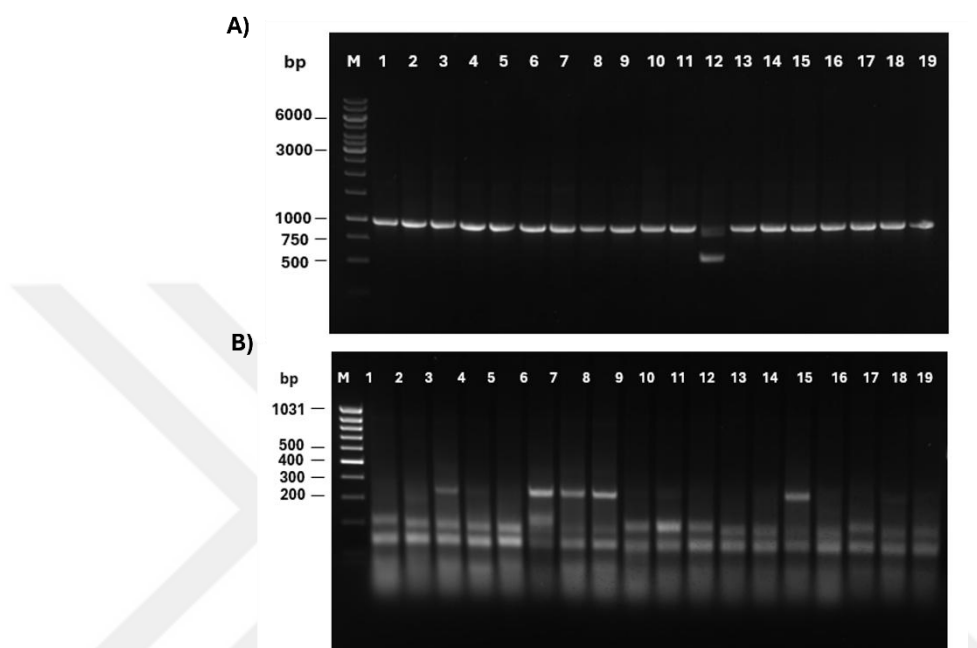


Figure 4.1: ScFv library integrity and diversity after the first panning round. (A) Colony PCR amplification of phage-scFv output from the first panning round. The expected scFv insert size is at ~1,000 bp. (B) DNA fingerprinting diversity analysis of PCR-amplified scFv inserts from the first panning round. M: DNA ladder; 1-19: randomly selected colonies.

As shown in agarose gel image of colony PCR amplification, nearly all randomly selected colonies from the first panning round harbored the scFv DNA library, except one colony (**Figure 4.1A**, colony 12). This colony had an additional DNA band at about 500 bp, possibly resulting from an incomplete insertion during scFv library construction. Nevertheless, the high rate of successful PCR amplification of all biopanning rounds shown in **Figure 4.1A-4A** indicates that 98.7% (75 out of 76) of the screened clones harbored intact scFv inserts.

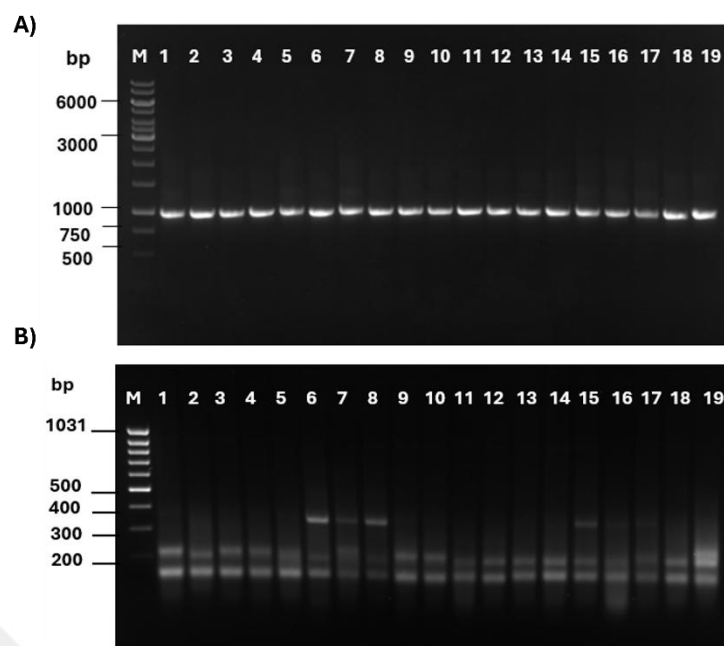


Figure 4.2: ScFv library integrity and diversity after the second panning round. (A) Colony PCR amplification of phage-scFv output from the second panning round. The expected scFv insert size is at ~1,000 bp. (B) DNA fingerprinting diversity analysis of PCR-amplified scFv inserts from the second panning round. M: DNA ladder; 1-19: randomly selected colonies.

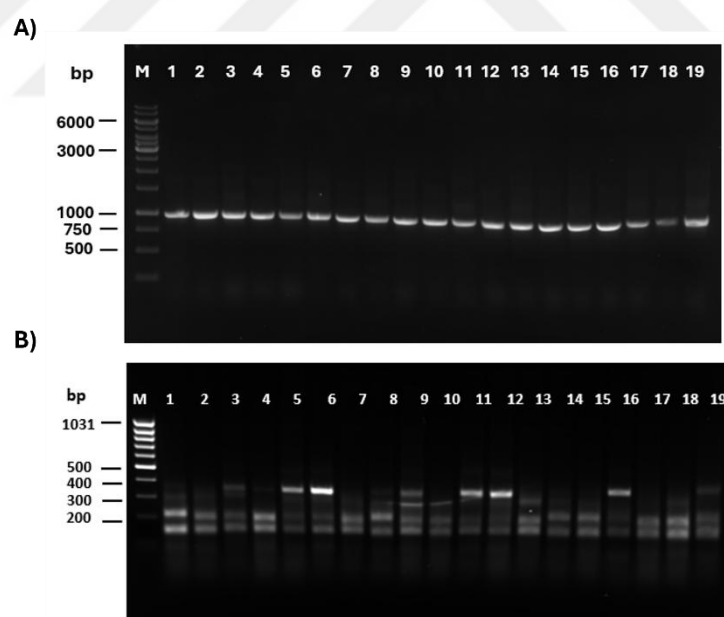


Figure 4.3: ScFv library integrity and diversity after the third panning round. (A) Colony PCR amplification of phage-scFv output from the third panning round. The expected scFv insert size is at ~1,000 bp. (B) DNA fingerprinting diversity analysis of PCR-amplified scFv inserts from the third panning round. M: DNA ladder; 1-19: randomly selected colonies.

*Bst*N1 restriction digestion of PCR-amplified scFv gene inserts demonstrated high diversity between selected clones from each panning round. The digestion produced a wide range of DNA fragment patterns, visualized on agarose gels in **Figure 4.1B-4B**. While several clones exhibited distinct fragments, some clones displayed identical or highly similar digestion patterns. These variations confirmed that the scFv library possess a high degree of sequence diversity.

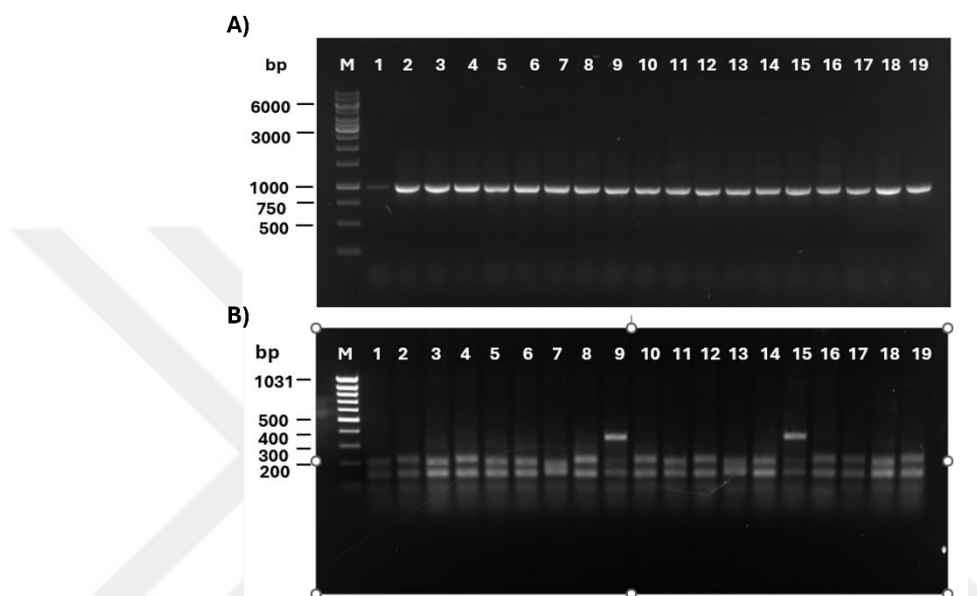


Figure 4.4: ScFv library integrity and diversity after the fourth panning round. (A) Colony PCR amplification of phage-scFv output from the fourth panning round. The expected scFv insert size is at ~1,000 bp. (B) DNA fingerprinting diversity analysis of PCR-amplified scFv inserts from the fourth panning round. M: DNA ladder; 1-19: randomly selected colonies.

The variability in digestion patterns was relatively conserved from the first to third panning round. Notably, the number of distinct patterns slightly decreased in the fourth round, shown in **Figure 4.4.B**. This is consistent with the lower phage output titer of fourth round. Overall, the diversity analysis confirmed that distinct VEGFR-2-specific scFv were enriched from the synthetic human scFv library.

4.3. Monoclonal Phage ELISA Screening Against VEGFR-2

To assess the binding specificity and affinity of individual phage-displayed scFv clones against VEGFR-2, monoclonal phage ELISA was conducted using 60 randomly selected clones from the fourth panning round. Each clone was cultured and superinfected with M13KO7 helper phage to generate monoclonal phage-scFv virions, as described in Materials and Methods.

Clarified supernatants of all 60 clones, containing monoclonal phage-scFv virions, were incubated with VEGFR-2-coated wells (target) and BSA-coated wells (negative control). Bound phages were detected using a primary mouse anti-M13 monoclonal antibody, followed by a secondary goat anti-mouse IgG conjugated to horseradish peroxidase. The binding was quantified by absorbance value at 450 nm using a microplate reader. The results demonstrated specific binding of several clones to VEGFR-2, as shown in **Figure 4.5**.

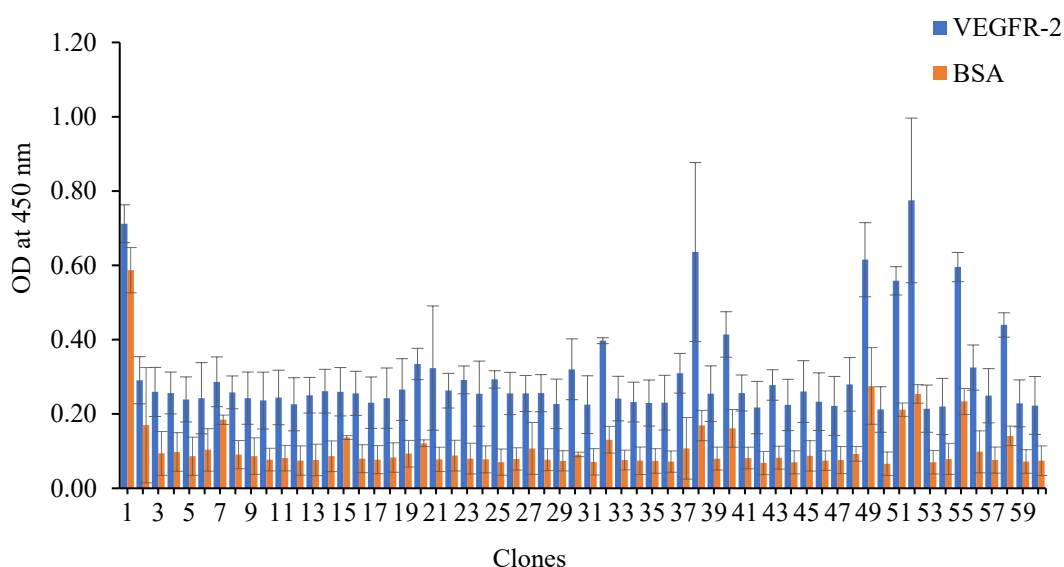


Figure 4.5: Monoclonal phage ELISA. Monoclonal phage-scFv supernatant from 60 clones were incubated with VEGFR-2-coated wells (target antigen) and BSA-coated wells (negative control). The binding was detected using anti-M13 mouse antibody, followed by anti-Mouse antibody-HRP. Colorimetric detection was performed using TMB substrate, and absorbance was measured at 450 nm. Bars represent the mean $\pm$ standard deviation of three independent experiments.

ScFv antibodies exhibiting more than 2-fold higher binding to VEGFR-2 with high absorbance value compared to BSA negative control were considered positive.

Thirteen out of sixty analyzed-scFv clones showed specific binding to the VEGFR-2 protein: clones 21, 23, 25, 30, 32, 38, 40, 49, 51, 52, 55, 56, and 58 (**Figure 4.5**). Due to sequencing limitations, seven scFvs with high-binding capacity, clones 25, 38, 49, 51, 52, 55 and 58, were selected for DNA sequencing and CDR diversity analysis.

4.4. Sequence Determination and CDR Diversity of Anti-VEGFR-2 scFv Antibodies

Monoclonal phage ELISA-positive anti-VEGFR-2 scFv antibodies were selected for DNA sequencing. Plasmid DNA from each clone was extracted from *E. coli* ER2738 cultures. The integrity of PCR-amplified scFv DNA insert in each selected clone was verified on agarose gel, shown in **Figure 4.6**. All selected clones harbor the scFv insert DNA at ~1,000 bp. The PCR products at 1,000 bp were extracted from the agarose gel to use as template for DNA sequencing PCR.

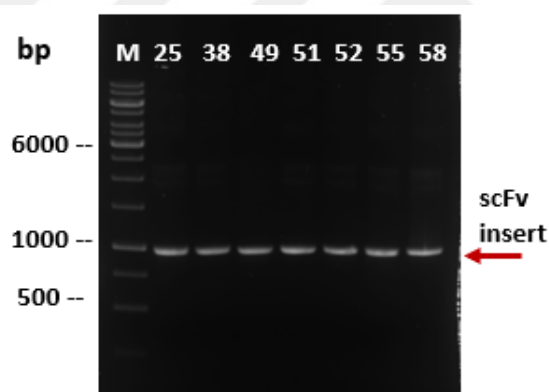


Figure 4.6: PCR product of anti-VEGFR-2 scFv clones. PCR amplification products of scFv clones 25, 38, 49, 51, 52, 55, and 58 were analyzed on 1% agarose gel before sequencing. The expected scFv DNA insert band at ~1,000 bp was observed in all clones.

Sanger DNA sequencing, also known as the chain-termination method, was employed to determine nucleotide sequences of selected anti-VEGFR-2 scFv clones. Sequencing was done using a GenomeLab GeXP Analysis System (Beckman Coulter), with two reads, both forward and reverse, for each clone. Raw sequencing data were processed and aligned using BioEdit, ApE and Chromas software to obtain consensus sequences.

Table 4.2: CDR diversity of anti-VEGFR-2 scFvs. Each amino acid is represented by a distinct color, while unintentional stop codons are indicated with an asterisk (*).

ScFv	Heavy Chain (VH)			Light Chain (VL)		
	CDR1	CDR2	CDR3	CDR1	CDR2	CDR3
25	GYTFTNSR	GIYRDDGNT	VRGGSRNDDAMDY	QGINNY	AAS	QQYSR*PLT
38	GFTFSGSG	KINSSSGDT	VRDDNDTGYSLDY	QGIGSN	AAS	QQPTHHPRT
49	GYTFTDSG	*ISPNNGNT	VRDHNDTSSGMDY	QGIDSN	AAS	QQPNSFPLT
51	GFSFSDSA	*MSTNGGST	VRQRNDNGDWIDY	QGINSY	AAS	QQPTHPLT
52	GYTFSSYS	*ISRSNGDT	VRGHSDYDDALDY	QNIGNY	AAS	QQSNHHPLT
55	GFTFTGSG	*IYTDGGTT	VRRDYDNDYALDY	QGISNN	AAS	QQPNGFPLT
58	GYTFTNYS	NINTSNGNT	VRNRTDYSADMDY	QNIDSN	AAS	QQYDDHPRT

CDR diversity assessment of scFv antibodies was performed using VBASE2 database. Based on VBASE2 database, CDR alignment revealed that all scFv antibodies are unique, shown in **Table 4.2**. Except CDR2 of VL chain, which was kept constant during the scFv library design, no identical CDRs were found in both VH and VL chains of scFv antibodies. This proves either that the library is highly diverse even after four rounds of affinity selection against VEGFR-2 or alternatively that the synthetic scFv library shows high specificity for VEGFR-2, which is consistent with the high output phage titers from panning rounds.

Interestingly, many of selected scFvs have a TAG stop codon in variable regions (**Table 4.2**, marked with an asterisk (*)). ScFv 25 harbored a stop codon in the VL chain, while scFvs 49, 51, 52, and 55 carried stop codon in the VH chain. Due to the potential impact on protein translation and functionality, all stop codon-containing scFv antibodies were excluded from further analysis, except scFv 49.

4.5. Protein Expression and Purification of Anti-VEGFR-2 scFv Antibody Fragments

To produce soluble scFv proteins, recombinant protein expression was carried out in *E. coli*. Due to limited sequencing capacity and to accelerate downstream analysis, initial protein expression was performed prior to sequencing verification of positive scFv antibodies. The pCANTAB 5E phagemids carrying each scFv insert were individually transformed into *E. coli* BL21(DE3) cells. The protein expression was induced with 1 mM IPTG at 25 °C and 30 °C for 2 and 16 hours.

SDS-PAGE and Western blotting analysis revealed that overall protein expression levels were low. ScFv 51, scFv 52, and scFv 55 (data not shown here) exhibited no detectable soluble and weak insoluble expression, while scFv 25 (Appendix, **Figure A.1**), scFv 49 and scFv 58 resulted in low soluble protein level, shown in **Figure 4.7A-7B**, respectively. As a note, scFv 38 was excluded from the initial protein expression study because the log-phase growth for IPTG induction was missed.

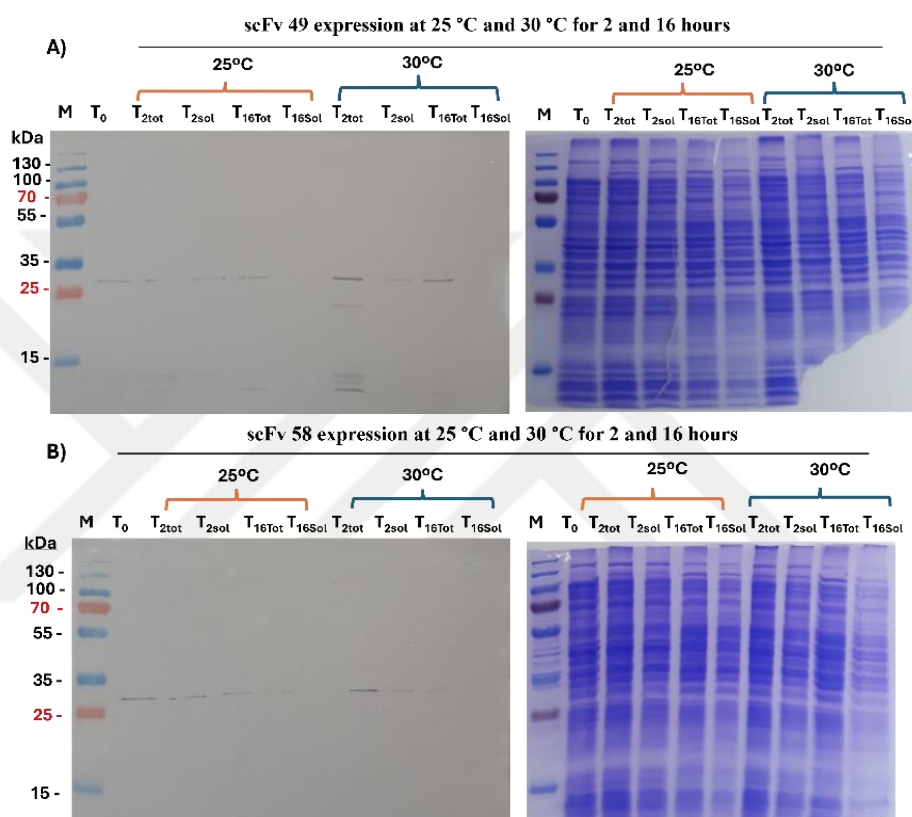


Figure 4.7: Protein expression of scFv 49 and 58 in *E. coli* BL21(DE3). (A) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 49 at 25 °C and 30 °C for 2 and 16 hours. (B) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 58 at 25 °C and 30 °C for 2 and 16 hours. The expected molecular weight of scFv proteins is ~27-28 kDa. The presence of scFv protein bands was detected using anti-E tag antibody HRP-conjugate. T; IPTG induction time, Tot: total cell lysate; Sol: soluble fraction.

Due to the weak expression profiles of the scFvs, the number of clones selected for further characterization was reduced. To focus on clones with the highest binding affinity for VEGFR-2, an ELISA was performed using the cell lysates obtained from each scFv expression culture (**Figure 4.8**). Equal amount of bacterial cell pellet for each scFv was lysed with B-PER lysis buffer. Total cell protein amount was quantified

by BCA and then incubated with VEGFR-2-coated (target antigen) and BSA-coated wells (negative control) in a 96-well plate.

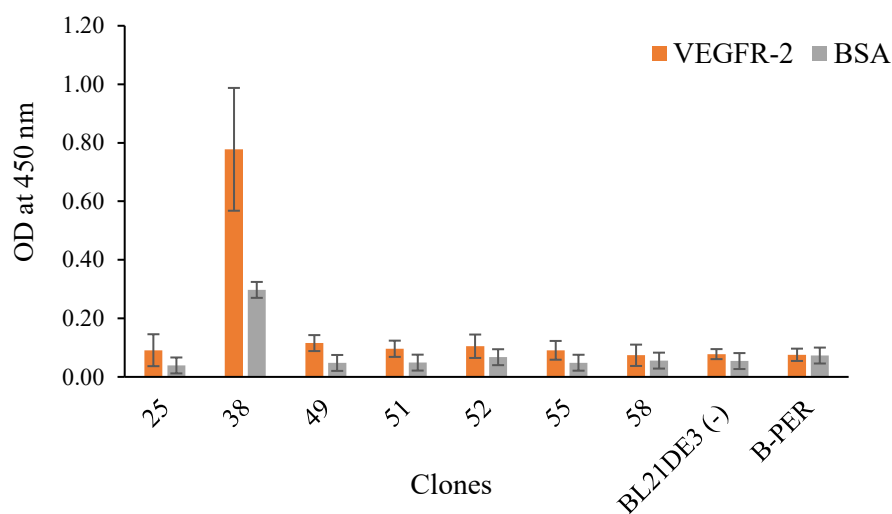


Figure 4.8: Cell-lysate ELISA of scFvs expressed in BL21(DE3) strain. Whole-cell lysates from each scFv-expressing culture were incubated in VEGFR-2-coated wells (target) and BSA-coated wells (negative control). Non-transformed BL21(DE3) cell lysate and B-PER buffer were also included as additional controls to assess background binding. The binding was detected using anti-E tag antibody HRP-conjugate. Colorimetric detection was performed using TMB substrate, and absorbance was read at 450 nm. Bars represent mean $\pm$ standard deviation of two replicates.

Based on the cell-lysate ELISA results, scFv 38 showed the highest binding affinity to VEGFR-2, shown in **Figure 4.8**. ScFv 38, therefore, was chosen for further protein expression studies and characterization.

The protein expression profile of scFv 38 was assessed at 16, 25, and 30 °C under 1 mM IPTG induction for 4, 16, 24, and 48 hours. The high-level expression of scFv 38 protein in both soluble and insoluble forms was verified by SDS-PAGE and Western blot, shown in **Figure 4.9** and **Figure 4.10**. ScFv 38 protein with an expected size of 27-28 kDa were clearly visible on Coomassie-stained gels (marked with a red rectangle) and were further validated by Western blot analysis. These results demonstrate that scFv 38 was soluble expressed in *E. coli* BL21(DE3) at 16 °C (**Figure 4.9**). However, the expression at 25 °C and 30 °C resulted in smaller protein fragments detected in Western blot analysis (**Figure 4.10A-B**).

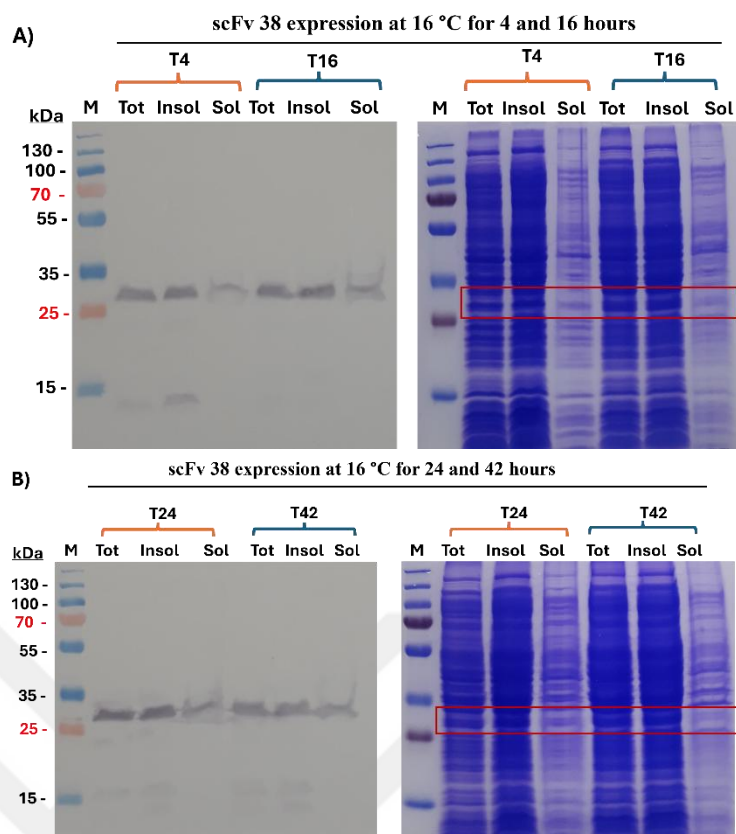


Figure 4.9: Protein expression of scFv 38 at 16 °C in *E. coli* BL21(DE3). (A) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 38 at 16 °C for 4 and 16 hours. (B) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 38 at 16 °C for 24 and 42 hours. The expected molecular weight of scFv proteins is ~27-28 kDa, marked with the red rectangular (right). The presence of scFv protein bands was detected using anti-E tag antibody HRP-conjugate (left). T; IPTG induction time; Tot: total cell lysate; Insol: insoluble fraction; Sol: soluble fraction.

Considering the higher yield of soluble protein without fragmentation, the expression condition of 1 mM IPTG induction at 16 °C for 24 hours was determined to be optimal for soluble scFv antibody production and purification in subsequent experiments.

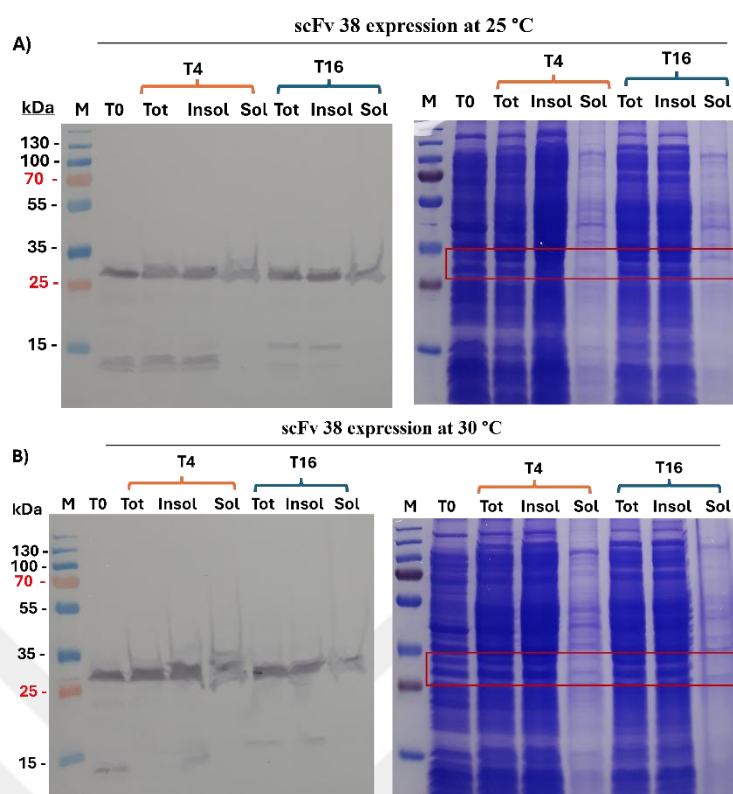


Figure 4.10: Protein expression of scFv 38 at 25 °C and 30 °C in *E. coli* BL21(DE3) strain. (A) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 38 at 25 °C for 4 and 16 hours. B) Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 38 at 30 °C for 4 and 16 hours. The expected molecular weight of scFv proteins is ~27-28 kDa marked with the red rectangular (right). The presence of scFv protein bands was detected using anti-E tag antibody HRP-conjugate (left). T; IPTG induction time; Tot: Total cell lysate; Insol: Insoluble fraction; Sol: Soluble fraction.

4.6. Purification of Soluble Anti-VEGFR-2 scFv Antibody Fragments

To obtain a large quantity of purified soluble scFv protein, scFv 38 was produced in 500 mL bacterial culture under 1 mM IPTG induction at 16 °C for 24 hours. Additionally, scFv 49 was also expressed to evaluate its binding affinity to VEGFR-2 as a soluble purified protein, despite its low-expression profile. Protein L affinity chromatography was employed for protein purification using an ÄKTA Pure 25 system. Since the synthetic human scFv library includes variable kappa light chain, this method was suitable for purification. The purification results were assessed by SDS-PAGE and Western blot (**Figure 4.11**).

As expected, scFv 38 was successfully purified with high yield of soluble protein, whereas scFv 49 yielded lower amount of soluble protein. The final amount of purified scFv 38 was 8.2 mg/L, while scFv 49 yielded only 28 μ g/L, showing more than a 200-fold difference in expression level.

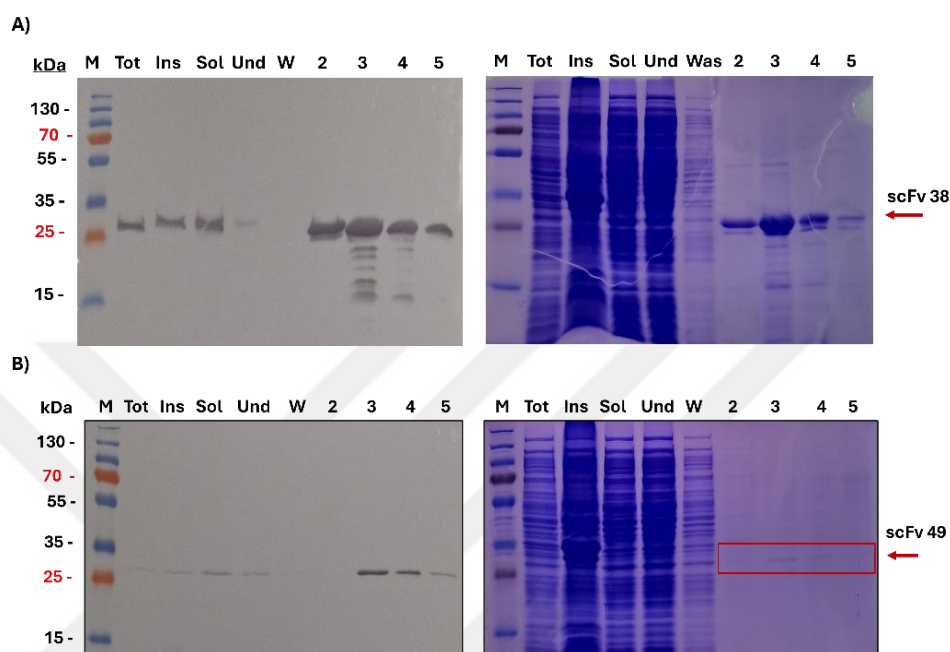


Figure 4.11: Protein purification of soluble scFv 38 and 49. Western blot (left) and Coomassie blue staining (right) analyses of soluble scFv 38 (A) and scFv 49 (B). The expected molecular weight of scFv proteins is ~27-28 kDa, marked with the red arrow. M: protein ladder; Tot: total cell lysate; Ins: insoluble fraction; Sol: soluble fraction; Und: unbound protein (flow through); W: wash ; 2-5: elution fractions.

The purification of scFv 38 resulted in at least 70% purity of soluble protein based on the gel densitometry analysis. However, lower molecular weight protein bands were observed in both the Coomassie-stained gel and Western blot of eluted fractions, excluding elution fraction 5, shown in **Figure 4.11**.

4.7. Soluble scFv ELISA

To investigate the binding ability of purified scFvs, 96-well plates were coated with commercial recombinant VEGFR-2 (extracellular domain 2 and 3, hFc-tagged) and VEGFR-2 (full extracellular domain 1-7, his-tagged). Elution fractions of scFv 38 and 49 were incubated with the antigen-coated plates, and the binding was detected using anti-E tag antibody HRP-conjugate (**Figure 4.12**).

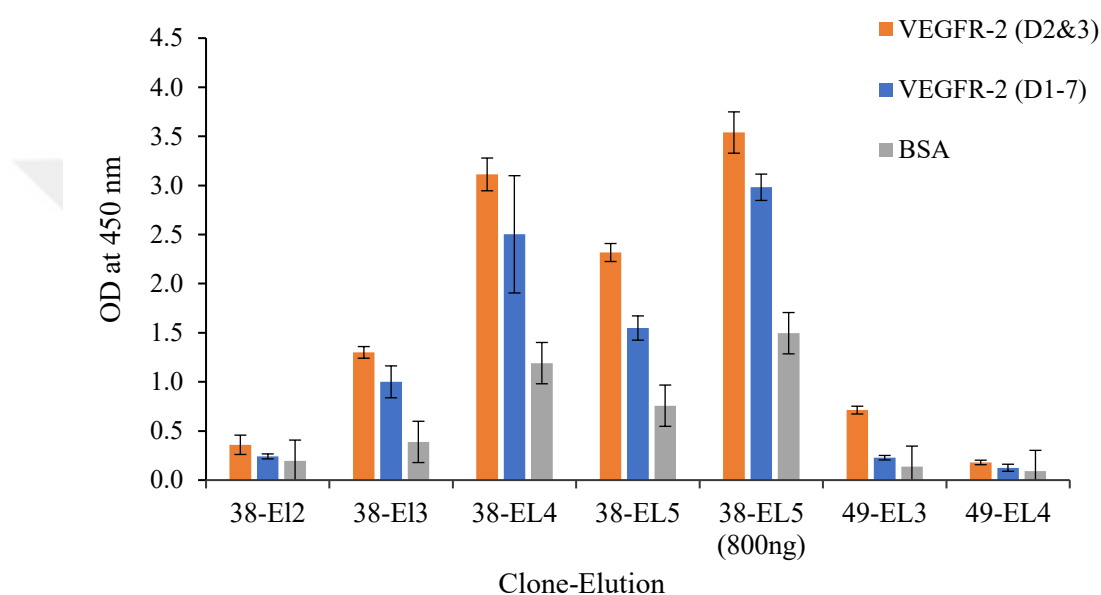


Figure 4.12: Soluble scFv ELISA. Elution fractions of purified soluble scFv antibodies were incubated with VEGFR-2-coated (extracellular domain 2&3), VEGFR-2-coated (extracellular domain 1-7) and BSA-coated wells (negative control). The binding was detected using anti-E tag antibody HRP-conjugate. Colorimetric detection was performed using TMB substrate, and absorbance was read at 450 nm. Bars represent mean $\pm$ standard deviation of three replicates.

Purified scFv 38 demonstrated strong and specific binding to both VEGFR-2 (extracellular domain 2 and 3), which was used as the selection target in biopanning, and the full-length extracellular domain (VEGFR-2 D1–7), shown in **Figure 4.12**. This confirms that pure scFv 38 antibody retains its antigen-binding ability not only to the selection target but also to the full-length extracellular domain of VEGFR-2.

Among the tested fractions, elution fractions 4 and 5 exhibited notably higher binding signals compared to fractions 2 and 3, despite equal scFv concentrations were incubated with the antigens. The difference between binding capacity may be explained with the presence of smaller protein impurities in elution fraction 3 and 4

(visualized on both SDS-PAGE and Western blot in **Figure 4.11**). When the concentration of elution fraction 5 (38-EL5) was doubled (800 ng), the binding was also significantly increased. Overall results suggest that the binding affinity of scFv 38 was high and specific to VEGFR-2, while the variability in functional or integrity across elution fractions were observed. Although low amount of scFv 49 was used in this ELISA, it displayed binding activity. Only elution fraction 49-EL3 showed moderate binding to VEGFR-2 D2&3, while elution fraction 49-EL4 did not demonstrate detectable binding to either form of VEGFR-2.

One week after purification, it was observed that the binding ability of scFv 38 was decreased, shown in **Figure 4.13**. This was hypothesized to result from partial degradation due to few reasons: (i) the low-pH conditions used in elution step during protein L affinity purification, (ii) mechanical or enzymatic cleavage or degradation, and (iii) improper folding due to the absence of disulfide bond formation.

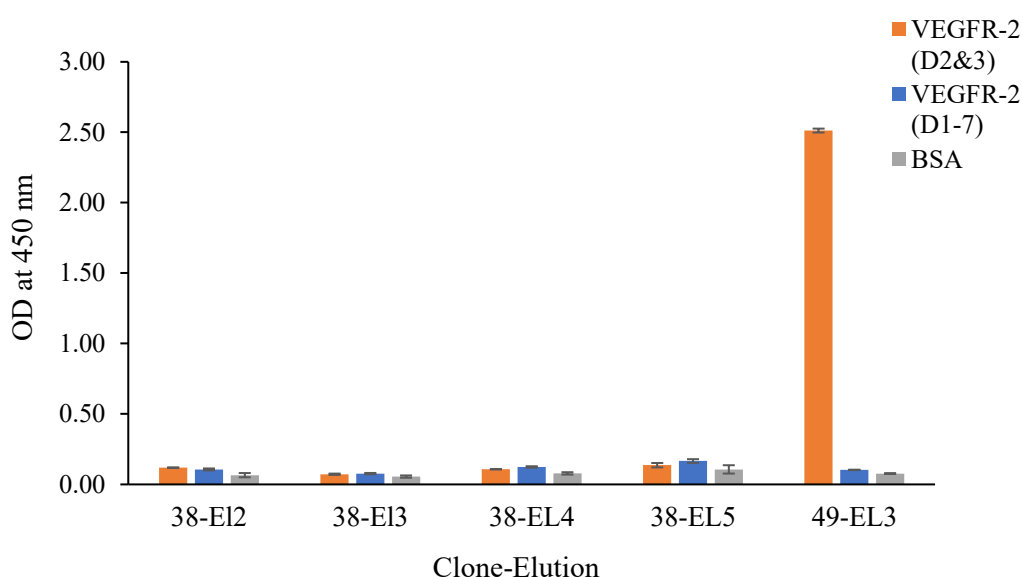


Figure 4.13: Soluble scFv ELISA done one week after scFv purification. Purified soluble scFv proteins were incubated with coated VEGFR-2 (extracellular domain 2&3), VEGFR-2 (extracellular domain 1-7) and BSA (negative control). The binding was detected using anti-E tag antibody HRP-conjugate. Colorimetric detection was performed using TMB substrate, and absorbance was read at 450 nm. Bars represent mean $\pm$ standard deviation of three replicates.

To overcome this problem, the protein expression was evaluated in *E. coli* HB2151 cells. HB2151 is an amber non-suppressor strain in which translation terminates upon encountering amber stop codons. Moreover, this strain is commonly employed to

enhance the stability and solubility of expressed scFv proteins. To verify the presence of amber stop codons previously detected in some scFv clones (see sequencing analysis), all scFv clones that tested positive in monoclonal phage ELISA were transformed into HB2151 cells. Protein expression was induced as described previously, and binding activity was assessed via cell-lysate ELISA (**Figure 4.14**).

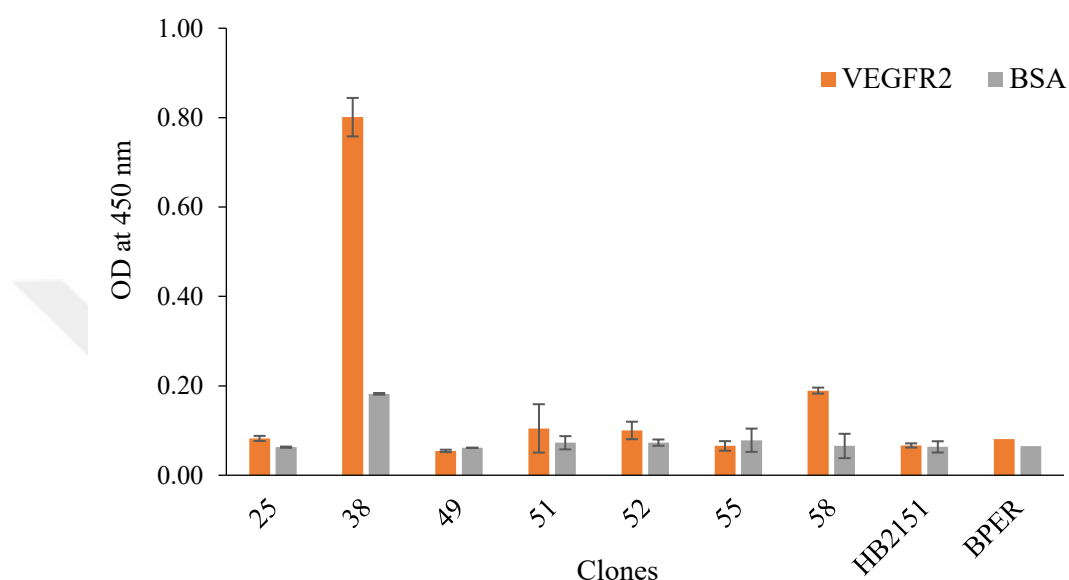


Figure 4.14: Cell-lysate ELISA of scFvs expressed in HB2151 strain. Whole-cell lysates from each scFv-expressing culture were incubated in VEGFR-2-coated wells (target antigen) and BSA-coated wells (negative control). Non-transformed HB2151 cell lysate and B-PER buffer were also included as additional controls to assess background binding. The binding was detected using anti-E tag antibody HRP-conjugate. Colorimetric detection was performed using TMB substrate, and absorbance was read at 450 nm. Bars represent mean $\pm$ standard deviation of two replicates.

As expected, scFv 38 retained its high binding affinity to VEGFR-2 when expressed in HB2151 strain, shown in **Figure 4.14**. Interestingly, scFv 58 also demonstrated a considerable binding activity to VEGFR-2, which was not seen when expressed in BL21(DE3) strain. Based on this result, scFv 58 was expressed in HB2151 strain and then successfully purified with protein L affinity, shown in **Figure 4.15**.

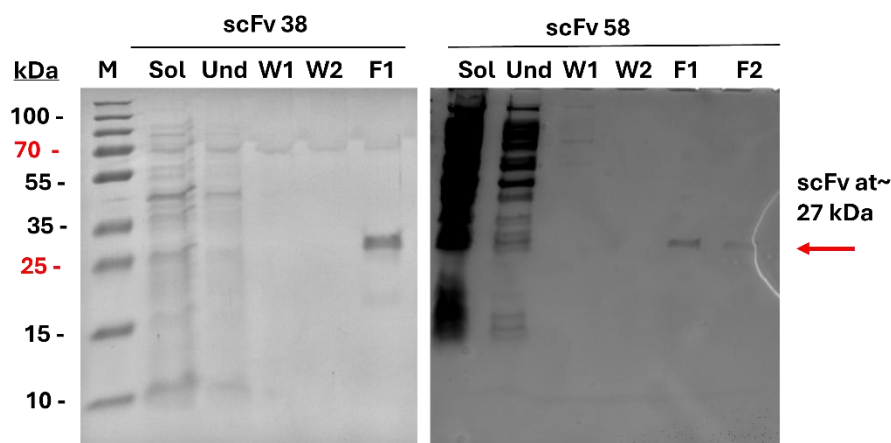


Figure 4.15: Purification of scFv 38 and scFv 58 expressed in *E. coli* HB2151. Coomassie blue staining analyses of purified soluble scFv 38 and scFv 58. The expected scFv protein size is at ~27-28 kDa marked with the red arrow. M: Protein ladder; Sol: Soluble fraction; Und: Unbound protein (flow-through); W: Wash; F: Elution fractions.

Before assessing anti-angiogenic effects of anti-VEGFR-2 scFv antibodies, the binding capacities of soluble scFvs purified at different time points were evaluated by ELISA (**Figure 4.16**). While the amount of VEGFR-2 used for coating the plates was kept constant, the concentrations of scFvs used in each assay varied.

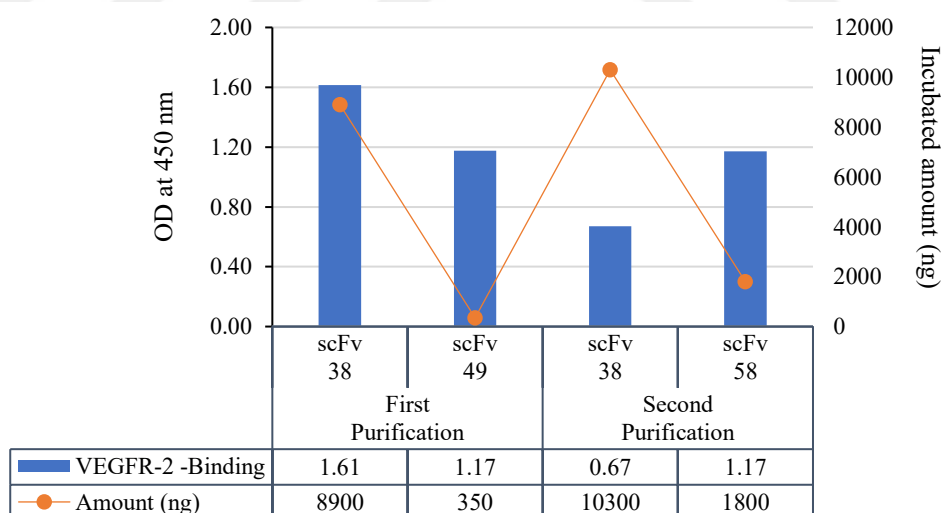


Figure 4.16: ELISA analysis of two independent purifications. Variable amounts of purified soluble scFv antibodies from the first and second purification batches were incubated in VEGFR-2 (Extracellular domain 2&3)-coated wells. The binding was detected using anti-E tag antibody HRP-conjugate. Colorimetric detection was performed with TMB substrate, and absorbance was read at 450 nm.

Essentially, scFv 49 maintained strong binding to VEGFR-2 despite being used at a relatively low concentration of 350 ng, shown in **Figure 4.16**. Similarly, scFv 58 demonstrated a strong binding at an intermediate concentration of 1,800 ng. In contrast, scFv 38 from first purification required a higher concentration (8,900 ng) to achieve strong binding activity. ScFv 38 antibody from the second purification exhibited lower binding activity than that the first purification despite being used at similar concentration (10,300 ng). These results suggest possible batch-dependent variability in functional integrity or stability of scFv 38 antibody.

4.8. Effects of Anti-VEGFR-2 scFv Antibodies on Endothelial Cell Survival

The effect of anti-VEGFR-2 scFv antibody fragments on endothelial cell survival was investigated using HUVECs, which endogenously express VEGFR-2. Following the cell attachment, HUVECs were serum-starved in starvation medium for 16-18 hours and subsequently incubated with VEGF throughout the scFv treatment to establish VEGF-VEGFR-2-dependent cell survival. Cells were treated with anti-VEGFR-2 scFv antibodies in starvation medium supplemented with VEGF for 72 hours. Starvation medium alone (VEGF-) was utilized as negative control, while starvation medium with VEGF (VEGF+) was used as positive control. Cell viability was measured after 72 hours using alamarBlue® reagent.

Cell viability (%) was calculated by normalization to positive control (VEGF+), which was set to 100%. The negative control (VEGF-) showed 97% viability, indicating a minimal VEGF-dependent cell survival under these conditions (**Figure 4.17**). ScFv 38 from the first purification exhibited a slight, dose-dependent inhibitory effect on HUVEC survival, with viability decreasing from 97% at 0.625 μ M to 88% at 5 μ M. However, no considerable difference was observed between the 2.5 μ M and 5 μ M scFv 38 treatments, likely due to full blockade of VEGF-driven survival at higher concentration.

In the absence of VEGF, treatment with 2.5 μ M scFv 38 resulted in 95% viability, slightly higher than the 89% observed at the same scFv concentration in the presence of VEGF. This may suggest that at 2.5 μ M, scFv 38 predominantly inhibits VEGF-VEGFR-2-mediated cell survival. At 5 μ M, scFv 38 resulted in identical cell viability with or without VEGF (88%), indicating that at this higher concentration, the

antibody effectively blocks VEGF-induced survival. It may also slightly reduce basal survival, potentially through inhibition of autocrine VEGF–VEGFR-2 signaling or minor nonspecific effects. Comparison of scFv 38 treatment without VEGF to the negative control (starvation alone, 97% viability) indicates that the antibody does not exert detectable nonspecific cytotoxic effects.

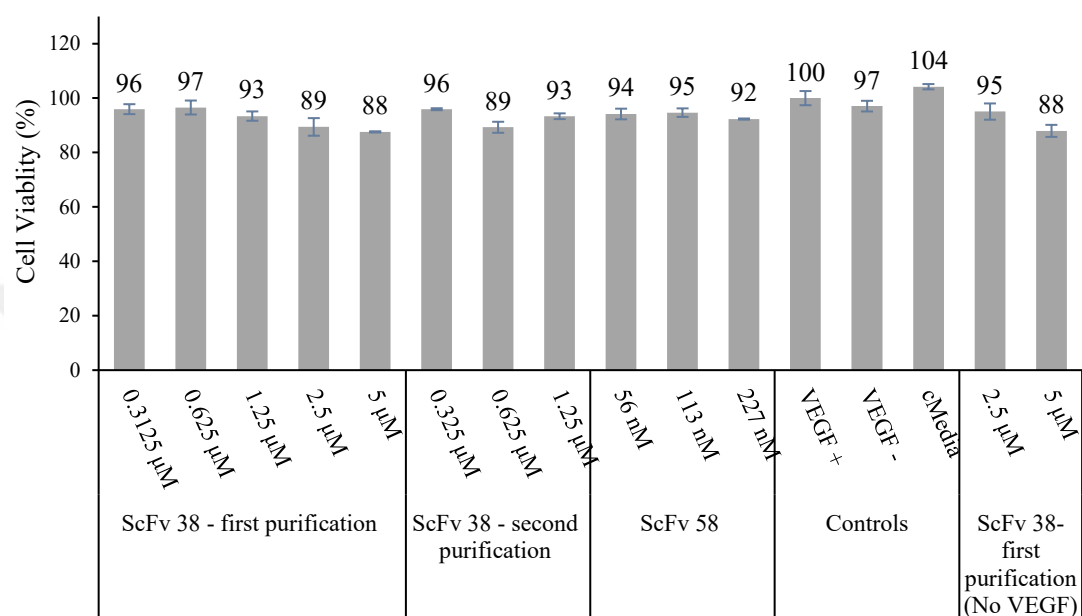


Figure 4.17: Endothelial cell viability assay. HUVECs were treated with various concentrations of anti-VEGFR-2 scFv antibodies in starvation medium supplemented with VEGF for 72 hours. Cell viability was assessed by alamarBlue® assay. Bars represent the mean $\pm$ standard deviation of three replicates. cMedia: complete media.

Similar minimal inhibitory effects on cell survival were seen with scFv 38 from second purification (**Figure 4.17**). The greatest reduction in cell viability occurred at 0.625 μ M, reaching 89%, yet no consistent inhibitory trend was observed across the tested concentrations. Due to the limited yield of purified soluble scFv 58, only lower concentrations were evaluated. At the highest tested concentration (227 nM), cell viability was reduced to 92%, while the two lower concentrations did not show considerable inhibitory effects.

4.9. Effects of Anti-VEGFR-2 scFv Antibodies on Endothelial Cell Migration

To evaluate the effects of the scFv antibodies on endothelial cell migration, a wound healing assay was conducted under the VEGF-dependent migration conditions. Confluent HUVECs were scratched and treated with various concentrations of scFv antibodies in presence of VEGF. Starvation medium alone (VEGF-) served as a negative control, while starvation medium with VEGF (VEGF+) was used as a positive control. Scratch images were taken at 0, 24, 48, and 72 hours. Wound closure (%) was quantified by normalizing wound area at each time point to the initial area (0 h) using ImageJ (**Figure 4.18**).

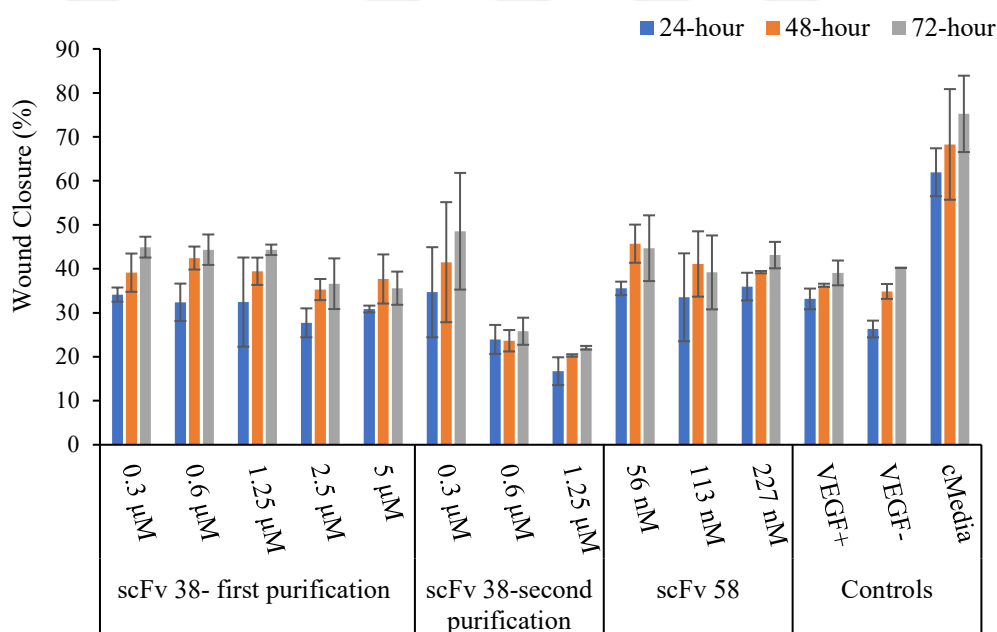


Figure 4.18: Wound closure following treatment with anti-VEGFR-2 scFv. The effects of anti-VEGFR-2 scFv antibodies on VEGF-dependent HUVEC cell migration and wound healing. Bars represent the mean $\pm$ standard deviation of two replicates. cMedia: complete media.

Although it was not considerable, VEGF treatment (VEGF+) promoted wound closure compared to VEGF- treatment (starvation-alone) at 24-hour of incubation, 33% vs. 26%, respectively (**Figure 4.18** and **Figure 4.19**). However, at 48- and 72-hours, wound closure was comparable between VEGF+ and VEGF- groups (36% vs. 35%, respectively). At 72 hours, starvation alone resulted in a slightly greater wound closure than VEGF treatment (40% vs. 39%).

ScFv 38 antibody from the first purification showed no cell migration inhibition compared to positive control. Only treatment at 2.5 μM and 5 μM concentration slightly reduced the wound healing. ScFv 58 had no inhibitory effect on cell migration in all three concentrations (228 nM, 114 nM and 57 nM). On the contrary, scFv 38 from the second purification exhibited a significant wound healing inhibition at 1.25 μM treatment compared to both positive control and negative controls. While 0.6 μM concentration resulted in moderate inhibition, the lowest concentration 0.3 μM showed no inhibition. Wound healing images of scFv 38 treatment from second purification are shown in **Figure 4.19**.

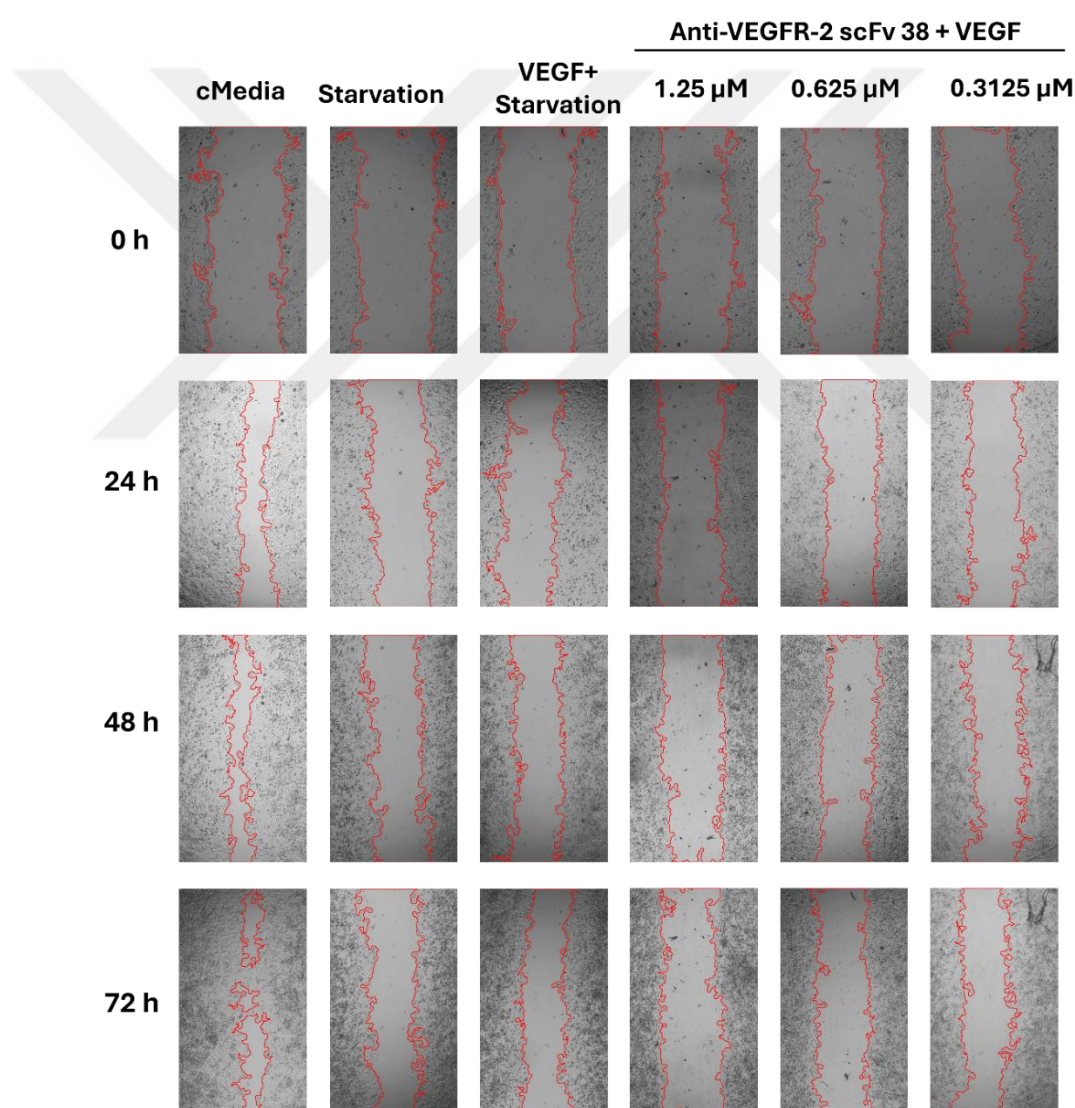


Figure 4.19: Wound healing images of anti-VEGFR-2 scFv 38 treatment. Wound images of HUVEC monolayers treated with scFv 38 from the second purification alongside control groups (Starvation, starvation + VEGF, cMedia (complete media)) captured at 0 h, 24 h, 48 h, and 72 h.

5. DISCUSSION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a crucial mechanism exploited by tumors to support their growth and metastasis. As the main pro-angiogenic factors, VEGFs and VEGFRs are overexpressed by tumor and tumor-associated cells to recruit blood vessels for required oxygen and nutrients supply [38]. Consequently, the VEGF–VEGFR signaling pathway represents a promising target in anti-angiogenic therapy of various solid cancers. Numerous therapeutics targeting the VEGF-VEGFR axis have been in use of cancer therapy, generally with the combination of chemotherapy. However, the major challenges, including tumor resistance, serious off-target activity, and restricted therapeutic outcome, highlight the need for novel and improved therapeutic candidates [19].

In this study, a synthetic human scFv library was screened against VEGFR-2 using phage display to develop novel anti-VEGFR-2 scFv antibodies with potential anti-angiogenic activity. VEGFR-2 was selected as the target due to its role as the main angiogenic signaling mediator of VEGF-VEGFR family [62]. By blocking the VEGF-VEGFR-2 binding, the study aimed to disrupt the pro-angiogenic signaling. For this purpose, VEGF-interacting IgD2 and IgD3 domains of extracellular region of VEGFR-2 were preferentially used as the target antigen in the biopanning process [52].

5.1. ScFv Library Screening and Selection Against VEGFR-2

The synthetic human scFv library used in this study was designed for high functional diversity and optimal protein solubility. It was previously constructed using human antibody VH and VL frameworks and synthetically engineered for extensive sequence variability in the CDRs, which mediate antigen recognition and binding [131]. Additionally, the VH-VL pairs were optimized for enhanced protein solubility, stability and functional expression in bacterial expression systems. Importantly, as a fully human antibody library, it minimizes immunogenicity and removes the need for a labor-intensive humanization process which may sometimes lead to reduced affinity and specificity [132]. Thanks to these design advantages, the library offers a high

probability of isolating novel scFv fragments against diverse human antigens, including VEGFR-2.

Antibody selection and screening of the scFv library against VEGFR-2 were carried out using the solution-phase biopanning strategy. This approach was deliberately chosen over classical solid-phase biopanning because it enables the full accessibility of target antigen surface to phage-scFv complexes [119]. Moreover, the solution-phase panning enables the antigen to retain its native structure in solution, which is often compromised during immobilization to a solid surface. This has shown to raise the chance of selecting high affinity scFv antibody specific to the antigen [133].

Notably, the phage output of first panning round (6.0×10^8 cfu) was higher than the titers typically reported in the literature (between 10^3 - 10^5), although similarly high outputs have also been documented (Table 4.1) [119], [134], [135]. Nonetheless, output titer can vary on the library diversity, antigen concentration, washing stringency, and the efficiency of helper phage rescue. Initial high output titer here may be explained by the broad diversity of the synthetic human scFv library and its intrinsic bias toward VEGFR-2. Supporting this interpretation, the integrity and diversity of the scFv library were preserved throughout the biopanning process, as confirmed by colony PCR and *Bst*NI restriction DNA fingerprinting analysis (Figure 4.1-4.4). The increasing phage output titers observed in the second and third panning rounds (1.0×10^9 cfu and 4.3×10^9 cfu, with enrichment factors of 1.66 and 7.17, respectively) further supported this interpretation. However, the phage output titer was unexpectedly decreased in the fourth panning round (1.2×10^8 cfu with 0.2 enrichment factor). As reported in previous studies, similar decreases during later rounds are often linked to reduced antigen concentration, increased washing stringency, and the potential loss of high-affinity binders under stringent conditions [136], [137].

Overall enrichment factors obtained in this work were lower than many prior phage display studies, which generally range from 10 to 1000 [138]-[140], although even lower values have been reported [134], [141]. Despite the modest enrichment factors, the strategy employed here successfully selected phage-displayed scFv clones with high affinity against VEGFR-2, as demonstrated by monoclonal phage ELISA (Figure 4.5).

Several scFv antibodies that demonstrated binding to VEGFR-2 were sequenced by Sanger sequencing. CDR alignment analysis using VBASE2 database revealed that all sequenced clones possessed unique CDR regions, except CDR2 of VL chain which

was deliberately conserved during the library design (**Table 4.2**). This finding strongly suggests that the synthetic human scFv library maintained high diversity even after four rounds of affinity selection.

However, some of scFv antibodies had amber stop codon (TAG) in their variable regions at the expected positions where high nucleotide diversity was introduced. This represents a well-recognized drawback of synthetic antibody libraries, in which the introduction of randomized sequences can unintentionally generate amber stop codons [142]. For instance, the widely used Tomlinson I and J scFv libraries were reported to contain clones with multiple internal amber stop codons [142]-[144]. Such premature stop codons can disrupt antibody selection and expression, potentially leading to the loss of high-affinity binders [143].

Importantly, phagemid vectors, including pCANTAB 5E used in this study, typically include an amber stop codon between scFv-encoding gene and bacteriophage pIII coat protein-encoding gene [145]. Such design enables the expression of antibody-pIII protein in amber suppressor *E. coli* strains including TG1 and ER2738 [142]. However, scFv clones bearing internal amber stop codon can still be displayed, but with limited efficiency. Moreover, amber codon-containing clones tend to have an amplification bias because of lower expression burden than fully expressed clones [146], [147]. This bias may lead to preferential enrichment of such clones. Indeed, 5 of 7 sequenced clones from the final panning round harbored an amber stop codon.

Amber codon-containing clones face challenges during protein expression, including limited expression level in amber suppressor strain and no expression in amber non-suppressor strains such as *E. coli* HB5121, as observed with scFv 49 [143]. These limitations can be overcome by repairing internal amber codons through site-directed mutagenesis or adopting novel strategies for functional screening of such clones [142], [143], [148].

As another limitation, the labor-intensive and time-consuming nature of random colony picking and Sanger sequencing restricted the number of colony evaluated. A total of 60 colonies were analyzed by monoclonal phage ELISA, whereas only 7 positive clones were sequenced. This relatively small sampling from only a single panning round may have limited the chance of isolating additional high-affinity clones. Typically, the identification of rare, high-affinity antibodies requires testing large numbers of phage-scFv clones, as the probability of success increases with the scale of screening. For example, studies that screened hundreds to thousands of colonies per

panning round have reported the recovery of antibodies with improved specificity and affinity [149]-[151]. With this insight, expanding screening sample size across all panning rounds would enhance the chances of isolating scFvs with superior binding characteristics.

As an alternative solution, phage display studies recently adopted next-generation sequencing (NGS) to address the limitations of traditional screening [152]-[154]. NGS enables simultaneous sequence analysis of millions of clones, assessing enrichment dynamics, CDR pattern usage, and overall library diversity across all panning rounds [152]. For future studies, NGS analysis of the synthetic human scFv library will provide a powerful platform for fast and comprehensive screening against diverse antigens.

5.2. Expression and Purification of anti-VEGFR-2 scFvs

The protein expression analysis of sequence-verified scFv antibodies in *E. coli* BL21(DE3) demonstrated different expression characteristics. Surprisingly, amber codon-containing scFv 49, included as negative control, displayed minimal expression in both soluble and insoluble forms (**Figure 4.7**). Although BL21(DE3) lacks amber suppression, this result aligns with previous reports describing low-level background expression of amber codon-containing proteins in BL21(DE3) [155], [156].

Interestingly, scFv 58, despite bearing no stop codon, also showed low-level expression in both soluble and insoluble forms (**Figure 4.7**). This outcome is likely due to the codon usage bias, inefficient folding, or low stability of scFv in *E. coli*, which are commonly reported limitations for recombinant antibody expression [157]. Unlike scFv 58, scFv 38 yielded high expression in both soluble and insoluble protein forms (**Figure 4.9-4.10**). These differences underscore the significant influence of sequence-specific features, especially CDR regions which vary among scFv clones, on expression efficiency and protein stability.

Moreover, ELISA analysis using whole-cell lysates showed that only scFv 38 retained strong and specific binding to VEGFR-2, confirming its functional expression and target affinity (**Figure 4.8**). However, protein expression optimization of scFv 38 at 25 °C and 30 °C revealed smaller protein fragments, which were initially considered bacterial impurities (**Figure 4.10**).

As expected, protein purification using protein L affinity chromatography further revealed differences in yield and integrity among the scFvs. ScFv 38 antibody purification yielded 8.2 mg of soluble protein per culture liter, whereas amber-containing scFv 49 yielded 28 µg soluble protein. This more than 200-fold difference reflects the inhibitory impact of internal amber stop codons on protein expression. These findings are in line with previous studies reporting low protein yields when expressing amber-containing scFv in amber suppressor strains [158], [159].

Again, lower protein band impurities were observed in elution fractions of scFv 38 (**Figure 4.11**). Literature review suggests that these additional protein bands likely resulted from scFv degradation. Possible reasons may include mechanical shear stress or enzymatic cleavage during the protein expression and extraction processes, or the low-pH elution step of protein L affinity purification. For instance, Petrus *et. al.* also reported the similar sized protein fragments during the protein L affinity purification of HIV-neutralizing scFvs expressed in *E. coli* BL21(DE3). They proposed that these fragments resulted from protein contaminants or scFv degradation caused by the low pH employed during elution step [160]. There are several other studies encountering the scFv degradation during protein expression [161], [162].

ScFv antibody expression was additionally assessed in amber non-suppressor *E. coli* HB2151 strain. ScFv 38 showed similar soluble protein expression profile. Surprisingly, scFv 58 displayed a higher level of soluble protein expression in HB2151 relative to BL21(DE3) (**Figure 4.15**). These results indicate that scFv expression profiles vary between strains, likely due to strain-specific factors such as folding environment, protease activity, or translation efficiency [157].

It should be noted that the expression of scFv antibodies was conducted using the pCANTAB 5E phagemid vector. While phagemid vectors enable functional display of antibody fragments and some studies have successfully expressed soluble scFv antibodies using pCANTAB 5E, they are not specifically engineered for high-level expression of stable, soluble protein [163]-[165]. Many studies have reported improved soluble expression profile by subcloning scFv inserts into dedicated protein expression vectors [161], [163], [166], [167]. For that, the soluble expression level and stability of novel anti-VEGFR-2 scFv antibodies developed in this study could likely be improved by subcloning into dedicated protein expression vectors, such as pET

vectors [148]. Additionally, using engineered *E. coli* strains such as SHuffle® T7 (NEB), which enable the proper folding of disulfide-bonded active proteins in cytoplasm, could significantly improve the yield and stability of anti-VEGFR-2 scFv antibodies [168].

5.3. Binding Activity of Anti-VEGFR-2 scFvs

Purified soluble scFv antibodies were subjected to ELISA for determining the binding to VEGFR-2. Immediately after purification, scFv 38 exhibited strong and specific binding to both the VEGFR-2 extracellular domain 2&3 (used as the biopanning target) and the full-length extracellular domain (D1–7) (**Figure 4.12**). This confirms that pure scFv 38 antibody retains its antigen-binding ability not only to the selection target but also to the full-length extracellular domain of VEGFR-2. However, binding activity of scFv 38 declined over time. In a subsequent ELISA, no binding was observed at the original concentration (**Figure 4.13**) but increasing the concentration ~20-fold restored strong binding (**Figure 4.16**). This loss of activity may have caused from partial degradation or loss of structural integrity due to few reasons: (i) the low-pH conditions used in elution step during protein L affinity purification, (ii) mechanical or enzymatic cleavage, (iii) partial improper folding due to the absence or break disulfide bond formation, and (iv) reduced protein stability over time. Similarly, Goffinet *et. al.* observed that scFvs derived from phage display often exhibit reduced binding activity when expressed in soluble form [159].

In contrast, scFv 49 maintained strong binding to VEGFR-2 even at relatively low concentrations, while scFv 58 demonstrated strong binding at intermediate concentrations (**Figure 4.16**). These findings suggest that scFv 49 is more resistant to external conditions and displays higher stability and affinity compared to scFv 38 and 58. The enhanced stability of scFv 49 probably stems from its unique CDR regions, which are the only difference between scFv antibodies. To further investigate the stability of scFv antibodies, thermal denaturation assays including differential scanning fluorimetry using SYPRO Orange could be employed in future studies [169], [170].

Binding capability of the anti-VEGFR-2 scFvs was primarily assessed using ELISA due to resource and technical limitations. While these results provided the initial evidence of specific VEGFR-2 binding by scFv 38, 49, and 58, further commonly used

analyses are required to comprehensively evaluate their binding specificity and affinities. For instance, ELISA assays against a broader panel of related and unrelated proteins would determine the specificity and functional binding capacity of the scFvs [133]. Moreover, the ability of scFvs to specifically bind the physiological conformation of cell-surface VEGFR-2 could be investigated by flow cytometry and immunostaining assays [133], [151]. Furthermore, quantitative measurement of affinity and binding kinetics using surface plasmon resonance or biolayer interferometry, would further confirm the specificity and affinity of scFvs against VEGFR-2 [171], [172].

In addition to experimental assays, *in silico* approaches using multi-step computational tools could be applied to analyze the interaction of anti-VEGFR-2 scFv antibodies with VEGFR-2. Three-dimensional structures of scFvs can first be modeled from the amino acid sequence using AlphaFold, SWISS-MODEL, or RoseTTAFold [173]-[175]. These models can then be subjected to molecular docking with VEGFR-2 using HADDOCK, ClusPro, or RosettaDock to predict binding orientations and identify key interface residues within the scFv-VEGFR-2 complex [176]-[178]. To complement docking, molecular dynamics simulations performed using GROMACS or AMBER would enable a quantitative evaluation of stability, affinity, and conformational flexibility of the complex [179], [180]. Collectively, these computational analyses would provide molecular-level insight into the binding characteristics of anti-VEGFR-2 scFvs and guide their optimization toward enhanced stability, specificity, and affinity.

5.4. Anti-angiogenic Activity of Anti-VEGFR-2 scFvs

The anti-angiogenic activity of novel anti-VEGFR-2 scFv 38 and 58 antibodies was assessed on VEGF driven *in vitro* endothelial cell viability and wound healing (migration) assays using HUVECs. These functional assays were chosen as VEGF-mediated VEGFR-2 activation promotes endothelial cell survival, proliferation and migration, which are fundamental in angiogenesis [181]. For instance, previous studies targeting VEGF-VEGFR-2 interaction have demonstrated inhibition in endothelial cell proliferation and migration [182]-[184]. Based on this rationale, various concentrations of purified anti-VEGFR-2 scFv antibodies were applied to HUVECs in presence of VEGF in both cell viability and wound healing assays. To

remove background proliferative and migratory signals, HUVECs were starved with 0.5% FBS before the treatment and during the treatment period.

In cell viability assay, VEGF supplementation, used as a positive control, did not significantly increase cell survival during the 72-hour incubation compared to the negative control (starvation medium alone) (**Figure 4.17**). The lack of VEGF-driven survival response likely limited the anti-proliferative and anti-survival effects of scFv antibodies. This is consistent with the minimal cell survival inhibition observed in scFv 38 and scFv 58 treatments, even at their highest tested concentrations. In contrast, more promising results were observed in wound healing assay. ScFv 38 from the second purification exhibited a considerable wound healing inhibition at 1.25 μ M treatment compared to both positive and negative controls (**Figure 4.18**). The cell migration inhibition was maintained during incubation of 24, 48 and 72-hour. A moderate inhibition was also seen at 0.6 μ M, whereas 0.3 μ M showed no detectable effect.

Overall results suggest that anti-VEGFR-2 scFv 38 antibody may possess anti-angiogenic potential. However, scFv degradation or lack of VEGF-driven angiogenic stimulation may have limited the ability to fully reveal their efficacy in inhibiting endothelial cell viability and migration. Another possible reason could be the use of late-passage HUVECs, as endothelial cell phenotypes and behaviors, including angiogenic stimulation, migration, are known to decline at higher passage numbers [185], [186]. Notably, Kiesworo *et. al* reported that late-passage HUVECs retain their VEGF-driven migration ability, while their reduced angiogenic potential stems from impaired VEGF-induced cell proliferation [187]. This may explain why scFv 38 exhibited stronger inhibitory activity on endothelial cell migration compared to cell survival and proliferation.

In future studies, several *in vitro* and *in vivo* angiogenesis assays could be employed to comprehensively assess the anti-angiogenic activity of scFv 38 once its expression and stability are optimized. For instance, *in vitro* tube formation assay using HUVECs can provide a rapid assessment of the ability of scFvs to block VEGF-induced capillary-like network formation [151]. An important consideration for *in vitro* angiogenesis assays is the endogenous VEGF and VEGFR-2 expression levels of endothelial cells. Although HUVECs are widely considered the standard endothelial cell model in angiogenesis studies, they represent heterogeneity depending on donor source, passage number, and growth culture [188]-[191]. Therefore, prior to assays,

VEGF and VEGFR-2 expression level should be quantified using Western blotting, ELISA, or flow cytometry [192]. Alternatively, other endothelial cell types such as human coronary artery endothelial cell, human aortic endothelial cell, or microvascular endothelial cell could be considered to validate the findings [193], [194]. For *in vivo* anti-angiogenic analysis, chicken chorioallantoic membrane, zebrafish angiogenesis, and corneal angiogenesis assays provide robust and complementary models to evaluate the therapeutic potential of anti-VEGFR-2 scFvs [125], [169], [195].



6. CONCLUSION

In this study, a novel synthetic human scFv library was screened against VEGFR-2 using phage display technology to develop potential anti-angiogenic scFv antibodies. Three anti-VEGFR-2 scFv antibodies were isolated via a solution-phase biopanning approach. Among them, scFv 38 emerged as a promising candidate featuring high soluble expression levels and strong binding affinity. ScFv 49 exhibited improved stability and binding activity despite its low expression, whereas scFv 58 resulted in moderate expression and binding. *In vitro* anti-angiogenic assays revealed that scFv 38 considerably inhibited endothelial cell migration but had limited effects on cell survival and proliferation. Future studies should prioritize enhancing protein expression and stability of scFv 38 and scFv 49 and quantifying their binding specificity and affinity. In addition, therapeutic potential of anti-VEGFR-2 scFv antibodies can be further evaluated with complementary *in vitro* and *in vivo* angiogenesis assays. In summary, this study demonstrates the first successful screening of the novel synthetic human scFv library against VEGFR-2 and provides a basis for future screening targeting a wide range of antigens.

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BIOGRAPHY

Yusuf EROL completed his Bachelor of Science degree in Molecular Biology and Genetics at Gebze Technical University in 2022. During his undergraduate studies, he developed a strong interest in immunology, drug discovery and development, and recombinant protein engineering. He gained valuable research experience through internships in several laboratories at Gebze Technical University and TÜBİTAK Marmara Research Center. He was awarded the TÜBİTAK 2247-C STAR Intern Researcher Scholarship and participated in a national research project focused on antibody development. In 2022, he enrolled in the Master's program in Biotechnology at Gebze Technical University. During his graduate studies, he joined the Medical Biotechnology Research Group at TÜBİTAK Marmara Research Center as a research scholar and contributed to national research projects. His research interests include antibody engineering, synthetic biology, and biotherapeutic development with a particular focus on phage display technology.



PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

[1] Erol Y., Uyar O. A., Kurnaz A., (2025), “Screening of a Synthetic Human scFv Library Against Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) for Identification of Potential Therapeutic Candidates”, 9th GTÜ Graduate Research Symposium, Gebze Technical University, Kocaeli, Türkiye, 26-27 May.



APPENDICES

APPENDIX-A: EXPRESSION OF scFv 25

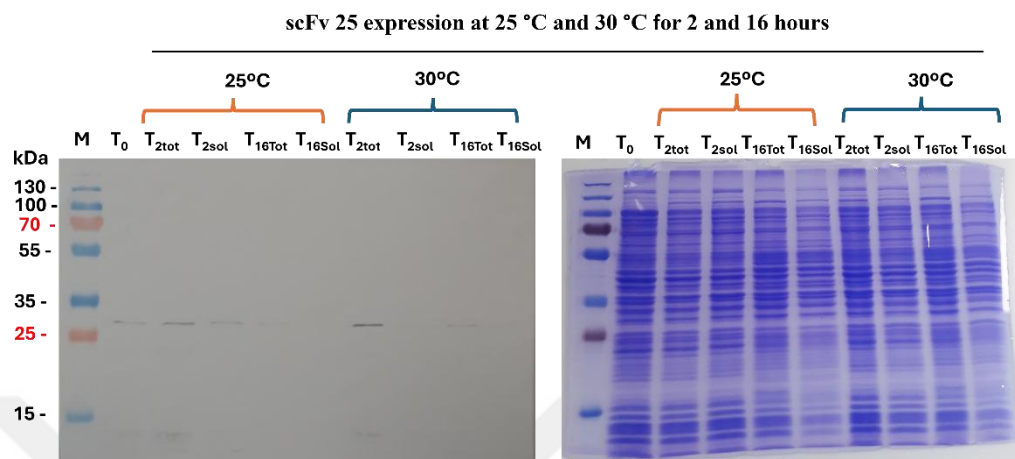


Figure A.1: Protein expression of scFv 25 in *E. coli* BL21(DE3). Western blot (left) and Coomassie blue staining (right) protein expression analyses of scFv 25 at 25 °C and 30 °C for 2 and 16 hours. The expected molecular weight of scFv protein is ~27-28 kDa. The presence of scFv protein bands was detected with anti-E tag antibody HRP-conjugate. T; IPTG induction time, Tot: total cell lysate; Sol: soluble fraction.