

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**DEVELOPMENT AND STRUCTURAL DETERMINATION OF
ANTIANGIOGENIC RECOMBINANT ANTIBODY STRUCTURES FOR
CANCER TREATMENT**



Ph.D. THESIS

Melis DENİZCİ ÖNCÜ

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

FEBRUARY 2022

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**Thesis Advisor: Prof. Dr. Gizem DİNLER DOĞANAY
Thesis Co-Advisor: Dr. Aylin ÖZDEMİR BAHADIR**

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**KANSER TEDAVİSİNE YÖNELİK ANTİANJİOGENİK REKOMBİNANT
ANTİKOR YAPILARININ GELİŞTİRİLMESİ VE YAPISAL TAYİNİ**

DOKTORA TEZİ

**Melis DENİZCİ ÖNCÜ
(521112104)**

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

**Tez Danışmanı: Prof. Dr. Gizem DİNLER DOĞANAY
Eş Danışman: Dr. Aylin ÖZDEMİR BAHADIR**

ŞUBAT 2022

Melis DENİZCİ ÖNCÜ, a Ph.D. student of İTÜ Graduate School student ID 521112104, successfully defended the thesis/dissertation entitled “DEVELOPMENT AND STRUCTURAL DETERMINATION OF ANTIANGIOGENIC RECOMBINANT ANTIBODY STRUCTURES FOR CANCER TREATMENT”, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Gizem DİNLER DOĞANAY**
İstanbul Technical University

Co-advisor : **Dr. Aylin ÖZDEMİR BAHADIR**
TÜBİTAK MAM GMBE

Jury Members : **Prof. Dr. Arzu KARABAY KORKMAZ**
İstanbul Technical University

Doç. Dr. Ceren ÇIRACI MUĞAN
İstanbul Technical University

Dr. Öğr. Üyesi Bülent BALTA
İstanbul Technical University

Prof. Dr. Eda TAHİR TURANLI
Acıbadem University

Prof. Dr. Berna SARIYAR AKBULUT
Marmara University

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To my husband Levent and daughter Eliz,



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Melis DENİZCİ ÖNCÜ
(Molecular Biologist)

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ABBREVIATIONS

aa	: Amino acid
Ab	: Antibody
anti-KDR 1.3G	: Grafted anti-KDR1.3
AP	: Alkaline phosphatase
APS	: Ammonium persulfate
B-PER	: Bacterial protein extraction reagent
BSA	: Bovine serum albumin
cDNA	: Complimentary DNA
CDR	: Complementarity determining regions
CH	: Constant region of heavy chain
CHO	: Chinese Hamster Ovary
CI	: Cell index
CL	: Constant region of light chain
CT	: C-Terminal domain
DMEM	: Dulbeccco's modified Eagle medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DOPE	: Discrete optimized protein energy
<i>E. coli</i>	: Escherichia coli
EBM-2	: Endothelial basal media
ELISA	: Enzyme linked immunosorbent assay
Fab	: The antigen binding fragment
FBS	: Fetal bovine serum
Fc	: Fragment crystallizable
FPLC	: Fast protein liquid chromatography
FR	: Framework
Fv	: Variable fragment
g	: Gram
GST	: Glutathione-S-transferase
HIS	: Histidine
His	: Histidine
hr	: Hour
HPLC	: High performance liquid chromatography
HRP	: Horse radish peroxidase
HUVEC	: Human umbilical vein endothelial cells
Ig	: Immunoglobulin
IgA	: Immunoglobulin A
IgG	: Immunoglobulin G

IgM	: Immunoglobulin M
IMGT	: The international ImMunoGeneTics information system
IPTG	: Isopropyl β - d-1-thiogalactopyranoside
KD	: Affinity constant
K_D	: Equilibrium dissociation constant
kDa	: Kilo dalton
KDR	: Kinase insert domain receptor
L	: Liter
LB Medium	: Luria bertani medium
mAb	: Monoclonal antibody
MBP	: Maltose binding peptide
MD	: Molecular dynamics
mg	: Miligram
min	: Minute
mL	: Mililiter
mm	: Milimeter
mRNA	: Messenger RNA
na	: Nucleic acid
ng	: Nanogram
nm	: Nanometer
nM	: Nanomolar
NPT	: Isothermal-Isobaric ensemble: moles (N), pressure (P) and temperature (T)
NT	: N-Terminal domain
ORF	: Open reading frame
PCR	: Polymerase chain reaction
PDB	: Protein data bank
PDGF	: Platelet derived growth factor
PEG	: Polyethylene glycol
Penstrep	: Penicilin streptomycin
pH	: Power of hydrogen
PIGF	: Placenta growth factor
PTM	: Posttranslational modification
RMSD	: Root mean square deviation
RNA	: Ribonucleic acid
Rpm	: Revolutions per minute
RTCA	: Real time cell analyzer
RU	: Responce units
scFv	: Single-chain variable fragment
SDS-PAGE	: Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
sec	: Second
SPR	: Surface plasmon resonance
TEMED	: Tetramethylethylenediamine
VEGF	: Vascular endothelial growth factor

VEGFR-2	: Vascular endothelial growth factor receptor 2
VH	: Variable heavy
VL	: Variable light
2xYT	: Yeast extract tryptone
4C1N	: 4-Chloro-1-naphthol





SYMBOLS

A	: Alpha
β	: Beta
t	: Time
Δ	: Delta
~	: Approximately equal
μM	: Mikromolar
nM	: Nanomolar
μg	: Mikrogram
μl	: Mikroliter



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DEVELOPMENT AND STRUCTURAL DETERMINATION OF ANTIANGIOGENIC RECOMBINANT ANTIBODY STRUCTURES FOR CANCER TREATMENT

SUMMARY

Vascular endothelial growth factor (VEGF) is a signaling molecule that plays a role in both vasculogenesis and angiogenesis. VEGFR-2/KDR (vascular endothelial growth factor receptor 2/ kinase insert domain receptor) is a tyrosine kinase that regulates a variety of angiogenesis-related cell responses. VEGFR-2/KDR is located on the surface of tumor cells and is heavily expressed in tumor-associated endothelial cells, where it modulates tumor-promoting angiogenesis. The suppression of VEGFR-2, which inhibits the angiogenic pathway in carcinogenesis, is thought to be a viable therapeutic method for the prevention and control of solid tumor growth. There are currently antibody (Ab) therapeutics targeting VEGFR-2 to treat cancer cells, but none of them is a final cure. Expectedly, the production of a novel effective anti-VEGFR-2 Abs would support cancer patients.

The advancement of recombinant antibody technology and their tailored fragments has resulted in the emergence of a diverse array of antibody molecules, including the antigen binding fragment (Fab), the variable fragment (Fv), and the single chain variable fragment (scFv). Compared to the large size of a full antibody, scFvs have greater tissue penetration, decreased immunogenicity due to small molecular masses and accelerated clearance time. However, there are also some drawbacks of production of scFv structure which has poor biophysical properties, such as increased aggregation tendency and decreased thermodynamic stability, resulting in low performance and application.

Protein solubility is critical in the synthesis and administration of therapeutic proteins. Inadequate solubility can lead protein aggregation, a detrimental phenomenon that can occur at any stage of recombinant protein synthesis. Numerous methods, extending from culture conditions to solubility enhancer tags, are being used to increase soluble expression. While fusing proteins to mostly soluble companions or expression with molecular chaperones may increase their total solubility, these techniques typically impair the proteins biological activity. There is no universal solution or technique for protein aggregation.

Anti-KDR 1.3 and anti-KDR 2.6 (anti-VEGFR-2) scFv molecules were formerly developed by phage display technique by TUBITAK MAM GMBE Immunogenetics Laboratory group. However, these antibodies are produced in inclusion bodies and form protein aggregates. Additionally, folding these molecules into active biological molecules is a laborious and uncertain process. Additionally, the protein yields are usually poor. The aim of this thesis is to address the expression and solubility problem of anti-KDR scFV molecules and to confirm the anti-angiogenic activities of the produced target molecules. In this manner, two different approaches have been adopted in the thesis, the first is the production and control of the target scFv molecules

in mammalian cells, and the second approach is to transfer the target scFv molecules to a different structure using the complementarity determining regions CDR-grafting method, ensuring their production in bacteria and controlling their activity.

In the first approach, the target scFv protein expression in mammalian cell culture was investigated. The existing scFv genes were utilized in the research. Different signal peptides were used for the construction of the plasmids including the target genes. Simultaneously, codon optimizations for the target cell line based on the target genes were performed. For this study, newly developed scFv genes were cloned into different mammalian expression vectors and transferred into competent cells by chemical transformation. Then, the cloned vectors were determined by colony polymerase chain reaction (PCR) studies. The DNA sequences of the newly cloned scFv constructs were controlled by a capillary electrophoresis system. With the results obtained, sequence analysis was performed, and the proper incorporation of the gene into the plasmid, as well as the presence of mutations, were examined. The sequence of the target genes obtained were translated using bioinformatics tools, and the protein to be generated was examined for frameshifts. In accordance with these findings, the resulting vectors were purified and transfected into mammalian cells. New scFv constructs were produced in mammalian cells and purification was made by using chromatography columns. Transient transfections with vectors encoding the regulated target genes were initially conducted in different mammalian cell lines. Then, stable transfections were generated using the optimum antibiotic concentrations. The serial dilution technique was used to produce monoclonal cells from stably transfected cells. Chinese Hamster Ovary (CHO-K1) cells were adapted and suspended in serum-free media. Several feeding strategies, temperature experiments, and high volume production experiments were carried out in the cell culture studies.

The resulting antibody structures were analyzed by SDS-PAGE and western blot methods. The binding of the samples to VEGFR-2 were then be checked by ELISA assays. After production and purification of the proteins, the activity and structure analysis were performed. Purified antibodies were examined by chromatographic methods based on HPLC (High Performance Liquid Chromatography). Real-time PCR method was used to investigate the presence of m-RNA in cells. In addition to these studies, Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS) studies were carried out to reveal the structural dynamics of the anti-KDR 1.3 protein obtained from bacteria.

Unfortunately, it was not possible to achieve better expression efficiency for the target anti-KDR molecules in mammalian cells. Overall results for poor expression or none were confirmed by monoclonal cells experiments, fed-batch experiments, codon optimizations, signal peptide alternatives. The selection of high-product yielding clones was requiring significant amount of time and effort in the development of cells producing recombinant molecules. As a result, the second half of this thesis focused on developing a unique technique for the quick and simple increase in solubility and production of the target proteins.

In the second approach, a CDR grafting technique was used to modify and upgrade the structure of the existing scFv with a more producible pre-existing scFv to improve the biophysical characteristics of these scFv molecules. The effect of the CDR grafting technique on the solubility and efficacy of the expressed protein was investigated in this study. Computational methods were used to model our antibody structure and physicochemical properties, followed by experimental confirmation.

CDR grafting technique was employed to modify and upgrade the structure of our scFv with a more producible pre-existing scFv. I used computational methods to model our antibody structure and its physicochemical properties, followed by experimental confirmation. Soluble expression analysis, affinity determination by surface plasmon resonance (SPR), *in vitro* activity assays were performed.

According to the computational data, CDR grafting enhanced the solubility of the grafted scFv protein in comparison to the native scFv protein. SDS-PAGE and western blot analysis showed the expressed protein's increased solubility. SPR analysis revealed that the grafted molecule exhibits comparable binding affinities to the VEGFR-2 receptor to the native molecule. Biological activity studies, including proliferation inhibition, migration and wound healing experiments studies on human umbilical vein endothelial cells (HUVEC), demonstrated that the newly synthesized grafted scFv protein possesses anti-angiogenic characteristics.

This study reveals that a soluble scFv can be created by grafting the variable regions from an intrinsically insoluble scFv onto constant sections derived from innately soluble molecule. The drug potency of a low yield anti-angiogenic scFv (anti-KDR 1.3) with inclusion body formation tendency was highly increased by employing CDR grafting strategy.



KANSER TEDAVİSİNE YÖNELİK ANTİANJİOGENİK REKOMBİNANT ANTİKOR YAPILARININ GELİŞTİRİLMESİ VE YAPISAL TAYİNİ

ÖZET

Vasküler endotelial büyüme faktörü reseptörü 2 (VEGFR-2/KDR) temel olarak endotel hücrelerde bulunmaktadır ve endotel hücrelerinin büyümesi, farklılaşması ve göç etmesini düzenlemektedir. VEGFR-2 özellikle tümörle ilişkili endotel hücrelerde yoğun şekilde eksprese edilir ve tümör invazyon ve metastazını stimüle eden anjiyogenezi düzenlemektedir. Anjiyogenez mevcut kan damarlarından yeni damar oluşmasıdır. Günümüzde kanser hücrelerini tedavi etmek için VEGFR-2'yi hedefleyen antikor (Ab) temelli tedavi yöntemleri bulunmaktadır, ancak bunların hiçbirisi nihai bir tedavi yöntemi değildir. Bu sebeple yeni ve etkili anti-VEGFR-2 antikor üretimi ile kanser hastalarının tedavisinde başarı elde edebilmek mümkün olacaktır.

Rekombinant antikor teknolojisindeki gelişmeler sayesinde; Fab fragmanları, Fv fragmanları ve ScFv fragmanları dahil olmak üzere çok çeşitli antikor molekülleri bulunmaktadır. Full bir antikorun ebatları ile karşılaştırıldığında, scFv'ler küçük moleküler kütleleri sayesinde daha büyük doku penetrasyonuna düşük immünojenisiteye ve düşük vücuttan atılım süresine sahiptir. Bununla birlikte, artan agregasyon eğilimi ve düşük termodinamik stabilite gibi zayıf biyofiziksel özelliklere sahip olan ve düşük performans ve uygulama ile sonuçlanan scFv yapısının üretiminin bazı dezavantajları da vardır. Çözünürlük ve stabilite sorunlarının üstesinden gelmek için çeşitli yöntemler geliştirilmiştir. Çözünürlük artırma çalışmalarında iki farklı genel yol tercih edilebilir. Bunlardan ilki moleküler mühendislik stratejileri kullanmadan; düşük sıcaklıklar, farklı besi yerleri, farklı suşlar kullanarak yapılabilmektedir. Çözünürlüğü artırmaya yönelik kullanılan diğer bir yöntem ise moleküler mühendislik yöntemleri ile yapılan füzyon, moleküler şaperonlarla birlikte ekspresyon, mRNA stabilizasyonudur. Bu yöntemler ile genel çözünürlükleri önemli ölçüde artırılabilirken, bu proteinlerin biyolojik aktivitelerini kaybedebildikleri gözlenmiştir. Çözünürlük tek başına proteinlerin aktif olduğu anlamına gelmemektedir.

Anti-KDR 1.3 ve anti-KDR 2.6 (anti-VEGFR-2) scFv molekülleri daha önce TUBITAK MAM GMBE Immunogenetik Laboratuvarı tarafından faj görüntüleme tekniği ile geliştirilmiştir. Ancak bu antikorlar inklüzyon cisimcikleri şeklinde üretilmekte ve protein agregatları oluşturmaktadır. Ayrıca bu proteinlerin aktif biyolojik formlara yeniden katlanması çok uzun süre ve emek istemekte ve tekrarlanabilir sonuçlar elde edilmemektedir. Ek olarak protein verimleri genellikle zayıftır.

Bu tez ile hedeflenen, anti-KDR scFV moleküllerinin ekspresyon ve solubilité sorununu çözmek ve üretilen hedef moleküllerin anti-anjiyojenik aktivitelerini doğrulamaktır. Bu doğrultuda, tez içerisinde iki farklı yaklaşım benimsenmiştir, ilki hedef scFv moleküllerin memeli hücrelerde üretimi ve Kontrolü, ikinci yaklaşım ise

hedef scFv moleküllerin CDR-grafting yöntemi ile farklı bir yapıya aktararak bakterilerde üretimini sağlamak ve aktivite kontrolünü gerçekleştirmek.

İlk yaklaşımda, memeli hücre kültüründe hedef scFv protein ekspresyonu araştırılmıştır. Çalışmalar için DNA sekansı belli olan ve genleri hali hazırda klonlama vektöründe bulunan scFv geni kullanılmıştır. Oluşturulan yapılarda farklı sinyal peptitleri ile tasarımlar gerçekleştirilmiştir. Aynı zamanda hedef genler üzerinde belirlenen hücre hattı için kodon optimizasyonları yapılmıştır. Yeni tasarlanan scFv genleri farklı memeli ekspresyon vektörlerine klonlanmış ve *E.coli* hücrelerine farklı transformasyon yöntemleri ile aktarılmıştır. Daha sonra bu hücrelerden koloni PCR yapılarak klonlanan vektörler tespit edilmiştir. Klonlanmış yeni scFv yapılarının DNA sekansları kapiller elektroforez sistemi yardımıyla gerçekleştirilmiştir. Elde edilen sonuçlar ile sekans analizi yapılmış, genin vektörün içine doğru yerleşimi ve mutasyon içerip içermediği kontrol edilmiştir. Dizilerden elde edilen genler bioinformatik programlar aracılığı ile translate edilmiş ve üretilecek protein de herhangi bir frame kayması olup olmadığı kontrol edilmiştir. Bu sonuçlar doğrultusunda, elde edilen vektörler saflaştırılarak, memeli hücrelerine transfekte edilmiştir.

Kontrolleri yapılan hedef genleri içeren vektörler farklı memeli hücre hatlarında ilk olarak transient transfeksiyonlar gerçekleştirilmiştir. Bu çalışmalar öncesinde, optimum koşulları belirlemek üzere transfeksiyon optimizasyon çalışmaları yapılmıştır. Daha sonra uygun koşulları belirlenen antibiyotik konsantrasyonları ile stabil transfeksiyon sağlanmıştır. Stabil olarak transfekte edilen hücrelerden seri dilüsyon yöntemi ile monoklonal hücreler elde edilmiştir. Hücre kültürü çalışmalarında CHO-K1 hücrelerinin serumsuz besi yerine adaptasyonu ve süspanse hale getirilmesi tamamlanmıştır. Aynı zamanda, hücre kültürü çalışmaları kapsamında farklı besleme stratejileri, farklı sıcaklık denemeleri ve büyük hacim üretim denemeleri gerçekleştirilmiştir.

Yeni scFv yapıları memeli hücrelerinde üretilmiş ve saflaştırma çalışmaları FPLC (Fast protein liquid chromatography) cihazı ile yapılmıştır. Elde edilen antikor yapıları SDS-PAGE ve western blot yöntemleri ile analiz edilmiştir. Daha sonra örneklerin VEGFR-2'ye bağlanmaları ELISA ile kontrol edilmiştir. Saflaştırılan antikorlar HPLC (High Performance Liquid Chromatography)'ye dayalı kromatografik yöntemlerle incelenmiştir. Hücreler de m-RNA varlığının araştırılması için gerçek zamanlı PZR yöntemi kullanılmıştır.

Bu çalışmalar yanında, bakterilerden elde edilen anti-KDR 1.3 proteinin yapısal dinamiklerinin ortaya çıkarılması için Hidrojen Deuterium Değişimi Kütle Spektrometrisi (HDX-MS) çalışmaları gerçekleştirilmiştir.

Tüm sonuçlar incelendiğinde hedef scFv molekülün memeli hücrelerinde ekspresyonunun solubilité ve üretim açısından avantaj sağlamadığı, üretimin memeli hücrelerde çok zayıf oranda elde edildiği veya üretimin gerçekleşmediğini göstermiştir. Tезin ikinci kısmında farklı bir yöntem benimsenerek daha etkili ve kısa sürede sonuç alınabilecek bir yaklaşım belirlenmiştir.

İkinci yaklaşımda, hedef scFv moleküllerinin çözünür bir biçimde eldesini sağlayabilmek için normal koşullar altında insansılaştırma çalışmaları için kullanılan bir yöntem olan CDR grafting yöntemi farklı bir amaçla kullanılmıştır. İnsansılaştırma (CDR grafting), özellikle kemirgen hayvanlar kullanılarak geliştirilen monoklonal antikorların (mAb'ler) immünojenitesini azaltmak ve bu yapıların insan bağışıklık sistemi aktivasyonunu artırmak için kullanılan bir tekniktir. Biz bu tekniği amacı dışında kullanarak, geliştirdiğimiz antikorun solubilitésinin artırılması için kullanmayı

hedefledik. Buradaki hipotezimiz de, çözünür bir biçimde üretim elde edilen scFv yapısının çerçeve bölgelerinin, inklüzyon cisimciği oluşturan bir scFv'yi oluşturan çerçeve bölgeleriyle değiştirilmesi durumunda, geliştirilen yeni scFv yapısının çözünür bir biçimde elde edilebileceğini gösterdik.

Grubumuz tarafından daha önce HbsAg'ye karşı geliştirilmiş ve çözünük bir biçimde eksprese edilen scFv'nin (Lig 7) yapısı ile değiştirmek için CDR-grafting yöntemi kullanılmıştır. Bu çalışmada, CDR-grafting tekniğinin eksprese edilen proteinin çözünürlüğü ve etkinliği üzerindeki etkisi araştırılmıştır. Bu çalışma için öncelikle hedef scFv (anti-KDR 1.3) ve (Lig7) moleküllerinin modellemeleri biyoinformatik programlarla yapılmıştır. Bu çalışmalar kapsamında, scFv yapıların sekanslarının karşılaştırması ve homoloji modellerinin karşılaştırmaları yapılmıştır. Bu kapsamda, scFv yapıların moleküler dinamik simülasyonları gerçekleştirilmiş ve oluşturulan yeni moleküller için çözünürlük değerleri belirlenmiştir. Bu çalışmalar ışığında, hedef molekülün CDR bölgeleri Lig7 molekülünün frame bölgeleri ile değiştirilmiştir. Elde edilen yeni oluşturulmuş scFv sekansı için yeni bir plazmit konstraktı tasarlanmış ve ticari olarak sentezlenmiştir.

Oluşturulan plazmit konstraktı bakteri hücrelerine transforme edilmiş, *Escherichia coli* (*E. Coli*) BL21 suşunda üretim gerçekleştirilmiş ve ekspresyon sonucu çözünür protein üretimi SDS-PAGE ve western blot analizleri ile gösterilmiştir. Elde edilen yeni scFv yapılarının saflaştırma işlemleri histidine kuyruğu kullanılarak FPLC cihazı ile gerçekleştirilmiştir. Saflaştırma sonrası hedef protein üretimi SDS-PAGE ve western blot çalışmaları ile doğrulanmıştır. Elde edilen moleküllerin SPR cihazı ile bağlanma analizleri gerçekleştirilmiş ve orijinal molekül ile karşılaştırılmıştır.

Yeni oluşturulan anti-KDR 1.3G moleküllerinin *in-vitro* aktivite testleri gerçekleştirilmiştir. Hücre deneyleri için HUVEC hücre hattı kullanılmıştır. İlk olarak proliferasyon inhibisyonu deneyi, xCELLigence RTCA DP gerçek zamanlı hücre analiz cihazı kullanılarak empedansa dayalı olarak gerçekleştirilmiştir. Yara iyileştirme deneyi, iki boyutlu alanada toplu hücre göçünü araştırmak için oluşturulmuş bir *in vitro* tekniktir. Bu deneyde, mekanik parçalama hücre yüzeyinde eşit bir biçimde hücresiz bir alan (bir yara alanı) oluşturulmuştur. Deney sonuçları, HUVEC hücrelerinin boş yara alanlarının yüzde değerleri olarak sunulmuştur.

Modelleme sonuçlarına dayanarak, CDR-grafting tekniği ile oluşturulmuş scFv molekülünün çözünürlüğü, orijinal scFv molekülü ile karşılaştırıldığında artmıştır. SDS-PAGE ve Western blot sonuçları, eksprese edilen proteinin çözünürlüğündeki artışı doğruladı. SPR sonuçları, anti-KDR 1.3G molekülünün, orijinal olana kıyasla VEGFR-2 reseptörüne benzer bağlanma afinitelerine sahip olduğunu göstermiştir. HUVEC proliferasyonu ve yara iyileştirme deneyleri dahil olmak üzere *in vitro* aktivite deneyleri, yeni oluşturulmuş scFv molekülünün anti-anjiyojenik özelliklere sahip olduğunu göstermiştir.

Bu çalışma, çözünür biçimde eksprese edilemeyen bir scFv yapısından, özünde çözünür dizilerden türetilen çerçeve bölgelerine CDR -grafting, bağlanma özelliğinde ve aktivitesinde herhangi bir kayıp olmaksızın çözünür bir scFv'nin geliştirilebileceğini göstermiştir. Moleküler mühendislik yaklaşımı kullanarak, bir scFv antikorunun inklüzyon cisimciği oluşturma problemini çözebilen ve zaman alan optimizasyon prosedürlerine olan ihtiyacı ortadan kaldırabilen umut verici bir stratejidir.



1. INTRODUCTION

1.1 Angiogenesis Inhibition

Angiogenesis refers to the development of non-existing blood vessels from already-existing ones. Angiogenesis occurs in two ways; physiological and pathological. Angiogenesis is a process that begins in the growing embryo and proceeds throughout adulthood to generate blood vessels in the body (Browder et al, 2001). In the normal circulatory system, blood veins are arranged regularly spaced and closely packed. In pathological angiogenesis, the stimulated new blood vessels branch irregularly and are structurally and functionally heterogeneous (Dvorak et al, 2003).

Following the uncontrolled proliferation of tumor cells, a rapid progression of tumors begins, especially with vascularization. Tumor cells can reach up to 2-3 mm in size without the need for new vessel formation in the tissue. These cells need new vascular formation (angiogenesis) to grow further. As a result, cancer cells release a huge amount of angiogenic factors and most of them bind to their receptors on endothelial cells by diffusing into neighboring small vessels and consequently activating mechanisms triggering new vessel formation (Browder et al, 2001; Liu Y et al, 2006).

When it comes to tumor progression, angiogenesis is a critical factor to take into consideration. Ongoing research has been performed to determine the best therapeutic technique for preventing tumor development by inhibiting angiogenesis. The most important molecule in angiogenesis is Vascular Endothelial Growth Factor (VEGF) (Takahashi and Shibuya, 2005). Therefore, the most important target in cancer treatment that can be applied by suppressing angiogenesis is suppression of VEGF and VEGF receptors (Gomez-Manzano et al, 2008). Three major VEGF receptors belonging to the receptor tyrosine kinase group that bind VEGF ligands have been identified; VEGFR-1 (Flt-1), VEGFR-2 (KDR, FLK-1), VEGFR-3 (Flt-4). Particularly VEGFR-2 molecule is mainly responsible for tumor angiogenesis in the signal transduction pathway. Blocking VEGFR-2 is known to reduce cell proliferation, migration, survival and vascular permeability (Figure 1.1).

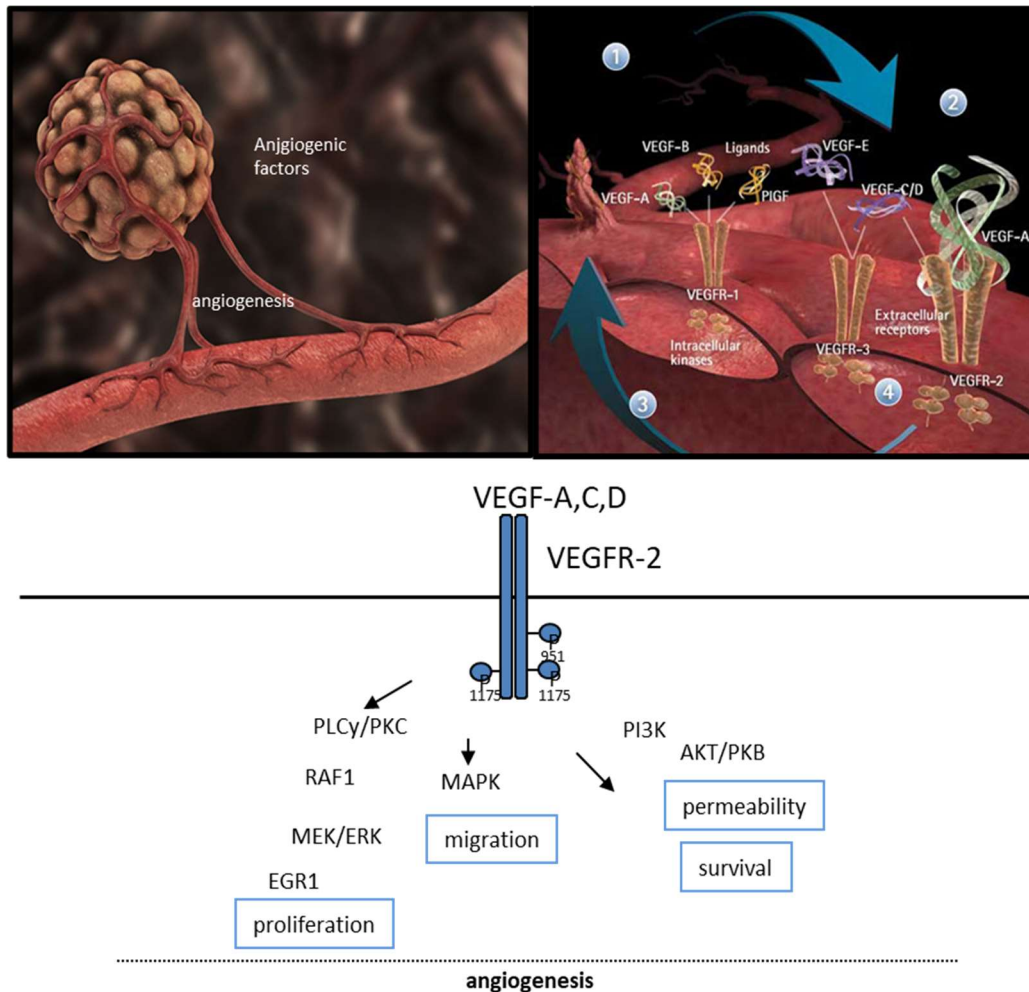


Figure 1.1 : Angiogenesis pathway adapted from Melincovici et al. (2018).

Antibodies against VEGF and VEGFR inhibitors were created and are now frequently utilized therapeutically. Bevacizumab, developed against vascular endothelial growth factor (VEGF) is a recommendation for first-line cure in patients with colorectal cancer (Hurwitz et al, 2005; Monk et al, 2005).

Ziv-aflibercept is a recombinant fusion protein formed by fusion of the Fc fragments of human immunoglobulin G1 with extracellular binding sites of human VEGF receptors 1 and 2 (Tang and Moore, 2013). Binding both VEGF and placental growth factor (PlGF) is a drug used in routine treatment with different combinations of drugs approved by the FDA since August 2012 (Sun and Patel, 2013).

The US Food and Drug Administration (FDA) authorized ramucirumab (Cyramza; Eli Lilly) in 2014 as a human VEGF receptor-2 antagonist to treat individuals with gastric cancer or GEJ adenocarcinoma. Ramucirab is a recombinant human immunoglobulin G1 monoclonal antibody that acts as an angiogenesis inhibitor (Lagassé et al, 2017).

1.2 Therapeutic Proteins

Studies are currently in progress to find proteins or peptides to cure diseases. Non-recombinant protein drugs are as old as modern medicine. Protein-based medicinal products have been used through the years including: blood transfusion, plasma products, hormones, vaccines, and many more. Insulin was the first non-antibody therapeutic protein isolated from animal pancreases and administered in 1922 for diabetes mellitus therapy. Animal-derived insulin's accessibility, price, and immunogenicity limited its use. It took more than 50 years to create the first recombinant protein therapy, humulin (human insulin) in the 1970s (Goeddel et al, 1979).

Proteins are macromolecules made up of chains of amino acids folded into constructions of three dimensions. They are involved in practically every biological function, from receptor signaling and locomotion to biocatalytic processes and cellular regeneration (Leader et al, 2008).

Peptides and proteins are currently the most important biological substances in terms of their clinical usefulness as therapeutics (Akash et al, 2015). Therapeutic proteins are gaining popularity as they are helpful in combating many highly fatal diseases (Hermeling et al, 2004; Akash et al, 2013; Ibrahim et al, 2013).

The development of therapeutic proteins began in the 1970s, when Kohler and Milstein created a method for producing monoclonal antibodies efficiently (mAbs) (Kohler and Milstein, 1975), with the emerging recombinant DNA technologies, PCR, and other molecular biology techniques and ended in the 1990s. Phage display techniques and other technologies advancement, including transgenic animal production, enabled the growth of fully human antibodies (Chames et al, 2009). The first clinically authorized therapeutic mAb, was a murine mAb Muromonab-CD3 (OKT3) produced by hybridoma technology in 1986 (Todd and Brogden, 1989). The proportion of biologic based approvals have been increased through years. The biggest and most widely sold therapeutic proteins at the moment is monoclonal antibodies (mAbs) and antibody derivatives mostly Fc fusion proteins (Dimitrov, 2012).

Protein therapeutics have complex structures that need to be maintained and can not be entirely synthesized by chemical procedures. Therefore, they should be produced in living cells or organisms (Zhu, 2012; Butler et al, 2012; Bandaranayake et al, 2014).

1.3 Antibodies

1.3.1 Structure and function

Antibodies are proteins associated with the immune system known as immunoglobulins. Immunoglobulins (Ig) are glycoproteins generated by plasma cells in reaction to foreign antigenic structures. The main role of antibodies is to bind foreign antigens specifically so that they can be disabled and/or marked for immune destruction (Johnson, 2013).

Immunoglobulins are heterodimeric proteins consisting of two heavy chain (approximately 55 kDa each) and two light chains (approximately 25 kDa each). These chains are linked by disulphide bridges and non-covalent bonds forming a Y-shaped protein of about 150 kDa. Each polypeptide chain can be functionally separated into variable (V) domains and constant (C) domains. Antigen binding is accomplished by variable domains that are unique to each antibody, while effector capabilities are defined by constant domains that do not differ significantly between antibodies. The region of the light chain variable is commonly referred to as VL. The region of the light chain constant is commonly referred to as CL. L chain is composed of either a κ or a λ chain. Surprisingly, a subgroup of antibodies seen in a small number of animals (camelids, nurse sharks) lacks light chains entirely and relies only on the heavy chain for antigen binding (Hamers-Casterman et al, 1993).

For the heavy chain variable (VH) and constant regions (CH), the notation is similar with CH1, CH2, and CH3 denoting the various constant heavy chain region domains. Carbohydrates are connected to the heavy chains' CH2 domains.

The molecule can be split into two parts as characterized by the enzymes papain and pepsin (Porter, 1959; Nisonoff et al, 1960). Two antigen-binding (Fab) fragments and a crystallizable fragment are produced by papain cleavage (Fc) (Jones and Gellert, 2004). The (Fc) region has solely constant heavy chain (HC) regions, whereas the (Fab) region comprises both a constant domain and variable heavy and light chain domains (VH and CL) (Figure 1.2).

Pepsin converts IgG into an Fc and a dimeric F(ab)₂ (Smith et al, 2004). The variable domains are created via a complex process. Four FRs and three CDRs exist within each V domain. A variable fragment (Fv) with VH and VL subdomains and a constant fragment (Fb) with CL and CH1 domains constitute the Fab (Paul, 2012). ScFvs are

made up of only immunoglobulin VH and VL connected together by peptide linkers (Figure 1.2). Their typical molecular weight is around 27 kDa. ScFv structures include the antigen-binding site and are as highly specific as full antibodies (Malm et al, 2016).

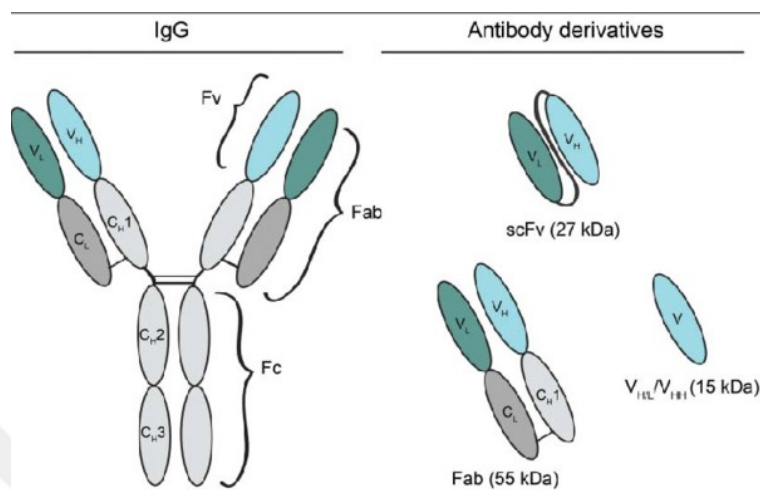


Figure 1.2 : Schematic representation of antibody (IgG) and antibody derivatives (scFv, Fab and VHL/VHH) retrieved from Malm et al, 2016.

Antibodies have five main groups, IgM, IgG, Iga, IgD, and IgE. IgG has become the most significant class of antibodies from the biotechnology point of view (Maynard et al, 2000).

1.3.2 Antibody engineering

Antibody development was principally dependent on animal immunization by the use of experimental mice, rabbits and other associated laboratory animals until the late 1980s (Wang et al, 2015). The use of novel molecular tools that utilized various model organisms as well as new methods of phage display technology, significantly enhanced the expression production of recombinant proteins. Engineered monoclonal antibodies have strong interaction and high specificity for their targets which are typically present in the disease environment.

scFv comprised of VH and VL domains linked by a flexible linker peptide can be produced via genetic engineering technologies (Malm et al, 2016).

Antibody fragments can give certain benefits over a full-size antibody. Antibody fragments are smaller compared to antibodies and frequently absent of glycosylation, saving labour and money. Furthermore, fragments are tiny enough to permeate areas that full-size antibodies cannot (Ahmad et al, 2012).

1.4 Expression of Recombinant Proteins

Traditionally, recombinant protein manufacturing requires transfecting cells with a vector expressing the target protein and then cultivating the cells. Proteins must be produced in a highly concentrated and soluble form in order to be used for biopharmaceutical applications (Esposito and Chatterjee, 2006).

Currently, there are various expression systems. The system to be employed is determined by proteins properties, its function, and the expected productivity. These expression systems are described in table below (Table 1.1). Each method has advantages and disadvantages, and selecting the appropriate system for the particular application is critical for efficient recombinant protein production.

Table 1.1: Different expression systems for recombinant therapeutic protein production.

<i>Expression system</i>	<i>PTMs</i>		<i>Production</i>	
	<i>Disulphide</i>	<i>Glycosylation</i>	<i>Cost of</i>	<i>Safety cost</i>
	<i>bonds</i>		<i>fermentation</i>	
<i>Bacterial</i>	+	x	Low-Moderate	Low
<i>Yeast</i>	+	+	Low	Low
<i>Plant cells</i>	+	+	Moderate	Low
<i>Mammalian cells</i>	+	+	High	High
<i>Animals</i>	+	+	Moderate	High

*Adapted from Dingermann (2008)

The key to successful recombinant protein production is selecting a suitable protein expression system. Numerous parameters must be considered, including the target protein properties, intended use, yield, and cost. Each system has their advantages and disadvantages. Here, two frequently used systems will be explained; bacterial and mammalian expression systems (Lai et al, 2013).

1.5 Bacterial Expression Systems

Bacteria may deliver several advantages for particular protein products, extending from fast accumulation of biomass to cheap scale-up manipulations (Baneyx and Mujacic, 2004).

One of the primary constraints in this expression platform is production of not soluble protein clumps (inclusion bodies, IBs). Bacteria have a significant disadvantage: unlike eukaryotic cells, they lack post translational modifications (PTMs). Plant and insect cell expression systems are also capable of producing proteins with complex patterns of glycosylation; however, the glycan structures produced differ significantly from those developed in humans (Ghaderi et al, 2012).

1.6 Mammalian Expression Systems

Enhanced productivity of mammalian cells, feeding and protein engineering strategies have turned mammalian cells into an outstanding expression system (Hacker et al, 2009). The ability of mammalian cells to guide protein folding, secretion and complicated PTMs makes them ideal choice (Running Deer and Allison, 2004).

In mammalian cells, recombinant proteins can be produced through transduction, transfection, or direct insertion into the host DNA. There are benefits and drawbacks for each of these methods. Typically, viral infection involves the spread of virus in the cells, virus isolation, and viral titer determination. Lipofection and polyfection are the newest and easiest methods for transfecting genes into various cells (Carter, 2011). Polyfection is a gene transfer mediated by cationic polymers and dendrimers, whereas lipofection is a technique for delivering genes into cells via cationic lipids. Electroporation and nucleofection is a quick and easy way of direct delivery of genes into cells (Tihanyi and Nyitray, 2020).

It is possible to produce a mammalian cell expression for both transient and stable. Transient expression systems are for high throughput screening, *in vivo* evaluation and early product analysis, whereas; stable expression systems are ideal for large scale production and characterization studies (Tihanyi and Nyitray, 2020). Protein expression concentrations rise around 48–72 hr after transfection in transiently transfected mammalian cells and decrease subsequently. Due to the fact that DNA is duplicated as an extrachromosomal component, production capability s quickly

diminished, allowing for just a minimal amount of output. Generating stable cell lines involves the incorporation of desired gene into the host cell's DNA (Liao et al, 2006). This results in expression over prolonged times, heterogeneous cell population and various expression levels.

Transfected cells undergo a selection process that begins with restoration of growth after following transfection and continues through single-cell cloning, proliferation, suspension, and serum-free adaption to ultimate clonal selection (Li et al, 2006a). It is a significant challenge to screen and select an efficient and stable clone from the transfectant population.

There are several ways of clone screening, however, the transfected cells are frequently selected by limiting dilution. In the process, cells are sequentially diluted on 96 well-plates, so that, original cell pool will be expanded through many cells. Thus, multiple rounds of serial subcloning will be needed to ensure monoclonality. ClonePix System (Molecular Device) is an automation colony selector tool for quickly scanning huge quantities of clones and detecting best expressing clones. Fluorescence-Activated Cell Sorting (FACS)-Based Screening can also be used for the selection of high producing clones.

The mostly preferred mammalian hosts for synthesis of recombinant proteins are baby hamster kidney cells (BHK21), murine myeloma cells (NS0, SP2/0), chinese hamster ovary (CHO) and human embryonic kidney (HEK293) cells. The Chinese ovarian hamster cell line CHO is an excellent expression system accepted as safe by regulatory authorities for contagious and pathogenic organisms (Hamdi et al, 2020).

Antibiotic selection has been used for a couple of years, but because of its costs and the necessity for antibiotic removal from the media, oriented the industry to metabolic selection. Different mammalian cell platforms are widely used by the pharmaceutical industry for expression of diagnostic and therapeutic molecules. Presently, the majority of pharmaceutical manufacturers choose cell lines using the dihydrofolate reductase (DHFR) or glutamine synthetase (GS) systems. Cells are grown under selection and following, limiting dilution or subcloning is performed to produce monoclonal cell lines.

Protein expression in mammalian cells is typically done by strong, constitutive promoters. The two most commonly used components were generated from Simian

virus 40 (SV40) and Cytomegalovirus (CMV) (Boshart et al, 1985; Carswell et al, 1989). For maintaining and strengthening transcripts various components can be used. The incorporation of a Shine-Dalgarno or Kozak sequence is considered as a significant factor to improve the translation initiation. The Kozak sequence is the ideal consensus sequence to initiate and facilitate the translation of mRNA (Kozak, 1986). Epitope tags are typically used for simple detection of the gene or fast purification of the protein. Epitope tags can be either in recombinant protein's N-terminus or C-terminus. To generate a viable cell line suitable for mammalian expression, an additional sequence encoding the desired gene is used. Post-translational modification (PTM) is the process of adding polyadenylation and secretory signaling motifs to recombinant proteins in order to control their localization (Khan, 2013).

In mammalian cells, complete antibodies with heavy and light chains can be co-transfected using independent vectors. However, this is a limited strategy and there is higher work load and chemical required. Instead, vectors with IRES elements are used to solve this problem. The IRES element allows the transcription of both genes into a single mRNA by same promoter. The IRES on the transcript helps the downstream gene to initiate the 5' cap-independent translation, while the upstream gene's transcription initiation is 5' cap-dependent. Therefore, a single mRNA is able generate 2 different proteins (Baird et al, 2006). By incorporating the selection marker as a descending gene in the vector, the transcription of the selection marker gene is adjusted conditional on the active transcription of the upstream target gene, so improving the efficiency of the selection procedure (Mancia et al, 2004; Ho et al, 2012).

1.7 Purpose of Thesis

Our previous studies led the discovery of two scFv molecules developed against VEGFR-2 that are already patented through Japan and USA under name of anti-KDR 1.3 and anti-KDR 2.6. The aim of this thesis was to solubly produce the target recombinant antibody structures developed against VEGFR-2 which is normally produced as inclusion bodies in bacterial cells. Then to evaluate the soluble scFv structure and its biological activity. Two different strategies have been used to solubilize inclusion body forming recombinant structures; first to develop cloning and

production methods in mammalian cell lines and the second was to develop a CDR – grafting approach for producing scFv in the soluble form in bacteria.



2. CHAPTER I - PROTEIN PRODUCTION IN MAMMALIAN CELLS

2.1 Purpose of the Chapter

The aim of this chapter is to produce the target recombinant antibody structures (scFv) developed against VEGFR-2 in mammalian cells which is normally produced in inclusion bodies in bacterial cells. Then to evaluate the biological activity and structure obtained by mammalian cells.

For this study, existing scFv genes will be modified to be optimally expressed in mammalian cells. Genes will be cloned into mammalian expression vectors and transferred into competent *E. coli* cells by transformation. Then, the cloned vectors will be determined by colony PCR. The DNA sequences of the newly cloned scFv constructs will be controlled by a capillary electrophoresis system. The resulting vectors will be purified and transfected into mammalian cells. New scFv constructs will be produced in mammalian cells and purification will be made with chromatography columns. CHO-K1 and Hek293 cells will be used as the mammalian expression system for this study. The resulting antibody structures will be analyzed by SDS-PAGE and Western blot methods. The binding of the samples to VEGFR-2 will then be checked by ELISA. The activity and structure of the proteins will be analyzed after they were produced and purified. HPLC-based chromatographic techniques will be used to analyze purified antibodies (High Performance Liquid Chromatography). The presence of m-RNA in cells will be investigated using the real-time PCR technique. In addition to these investigations, Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS) will be used to determine the structural dynamics of the anti-KDR 1.3 protein produced from bacteria.

2.2 Literature Review

2.2.1 Optimization of protein production in mammalian cells

Numerous approaches for improving recombinant protein expression in mammalian cells have been explored. This can be accomplished through proper vector design. It

involves the use of powerful promoters, appropriate signal peptides, and codon optimization (Jalah et al, 2007). In order to attain elevated concentrations of production, plasmids generally possess powerful promoters like cytomegalovirus (CMV) to obtain maximum RNA messenger (Running et al, 2004). It has been demonstrated that the use of additional signal peptides results in greater amount of secreting molecules (Knappskog et al, 2007; Kober et al, 2013). The proteins are transferred through the ER and Golgi apparatus following translocation and cleavage of their signal peptides and subjected to assembly, folding and post-translational modifications among this passage. Eventually, the modified proteins are bundled into secretory vesicles and exported through the plasma membrane by merging.

The length of residues of mammalian signal peptides varies mostly from 15 to 30 amino acids (Johnson, 2013). Signal peptides comprise of a polar N-terminal region (n-region), a hydrophobic core (h-region) and a C-terminal region (c-region). Signal peptides lack a well-defined consensus sequence and exhibit high variability in terms of size and content.

The hydrophobic core of a signal peptide is critical because the SRP connects this area and controls the translation inhibition needed for preventing the release of synthesized proteins into cytosol. The increase in positive charges in n-region also related to improved secretion of protein (Liu et al, 2018). Translocation efficiency is dramatically lowered by introducing non-hydrophobic residues in the h-region (Rapoport, 1986). For expressing a desired protein in a specified cell, signal peptide from other species may also be used. Human albumin, azurocidin, cystatin signal peptides were shown to be quite strong (Kober et al, 2013).

Optimizing DNA sequence often involves removing repeated sequences, undesired structures like stem-loops and splice sites, AT or GC rich content, ribosomal entry sites and optimizing the use of tRNA (tRNA) in a specified organism, all for optimizing translation and mRNA stability (mRNA) (Presnyak et al, 2015). Even though the final protein transcribed by an optimized DNA sequence has the similar amino acid composition, the changes introduced may alter the proper protein folding, solubility and activity by altering the dynamics of protein translation.

Cell culture process optimization is considered as cell line-dependent and based on a particular cell line's need. In order to optimize a feeding approach, nutrient

consumption, accumulation of by-products and the equilibrium between development and manufacturing should be considered. Additives can be used to supplement growth media. Growth factors and feed solutions have been proven to raise yields and growth times by adding vital elements. Cell growth and protein yields can also be enhanced through regulated growth settings such as temperature. A basic and very successful method of controlling cell proliferation is to lower temperature. Mammalian cells cultivated in low temperature have been demonstrated to exhibit reduced growth rate, increase in specific productivity and maintain high viability (Reuveny et al, 1993; Kaufmann et al, 1999; Yoon et al, 2003).

MAbs are typically developed on a large scale in bioreactors with suspension cells as they allow improved cell densities. Due to its efficiency in upstream/downstream processing, serum-free suspension cells are ideally necessary for recombinant protein production. Chemically defined serum-free media provide reliability, lower production costs, decrease immunogenicity and free from blood-borne pathogens (Costa et al, 2013). There are different approaches for serum-free media adaptation including a) direct adaptation b) sequential adaptation c) reduction of serum content (Caron et al, 2018).

2.3 Materials and Methods

2.3.1 Molecular cloning

For molecular cloning studies, both standard restriction digest and ligation cloning and In-Fusion® HD Cloning Kit (Clontech, 011614) were used. For the traditional system, insertion of the target DNA sequence into the chosen vector was accomplished by “cut and paste” method using indicated restriction enzymes determined for each vector. The reactions were prepared according to the instruction’s manual. The gene of interest and ligated vector were isolated and purified from both digestions using gel electrophoresis. The plasmid and insert were then ligated together and transformed into *E. coli*. For the In Fusion system, In-Fusion enzyme recognizing 15 bp overlapping sequence at both 5’ and 3’ ends of the PCR product and linearized vector was used to re-combine them. Overlapping sequences were generated with the addition of nucleotide sequences to the 5’ end of each primer designed for the amplification of target sequence. The target sequence was amplified with extra sequences at both ends that were homologous to the linearized plasmid ends using PCR. The linearized vector

pGFP1 is IRES-containing bicistronic vector (Figure 2.1) that express together with the fluorescent protein in mammalian cells. NheI and SmaI sites were used for both the vector and the scFv insert region. Interleukin-2 (from pFUSE-hIgG1-Fc2 vector), Kozak sequence, Histidine tail were added to the N terminal region.

Different plasmids with different plasmid designs were used for cloning studies. Plasmids used in the thesis study can be listed as; pIRES2-AcGFP1 (Takarabio), pUC57 (Genscript) (cloning vector), pcDNA3.1+ (Genscript), pDream2.1/MCS (GenScript), pET-42a (+) (Novagen), pmaxGFP plasmid (AMAXA Biosystems, Gaithersburg, MD) (control vector).

pGFP1 is IRES-containing bicistronic vector (Figure 2.1) that express together with the fluorescent protein in mammalian cells. NheI and SmaI sites were used for both the vector and the scFv insert region. Interleukin-2 (from pFUSE-hIgG1-Fc2 vector), Kozak sequence, Histidine tail were added to the N terminal region.

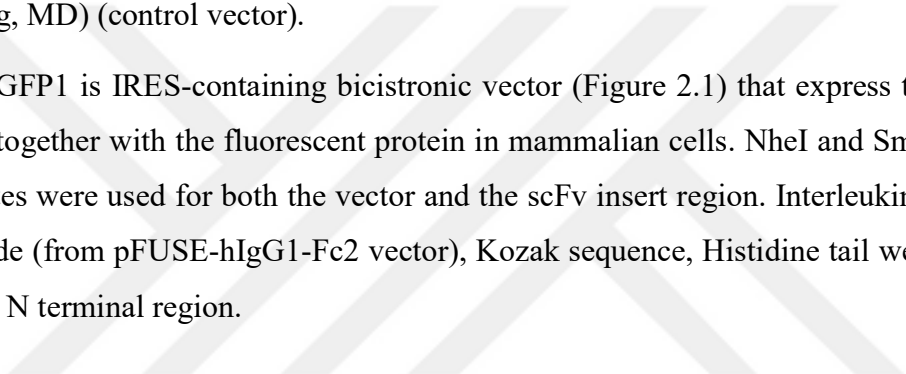


Figure 2.1 : pIRES-AcGFP1 expression plasmid.

PIRES2-AcGFP1-Kozak_NHis-anti-KDR 2.6 and PIRE2-AcGFP1-Kozak_NHis-anti-KDR 2.6 constructs were designed for the anti-KDR 2.6

Following that, a hairpin structure was noted in the results produced by employing the optimized sequence (<https://eu.idtdna.com/site/order/geneentry>), and in order to rectify this structure, the bases in the repetition areas were organized in such a manner that they would not modify the sequence. A repeat in the sequence at the following locations: 387,372 has been modified to reduce the length of these repeats to less than 14 bases. The sequence that contains a window of 100 bases starting at base 363 with GC content of 81% has been modified to have less than 69%. Optimized genes were obtained in pUC57 cloning vector from Genscript (Figure 2.2).



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from the protein. A different signal peptide "modified serum albumin, MSA" (MKWVTFISLLFLFSSSSRA) was added to N terminal region (Attallah et al, 2017).

Additionally, the anti-KDR 2.6 ScFv gene structure has been codon optimized by the Genscript Company and named as anti_KDR 2.6_Opt-I. The native gene employs tandem rare codons, which can impair translation efficiency or possibly deactivate the translational machinery. By lowering the CAI from 1.00 to 0.94, I were able to reduce the codon use bias in *Cricetulus griseus* (CHO).

To extend the half-life of the mRNA, the GC content and unfavorable peaks have been modified. The Stem-Loop structures, which influence ribosomal binding and mRNA stability, were disrupted. Furthermore, negative cis-acting regions were modified in order to avoid alternative transcription start sites.



Figure 2.3 : pcDNA3.1 expression plasmid.

pDream2.1/MCS (GenScript) is an expression vector (Figure 2.4) in which three major protein expression systems: Bacteria, Insect cells and Mammalian cells can be used. pDream2.1/MCS (GenScript) vector is used for both bacterial expression and mammalian expression studies. Two restriction digestion sites were used for linerization of the vector which is BamHI and NotI.

2.3.3 Plasmid amplification and purification

Plasmid DNA were extracted from overnight cultures. The ZymoPure Plasmid Mini and Midi Kits were used to purify plasmid DNA on a small and large scale, according to manufacturer's instructions.

2.3.4 Polymerase chain reaction (PCR)

Unless otherwise mentioned, PCR amplification reactions were performed using Taq polymerase. The primers used in the PCR reactions are given in the table below; Table 2.1.

Table 2.1 : Primers used for cloning reactions.

	5'-3' Sequence
KDR 1.3 _pDream 2.1_For	CGACGATAAGGGATCATGGCCCAGGTGAAGCTG
KDR 1.3 _pDream 2.1_Rev	ACACTATAGGCGGCCTCAGTGATGGTGATGGTGATGTG
KDR 1.3 _pET 42a_For	GGTGGTGGTGCTCGAATGGCCCAGGTGAAGCTGC
KDR 1.3 _pET 42a_Rev	GGACGCGGGTCCATGTCATGCGGCCGCCCCGTTTCAGC
Forward KDR2.6 pIRES	TTATGCTAGCATGGCCCAGGTCAAAGCTGC
Reverse KDR2.6 pIRES	TTGACCCGGGCTAGTCATTTTATTTCCAGCTTGGTCCCAG
Forward KDR1.3 pIRES	TTATGCTAGCATGGCCCAGGTGAAGCTGC
Reverse KDR1.3 pIRES	TTGACCCGGGCTAGTCACGCCCGTTTCAGCTCCAGC
For_pcDNA 3.1	CTAGAGAACCCACTGCTTAC
Rev_pcDNA 3.1	GCTGGCAACTAGAAGGCACA
For_HindIII_infusion_opt1	GTTTAAACTTAAGCTTCGCCACCATGAAATGGGTAC
Rev_XhoI_infusion_opt1	GCCCTCTAGACTCGAGTTATTTGATTTCAGCTTTGTTCCG
For_HindIII_infusion_opt2	GTTTAAACTTAAGCTTTGCCACCATGAAGTGGGT
Rev_XhoI_infusion_opt2	GCCCTCTAGACTCGAGCTATTTGATTTCAGTTTAGTTCCA
For HindIII KDR2.6_optI	TTATAAGCTTCGCCACCATGAAATGGGTCA
Rev XhoI KDR2.6_optI	ATAACTCGAGTTATTTGATTTCAGCTTT
For HindIII KDR2.6_optII	TTATAAGCTTTGCCACCATGAAGTGGGTT
Rev XhoI KDR2.6_optII	ATAACTCGAGCTATTTGATTTCAGTTT
CMV-For-out	CGCAAATGGGCGGTAGGCGTG
CMV-F1-out	CAACTCCGCCCCATTGACGC
IRES2-R1-out	ACATATAGACAAACGCACACCGG
IRES2-R2-out	AAGACGGCAATATGGTGG

The reaction mixture contained 10X PCR Buffer, 10 mM dNTPs, 10 μ M forward and reverse primers, 100-200 ng DNA template, 1 unit Polymerase and nuclease free dH₂O. Reaction parameters were 99°C for 30 sec for initiation, 30 cycles; 50-60°C for 20-45 sec (depending on primers), 30 sec per kb of DNA extension, with a final extension of 5 mins at 72°C and an infinite stay at 4°C.

2.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using 50 or 100 mL. 1-1.5 % Agarose (Sigma Aldrich) was dissolved in 1X TAE Buffer. The solution was heated in a microwave until it was completely dissolved before the addition of 2.5-5 μ L of RedSafe DNA stain (Chembio). Solution was then poured into the Bio-Rad gel system to solidify and a plastic comb was inserted. Gels were transferred into the tank (Bio-Rad) and filled with same TAE buffer. DNA samples were mixed with 6x Loading Dye (NEB) and loaded into the wells along with a DNA size standard. Gels were run on 80 Volt 40 min in the gel electrophoresis system (Bio-Rad). Gels were visualized under UV light with the Azure c600 Imaging system (Azure Biosystems). Desired DNA segments were excised with surgical blades if necessary, extracted and purified.

2.3.6 Restriction enzyme digestion

Restriction enzyme digestion for cloning studies was performed by using the enzymes of New England Biolabs (NEB, USA). Enzyme reactions were conducted using the buffers and conditions as recommended in the manufacturer's instructions.

2.3.7 Transformation of bacteria

For the standard restriction digest and ligation cloning, *E. coli DH5 α* cell line was preferred with CaCl₂ transformation. To prepare competent cells, one of the bacterial colonies was selected and seeded into 5 ml of LB medium. Cells were incubated at 37°C at 220 rpm overnight. Cells cultured overnight were transferred to 40 ml LB medium. Cells were grown at 37°C 220 rpm until the OD₆₀₀ value was 0.5. At OD₆₀₀ 0.5, the culture flask was kept on ice for 10 min. The supernatant was then removed by centrifugation at 3000 rpm for 10 min at 4°C. The pellet was thawed on ice with 20 ml of 50 mM CaCl₂ solution. After 30 min on ice, it was centrifuged for 10 min at 3000 rpm at 4°C. The supernatant was discarded and the pellet was redissolved with 4 ml of 50 mM CaCl₂ solution. Cells were ready for transformation at this stage.

Ligation mixtures were completed to 50 µl with sterile 50 mM Tris pH: 7.2 and transferred to sterile glass tubes. 300 µl of *E. coli DH5α* competent cells were added to the ligation mixture and mixed gently and incubated on ice for 40-60 min. The tubes were then incubated at 42°C for 3 min and then at room temperature for 10 min. 1 ml of LB medium was then added to the tubes and shaken at 37°C 220 rpm for 60 min. Then 200 µl of each mixture was spread on LB / Antibiotic plate and allowed to incubate overnight at 37°C. The remaining transformation mixture was transferred to 5 ml of liquid LB / Antibiotic medium and shaken at 37°C 220 rpm overnight. The next day, colony PCR was performed to check whether colonies in the transformation plates contained the target gene and the results were checked on agarose gel.

In-Fusion HD cloning procedure was followed according to manufacturer's instructions. 2.5 µl of the In-Fusion reaction mixture was added on the 50 µl of Stellar competent cells. 1/5th of each transformation reaction was placed in separate tubes and completed to 100 µl with SOC medium. The reactions were spread on LB plate containing the appropriate antibiotic and incubated overnight at 37°C for colony formation. The next day, individual colonies were analyzed for target DNA by colony PCR screening.

2.3.8 Bacterial stock preparation

Once overnight bacterial culture was ready, same amount from the bacterial stock and 60% glycerol was mixed on ice carefully, finally, obtaining a 30% glycerol stock. Glycerol stocks were immediately transferred to -80°C and were thawed on ice when needed.

2.3.9 Colony PCR

15 µL of distilled water was prepared in 0.5 ml eppendorf tubes. 5 ml LB/Antibiotic tubes were also prepared for each pcr sample. Colonies grown in petri dishes containing LB/Antibiotic were enumerated and by touching the tip of the loop and dip and mixed in distilled water. The remaining 5 ml of LB/Antibiotic were then placed in culture tubes. The culture tubes were allowed to shake at 37°C 220 rpm.

Bacteria were incubated at 95°C for 3 min and exploded cells were centrifuged and supernatant was transferred as the DNA template into a fresh tube. PCR reaction was performed using this as the DNA template.

PCR amplification reactions were performed using 2.5 µl 10X PCR Buffer, 0.5 µl 10 mM dNTPs, 2 µl 10 µM forward and reverse primers, 2 µl DNA template, 0.5 µl 1 unit Polymerase and 17.5 µl nuclease free dH₂O. Reaction conditions were 95°C for 5 min for initiation, 30 cycles; 94°C for 1 min, 50-60°C for 2 min, 72°C for 2 min, and a final extension of 10 min at 72°C and an infinite stay at 4°C. Colony PCR results were checked on 1.5 % agarose gel.

2.3.10 DNA sequencing for confirmation of target gene insertion

DNA sequencing were performed with inner and outer primers to control the target gene regions and to ensure that they were correctly aligned on the vector. Sequencing reactions were performed with the Beckman Coulter CEQ8800 Sequence Analyzer System using the CEQ DTCS Quick Start Kit. The sequencing reactions were performed according to the manufacturer's instructions, as shown in Table 2.2.

Table 2.2 : Sequencing reaction using DTCS Quick Start Kit.

PCR Reaction	
dH ₂ O	9 µl
DNA template (100-150 ng)	1 µl
Primers (1.6 pmol)	2 µl
DTCS Quick Start Master Mix	8 µl
Total	20 µl

After the sequencing reaction, an ethanol precipitation was performed. Sterile 0.5 mL microcentrifuge tubes were labeled for each sample. To each labeled tube, 5µL Stop Solution (equal volumes of 3M NaOAc pH 5.2 + 100mM EDTA pH 8.0, 1µL of 20mg/mL glycogen) were added. Immediately after, 60 µL cold 95% ethanol were added to the mix and stirred thoroughly. The mix were centrifuged immediately at 14,000 rpm at 4°C for 15 min. The supernatant was removed with a micropipette carefully. The pellet was washed twice with 200 µL 70% cold ethanol and centrifuged for a minimum of 2 min at 14,000 rpm and 4°C. The supernatant was removed using a micropipette after centrifugation. The samples were vacuum dried for 10 min. and then resuspended by 40 µl of sample loading solution (SLS). Samples were then carefully pipetted to the sequencing plate and one drop of mineral oil was added on top of each sample. The buffer plate was prepared for the same sample numbers. Then

the plates were loaded on the CEQ 8800 Genetic Analysis System using the run method LFR-a. After the sequence was completed, the raw data were analyzed by sequencing module of CEQ8800 software.

After data were collected and analyzed, the chromatograms were further assembled and checked by using the ChromasPro tool and a consensus sequence was obtained. The sequences were then aligned with the BioEdit tool, and compared with the original gene sequences.

2.3.11 Cell culture maintenance, storage and recovery

2.3.11.1 Adherent cells and maintenance

Critelus griseus, Chinese Hamster Ovary (CHO-K1) cells obtained from ATCC (CCL-61) was used. Cells were stored in liquid nitrogen with complete growth medium and 5% DMSO at a 1×10^6 cell density. Cells were thawed in 37°C water bath and suspended in a prewarmed F-12 Ham Media with 10% fetal bovine serum (FBS). Cells were then centrifuged at 1300 rpm for 5 min and excess medium aspirated by gently pipetting. Cells were resuspended by appropriate amount of complete media (5 mL) and transferred to a T25 tissue culture flask. Cells were incubated in 37°C and 5% CO₂.

For cell maintenance, cells were subcultured every 2-3 days. The medium was aspirated from the cell culture flask and 2-3 mL PBS was added and gently mixed. PBS was withdrawn, 2 mL trypsin was applied, and the mixture was incubated at 37°C for 5 min. Detached cells were neutralized with media and transferred to a falcon tube. Cells were centrifuged at 1.3 rpm 5 min. Supernatant was aspirated and cells were resuspended and seeded in a fresh medium.

Human Embryonic Kidney 293 (Hek 293) cells were maintained in Dulbecco's modified Eagles/F12 medium supplemented with 10% fetal bovine serum. Cells were incubated in 5% CO₂ humidified air at 37°C incubator. Cells were split upon reaching 80% (2-3 days) confluency using 0.25% Trypsin-EDTA.

2.3.11.2 Serum free and suspension adaptation

For serum free adaptation, reduction of serum content method was followed and cells were cultivated in lower concentration of FBS for at least two successful passages. Cells were transferred to Excell CD CHO medium (Merck) with supplementation

(Cholesterol, MEM NEA, Glucose) at 1% FBS, and then serum levels were gradually reduced. Cells stopped adhering at 0.5-1 % and the adaptation was continued in the suspension (Figure 2.6).

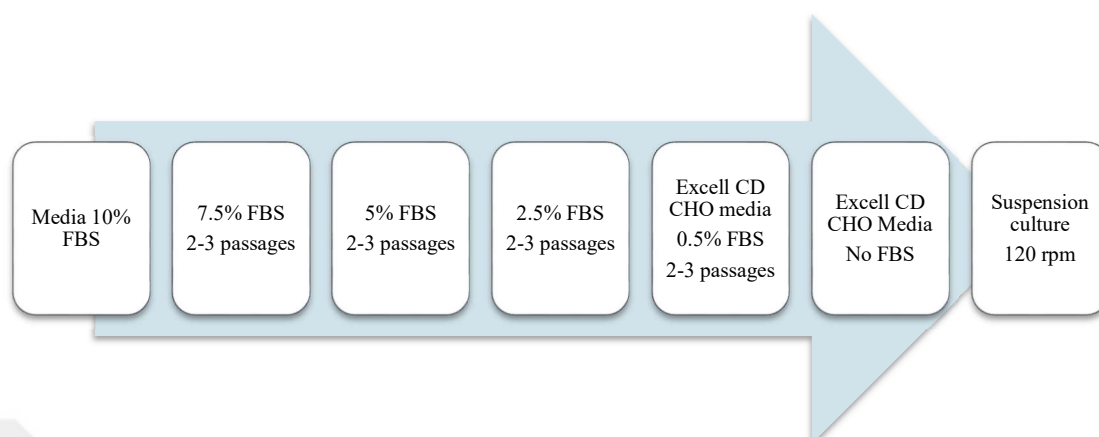


Figure 2.6 : Serum free adaptation steps by reduction of serum content.

2.3.12 Suspension cells

Serum free and suspension adapted CHO-K1 cells obtained from ATCC (CCL-61) was used and named CHO-K1S afterwards. Cells were stored in liquid nitrogen with complete growth medium and 10% DMSO at a 2×10^6 cell density. Cells were thawed in 37°C water bath and suspended in a prewarmed Excell CD CHO Media (Merck). Cells were then centrifuged at 800 rpm for 5 min and excess medium aspirated by gently pipetting. Cells were resuspended by appropriate amount of media (20 mL) and transferred to 125 mL shake flask. Cells were incubated in 37°C, 120-130 rpm, 5% CO₂.

For cell maintenance, cells were subcultured every 2-3 days. Cells were pelleted by centrifugation at 800 rpm for 5 min and excess medium aspirated by gently pipetting. Cells were resuspended and seeded in a fresh medium.

2.3.13 Transient transfection

First of all, transfection efficiency experiments were carried out with a 96-well plate. Lipofectamine 3000, Lipofectamine 2000, Turbofect, iN-fect chemicals were used. Different amounts of chemicals and different amounts of insert DNA were tried (100 ng, 175 ng, 250 ng DNA Lipo 3000, Lipo 2000, turbofect; 50 ng, 100 ng, 200 ng DNA iN-fect). The different chemical values studied are shown in the Table 2.3.

Table 2.3 : Transfection efficiency reagents and plasmid concentration optimization.

lipofectamine3000 /DNA (ng)	lipo3000 / rgnt (μl)	rgnt (μl)	rgnt (μl)
100	0.15	0.2	0.3
100	0.15	0.2	0.3
100	0.15	0.2	0.3
175	0.15	0.2	0.3
175	0.15	0.2	0.3
175	0.15	0.2	0.3
250	0.15	0.2	0.3
250	0.15	0.2	0.3
250	0.15	0.2	0.3
lipofectamine2000/DNA (ng)	lipo2000/ rgnt (μl)	rgnt (μl)	rgnt (μl)
100	0.2	0.3	0.5
100	0.2	0.3	0.5
100	0.2	0.3	0.5
175	0.2	0.3	0.5
175	0.2	0.3	0.5
175	0.2	0.3	0.5
250	0.2	0.3	0.5
250	0.2	0.3	0.5
250	0.2	0.3	0.5
turbofect/DNA (ng)	turbofect/rgnt (μl)	rgnt (μl)	rgnt (μl)
100	0.3	0.4	0.6
100	0.3	0.4	0.6
100	0.3	0.4	0.6
200	0.3	0.4	0.6
200	0.3	0.4	0.6
200	0.3	0.4	0.6
300	0.3	0.4	0.6
300	0.3	0.4	0.6
300	0.3	0.4	0.6

All the constructs (7 constructs explained in detail) were transfected to the CHO-K1, CHO-K1S, and Hek293 cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were seeded 0.7×10^6 cells/well into 6well plates. The next day, cells that were confluent were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) chemicals.

According to the obtained results; using the manufacturer's instructions, 2 independent tubes were prepared. Diluted lipofectamine: Opti-MEM reduced serum medium (Invitrogen) 150 μl mixed with 6 μl lipofectamine 2000 reagent and Diluted DNA:

150 μ l Opti-MEM medium mixed with 2 μ g DNA. The two solutions prepared were mixed gently in a 1:1 ratio and incubated for 10 min to allow the DNA-Lipofectamine 2000 complexes to form. After incubation, 250 μ l was added to the wells. DNA was not added to the control sample. The results were visualized with a fluorescent microscope / Biotek Cytation 5 device. The cell culture media was then collected for protein determination. The media was centrifuged at 4000 g for 5 min at 4°C. And the supernatants were stored at -20°C for further use. In addition, for the pcDNA 3.1_opt I_anti-KDR2.6 construct double transfection was performed in order to observe any positive results.

Transfections via 4-D Nuclofactor (Lonza) device has been performed additionally. 1×10^6 cells were resuspended in 100 μ l of transfection buffer for the nucleofection studies, as indicated in the procedure. Positive control was the pMaxGFP plasmid (AMAXA Biosystems, Gaithersburg, MD), which included an enhanced green fluorescent protein (EGFP) gene with a cytomegalovirus (CMV) enhancer/promoter (2 g/transfection sample). A particular preset software was used to nucleoporate the cell/DNA mixes in 1 cm transfection cuvettes. After electroporation, the cells were pre-heated to 37°C for 10 min in their respective culture media before being cultured in cell type-specific growth medium on a 6 well plate.

2.3.14 Stable transfection

Antibiotic killing experiments were conducted using G418 antibiotics to establish a stable cell line. In the experiment, cells were added to the 24 well plates at a concentration of 3×10^5 cells/mL. The next day, the media on the samples were removed and the media was replaced with different concentrations (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 1000 and 2000 g/mL, can be seen in Figure 2.7) of antibiotics, and the cell growth was observed by Clone Image Selection device.

	1	2	3	4	5	6
A	0	0	50	50	100	100
B	200	200	300	300	400	400
C	500	500	600	600	700	700
D	800	800	1000	1000	2000	2000

Figure 2.7 : G418 concentrations on a 24 well plate for antibiotic killing curve.

2.3.15 Feeding strategy, temperature shift and fed-batch experiments

In the fed-batch culture studies the cells were cultivated at 37°C for the first days. On day 5-7, culture temperature was reduced to 31°C and cultures were terminated in the 12-16 day. The cell cultures were fed with Feeding Media as stated in the Table 3.1. Every day, samples were taken and biochemical parameters were determined. Afterwards, media was harvested by centrifugation and cell pellets were lysed with RIPA buffer (Sigma Aldrich) according to the manufacturer's instructions and samples were analysed for target protein expression.

Initially (Set 1), cells seeded in T175 plates were observed for their survival without supplementation. The experiment was terminated on the 9th day.

For determining the feeding strategy, different concentration of cell boost 7a & 7b supplements were added to the cells (Set 2) grown in 6 well plates. Cells were counted with Biotek Cytation 5 device. The experiment was terminated on the 12th day. The same set of experiment was also investigated for temperature shift which includes transferring the cell culture to 31°C at 5th day.

After the nutrient concentration was determined (Set 3), 2×10^6 cells were seeded in T175 cell growth plates. Cells were fed with Cell boost 7a & 7b supplements between 3-4 day 1.5%, between 5-15 day was 2%. For monitoring of the key parameters such as pH, glucose and lactate MetaFLEX Bioanalyte Analyzer (Beckman) device was used. On the 5th day, the samples were taken to 31°C.

After the feed concentration was determined (Set 4), 2×10^6 cells were seeded in 4 T175 cell growth plates. Feedings with Cell boost 7a & 7b supplements were made from 1.5% on the 3rd day. Measurements were made with Metaflex. On the 5th day, one of the samples was taken to 35°C and one set was left at 37°C. The experiment was terminated on the 16th day.

2.3.16 Monoclonal cell development

In this thesis, manual limiting dilution was employed for generating a monoclonal cell line. After obtaining stable cell lines with antibiotics selection, two 100 mm petri dishes were inoculated with a cell concentration of 0.5×10^4 cells/plate.

Colony formation was observed day by day. After colony formation, colonies with GFP were marked under fluorescent microscope. Sterilized and cut pipette tips were

pressed into vaseline and inserted on marked colonies. 50 µl of trypsin was added into the rings. 500 µl of media was added to 24 well plates. After the cells were detached, the selected colonies were seeded on the 24 cell plates.

Colony formation was observed by a GFP microscope in the following days. Fluorescently labeled cell colonies were removed and counted and their viability were checked. Dilutions were made at a concentration of 1 cell/mL, 0.1 cells/mL, and 0.2 cells/mL and inoculated into 24 well plates.

After seeding with the lowest densities for single clone formation, the plates were scanned with Clone Select Imager (Molecular Device) for 10 days, twice a day for the first 4 days, and every other day in the following days. Monoclonal cells were analyzed and determined with the same instrument.

2.3.17 scFv production and purification of cells

Preliminary purification studies were performed using the culture medium of the polyclonal anti-KDR 2.6 transfected cell population. Protein was purified using 1 mL His tag column in AKTA pure FPLC. Na-phosphate buffer pH 7.4 containing 150 mM NaCl was used as mobile phase. Na-acetate pH 3.6 buffer was used in the elution step.

2.3.18 SDS-PAGE

To evaluate protein expression, SDS-PAGE analysis was performed. SDS-PAGE gels were prepared according to the Table 2.4. After polymerization isopropanol was removed the comb was placed for well formation.

Table 2.4 : SDS-PAGE gel preparation.

Reagent	Seperation Gel (12%)	Stacking Gel (4%)
dH ₂ O	5.25 mL	3076 µl
%30 Acrylamide, 0.8 % Bis-Acrylamide	6 mL	670 µl
4x 1.5 M Tris-Hcl (Ph:8.8); 0.4 % SDS	3.75 mL	-
4x 1.5 M Tris-Hcl (Ph:6.8); 0.4 % SDS	-	1225 µl
%10 Ammonium Persulfate	100 µl	40 µl
Temed	10 µl	4 µl

After the gel was completely polymerized, the comb was removed, placed in the electrophoresis tank and filled with running buffer. The samples were denaturized at 95°C for 5 min (8 µl loading buffer (SDS, β-mercaptoethanol, bromophenol blue), 24 µl sample). The samples (12 µl) were loaded on a 12% polyacrylamide gel. Electrophoresis was initiated by applying 100 volts to the system for 10 min, then increased to 120 volts and carried out for 50 min. The proteins were observed with Coomassie Brilliant Blue (Applichem) staining. The gels were gently agitated in the staining solution (0.1 percent Coomassie Brilliant-Blue R250, 25% isopropilic alcohol, and 10% acetic acid) for 30 min. To remove the excess Coomassie, the gels were washed with 30% ethanol and 10% acetic acid destaining solution.

2.3.19 Western Blot

The SDS-PAGE gel prepared was transferred to nitrocellulose membrane by electroblotting under electric field. The gel was sandwiched between two electrodes and 3MM filter paper wetted with transfer buffer. The transfer was carried out at 15 volts in 30 min by Trans-Blot Turbo transfer system (BioRad).

After transfer, the nitrocellulose membrane was blocked by BSA (0.1% Tween 20) for 1.5 h. and incubated with diluted antibody in blocking solution for 2 h at room temperature. Membrane was washed 3 times with TPBS for 5 min. Color formation was observed by adding 4C1N (4-Chloro-1-naphthol) substrate solution to the antibody labeled with horse radish peroxidase (HRP). In chemiluminescence imaging samples, 1/1 working solution was prepared and 1 mL was poured onto the membrane and incubated for 1 min. Western blot images were then recorded using the Azure c600 Imaging system (Azure Biosystems).

2.3.20 Enzyme linked immunosorbent assays (ELISA)

96 well plate was coated with 50 µl of coating buffer containing VEGFR-2 antigen (250 ng per well overnight at + 4°C. After washing with TPBS 8 times, 100 µl of blocking buffer (0.5 % BSA) was added to the wells and allowed to stand at room temperature for at least 2 hr. After washing the wells 8 times with TPBS, 50 µl controls or samples to be analyzed were added to each well and incubated for 2 h. After washing the wells 8 times with TPBS, antibody was added in 50 µl blocking buffer at the dilution as recommended in the protocol. After 1 hr incubation at room temperature, the wells were washed 8 times with TPBS and 50 µl of pNPP (alkaline phosphatase) /

ABTS (horse radish peroxidase) substrate solution was added to each well to produce a color reaction. After incubation for half an hour at room temperature, absorbance values at 405 nm (pNPP) -410 nm (ABTS) were determined in the ELISA reader (Bio-Tech EIA Reader).

2.3.21 High pressure liquid chromatography (HPLC)

The HPLC equipment was used to perform the Size Exclusion Chromatography (SEC) examination of the materials. It is based on chromatographic separation of antibodies in dissolution based on their size. It is used to remove impurities from pollutants such as aggregates, intermediates, and dead cell debris. The Agilent Bio SEC-3 column was utilized as the column. P2K18 BY, P2K18 in 1% FBS BY, BSA, Lig 7 bacterial isolate, P1K15. BSA was used to compare with FBS in media content.

2.3.22 Total mRNA isolation and cDNA generation

The sample preparation for total RNA isolation for Real-Time PCR was carried out using kit (MACHEREY-NAGEL) according to manufacturer's instructions. Firstly, the transfected and wild type CHO-K1 cells were precipitated (1×10^6 cells). The cells were lysed and homogenized following the addition of the carrier RNA. The lysate was filtrated at 11000 g 30 s. The lysate was loaded on the membrane and centrifuged at 11000g for 30 s to bind RNA. The silica membrane was desalted and DNase was added to digest DNA at room temperature for 15 min. The silica membrane was washed three times and finally, highly pure RNA was eluted by RNase free dH₂O. The quality and quantity of the RNA was determined by Thermo Scientific Nanodrop 1000 Spectrophotometer. The extracted RNA was converted to cDNA by using SensiFast cDNA synthesis kit (Bioline) according to the manufacturer's instructions. The reaction mixture included mRNA (1µg), 5x TransAmp Buffer (4 µl), Reverse Transcriptase (1 µl) in a total of 20 µl reaction run in the stated conditions (Table 2.5).

Table 2.5 : cDNA synthesis PCR cycle.

Steps	Temperature	Duration
1	25°C	5 min
2	42°C	30 min
3	85°C	5 min
4	4°C	-

The reaction was prepared on ice and mixed by gently pipetting and synthesized accordingly as stated in Table 2.5. The quality and quantity of the cDNA was determined by Thermo Scientific Nanodrop 1000 Spectrophotometer.

2.3.23 Real-Time RT-PCR

Primers pairs were generated using an online primer design tool (GenScript Online PCR Primers Designs Tool). All primer pairs (Table 2.6) were tested before used in the Real-Time PCR reaction.

Table 2.6 : Real time PCR primers.

Primers	5'3'
KDR 1.3 RT F	TGGATCCGGAAATTCCCAGGGCATAA
KDR 1.3 RT R	CGTGGTCCCTTGGCCCCAGT
KDR 2.6 RT F	TGCAAGGCTTCCGGTTACGCA
KDR 2.6 RT R	GCTGCATGAAGGCTGTGCTGGA
Opt I_RTPCR_F	TGGGTGAAGAAGTCCAACGA
Opt I_RTPCR_R	CACTGTCATGGTAGCCTTGC
Opt II_RTPCR_F	CTGCTATCCTCTCCGCTTCA
Opt II_RTPCR_R	GCTTCTGCTGATACCAAGTGC
CHO GapDH Fwd	CATCACCATCTTCCAGGAGC
CHO Rev GapDH	CTTGGTTCACACCCATCACA

PCR reaction was performed using iTaq Universal SYBR Green Supermix. The reaction protocol and thermal program used are given respectively in Table 2.7 and 2.8. GAPDH gene was used as housekeeping gene and dH₂O was employed as negative control.

Table 2.7 : Real Time PCR reaction mix.

Components	(μ l)
dH₂O	7
Primer	1+1
DNA	1
iTaq	10

Table 2.8 : Real Time PCR conditions.

Steps	Temperature	Duration	Cycle
Denaturation	94°C	5 min	1
Amplification	94°C	40 sec	40
	56°C	30 sec	
	72°C	40 sec	
	94°C	1 min	1
	55 °C	1 min	1
Hold	4°C	∞	--

Normalization of expression levels was done by comparing with the GAPDH expression levels as an internal control. The gene expression levels were determined as relative units with respect to mRNA levels of GAPDH used as reference. Each sample was analyzed in triplicates and relative quantification of mRNA levels were calculated by using comparative $\Delta\Delta C_t$ method. Obtained C_T values were converted to $2^{-\Delta\Delta C_P}$ values as suggested by Pfaff (2001) and reflected in the Figure 2.8.

$$\text{ratio} = 2^{-[\Delta C_P \text{ sample} - \Delta C_P \text{ control}]}$$

$$\text{ratio} = 2^{-\Delta\Delta C_P}$$

Figure 2.8 : Comparative $\Delta\Delta C_t$ calculation according to Pfaffl (2001).

2.3.24 Flow cytometry

CytoFLEX Flow Cytometer (Beckman Coulter) was used to investigate the intra-cellular expression of target protein by using a fluorescent labelled Rat anti-Ms IgG (Kappa light chain) antibody. The ability of the target protein to bind the anti-Ms IgG antibody was analyzed indirectly. Transfected cells and un-transfected control cells were seeded with a viable cell density of 2×10^6 cells/mL and incubated for 3 and 20 days respectively. The cells were centrifuged at 350 g, and re-suspended in PBS twice before the samples were permeabilized with 0.1% Tween 20. 1 μ g/mL antibody was incubated with Rat anti-Ms IgG (Kappa light chain) conjugated with R-phycoerythrin

according to the manufacturer's instructions. Before analysis, transfected culture samples, un-transfected control cells were loaded to accomplish gating of flow cytometry data. CytExpert for CytoFLEX Acquisition and Analysis Software was used to analyze flow cytometry data.

2.3.25 HDX-MS studies

In order to better understand the dynamic structure of the proteins and their binding regions for VEGFR-2, I have used the hydrogen/deuterium exchange mass spectrometry (HDX-MS) analysis. Proteins were dissociated into peptide fragments in the HDX system by passing through the column containing the pepsin enzyme to allow analysis at the atomic level. All peptides obtained in the LC-MS (NanoAcquity, Synapt G2si Q-TOF system, Waters, Milford, USA) system was examined at high resolution. HDX-MS calculates the interchange levels of the amide groups D-for-H along the backbone in a D₂O solution. The exchange rate is determined by gathering MS data at various intervals following D₂O exposition, leading in 'deuterium uptake vs. time' plots that represent conformation and dynamics under certain circumstances.

H/D exchange was initiated by diluting 20 pmol target protein in 37.5 µl labeling buffer (20 mM HEPES, 150 mM NaCl, pD 7.4) prepared using D₂O instead of H₂O and DCl instead of HCl. At various time points, the exchange reaction was stopped with 1:1 ratio quenching buffer (20 mM HEPES, 150 mM NaCl, 3 M guanidine hydrochloride, 200 mM TCEP, pH 2.3) and incubated on ice for 30-60 sec (longer times might cause back-exchange). To localize the exchange of deuterium to different regions of the polypeptide backbone, pepsin was used to digest the protein into peptides. DynamX, advanced HDX-MS software, was used for peak detection and quantification, as deuterium uptake was determined by centroiding isotope peak envelopes for hundreds of peptides over several time points and replicates to process results.

In terms of reproducibility, each HDX-MS assay was performed as four replicates. At the end of this analysis the regional variations were observed at peptide level in the HDX-dependent protein surface as the hydrogenation of the surface-interacting protein complex regions with the deuterium isotope is slower than the hydrolysis of the completely hydrogenated hydrogens.

2.4 Results and Discussion

2.4.1 Molecular cloning and transformation

To overcome the inclusion body problem in the bacterial expression system and anti-KDR 1.3 or anti-KDR 2.6 structures were cloned into different constructs for both bacterial and mammalian expression systems as explained in detail in the material methods part. First of all, the target gene was amplified by PCR reaction and the gene products were confirmed by agarose gel electrophoresis (Figure 2.9).

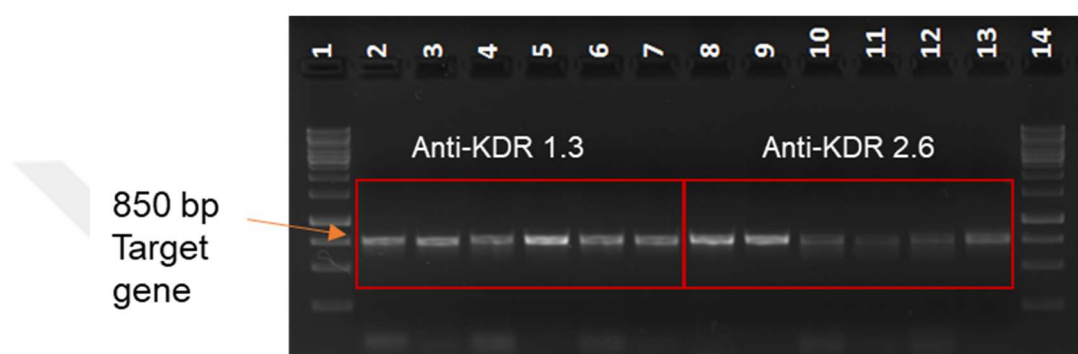


Figure 2.9 : PCR amplification of the target genes (anti-KDR 1.3 and anti-KDR 2.6).

Then the bands were purified by gel extraction and results were confirmed by agarose gel electrophoresis (Figure 2.10) and concentrations were determined by Nanodrop measurements.



Figure 2.10 : Agarose gel purified target gene constructs.

After the digestion of specified plasmids with the pre-determined restriction enzymes and agarose gel extraction of the target bands, the cloning reactions were performed. Initially, the resulting novel gene constructs that were cloned into the pIRES2-AcGFP1 vector were checked for target gene insertion.

According to colony PCR results, 16 positive colonies (20 colonies screened) were obtained from pIRES2-AcGFP1-Koz-NHis-anti-KDR2.6 samples. According to colony PCR results, 5 positive results (37 colonies screened) were obtained from pIRES2-AcGFP1-Koz-IL2-NHis-anti-KDR2.6 samples (Figure 2.11 and Figure 2.12).

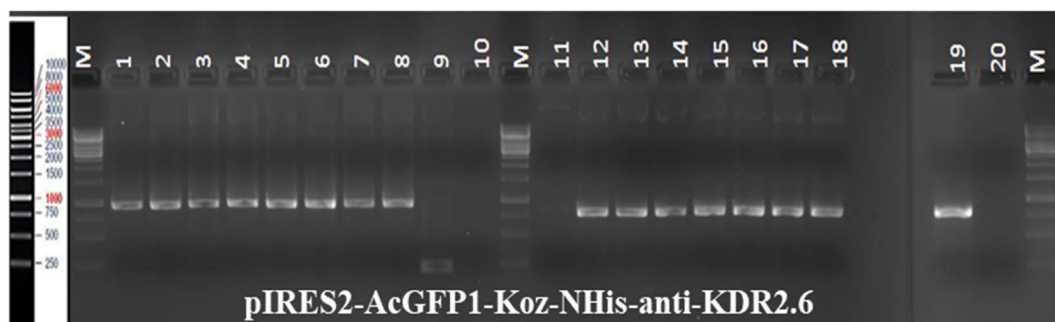


Figure 2.11 : Colony PCR results for pIRES2-AcGFP1-Koz-NHis-anti-KDR2.6 construct.

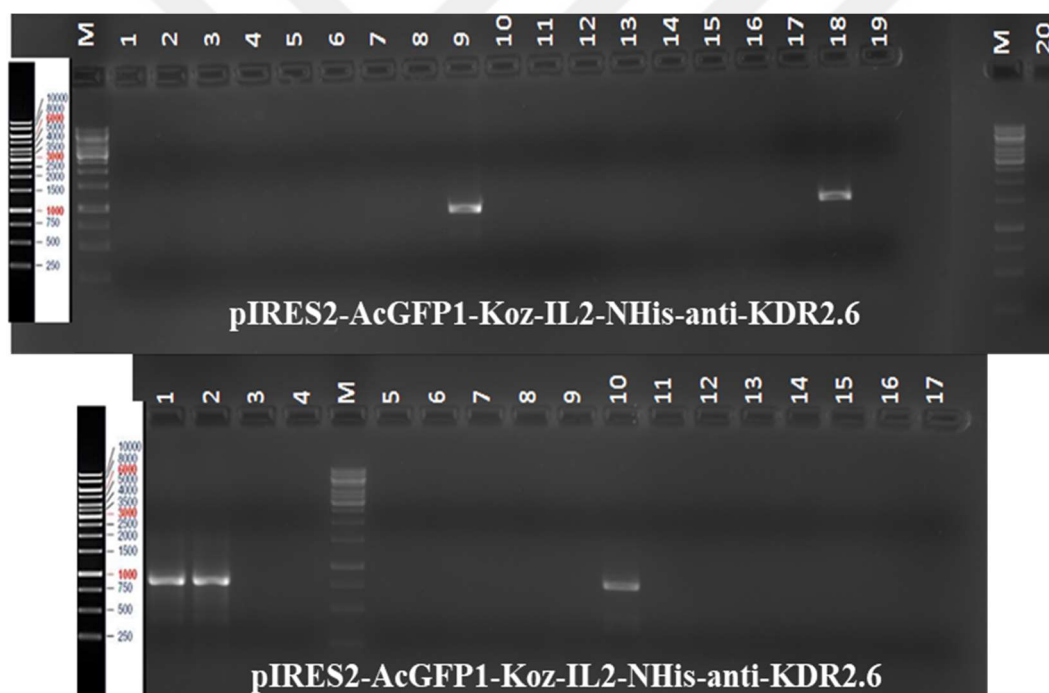


Figure 2.12 : Colony PCR results for pIRES2-AcGFP1-Koz-IL2-NHis-anti-KDR2.6 construct.

Sequence PCRs were performed using inner and outer primers to control the correct alignment of target gene sequences on the vector. After the analysis of the target sequences using ChromasPro program, a consensus sequence was obtained by assembly of all the forward and reverse sequences and the replicates (Figure 2.13).

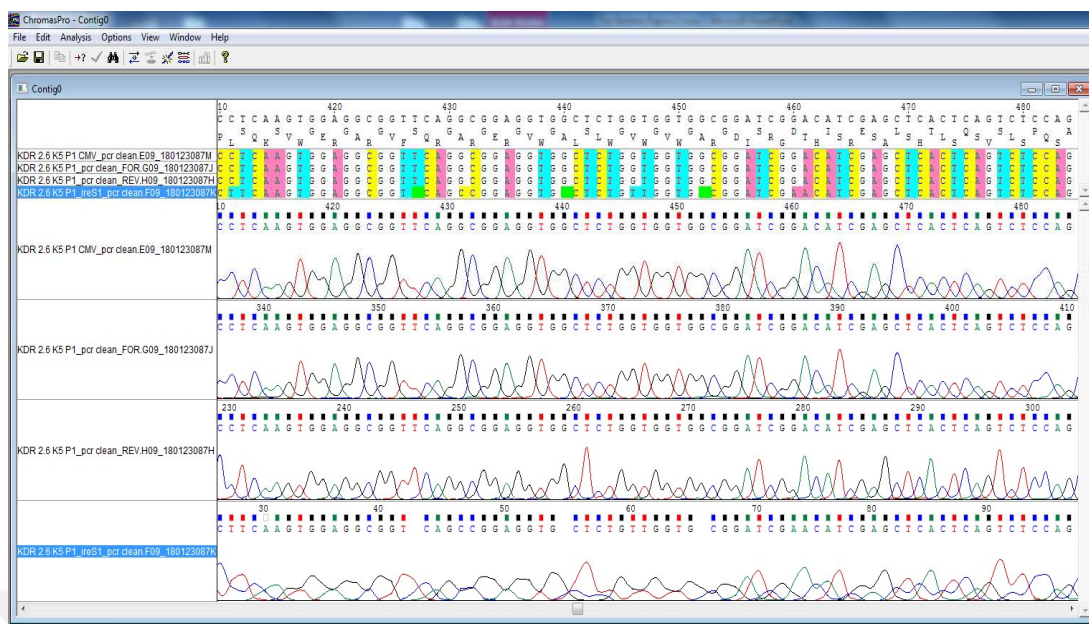


Figure 2.13 : Target genes analysis on the ChromasPro program.

Target gene sequences were confirmed by alignment with the known constructs by using the BioEdit program (Figure 2.14).

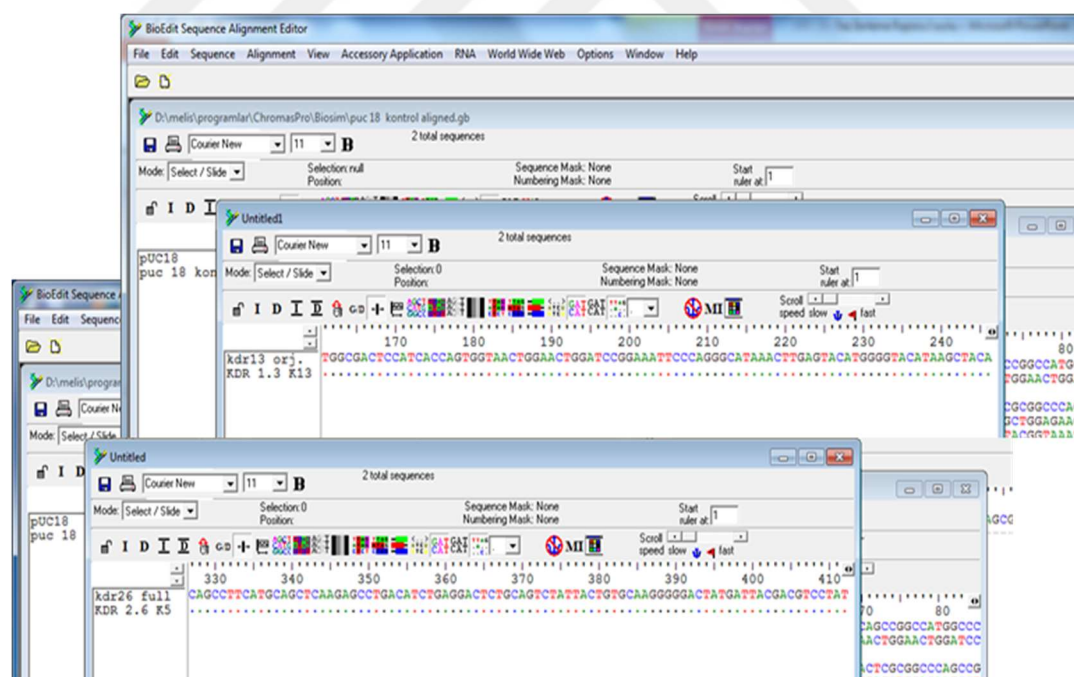


Figure 2.14 : Target genes analysis in the BioEdit program.

The alternative novel gene constructs that were cloned into the pcDNA3.1+ vector was also analyzed for correct target gene insertion. According to colony PCR results, 29 positive results (46 colonies screened) were obtained from pcDNA 3.1+-Pre_MSA-anti-KDR2.6 samples (Figure 2.15).

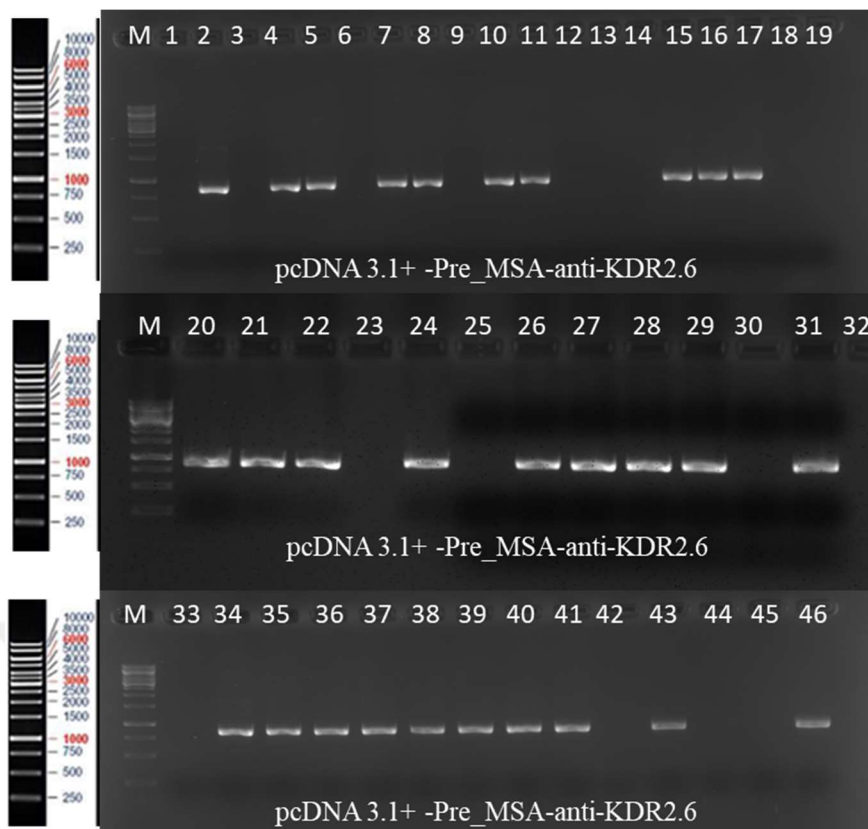


Figure 2.15 : Colony PCR results for pcDNA 3.1+ -Pre_MSA-anti-KDR2.6 constructs.

Extensive attempts have been made to address the challenges of solubility and stability. Protein tags such as glutathione-*S*-transferase (GST) and maltose-binding protein (MBP) have been generally used as affinity tags for purifying recombinant proteins. When compared to their original ones, fusion proteins have been shown to produce recombinant better and have improved solubility and sample stability.

The alternative novel gene constructs that were cloned into the pET42a-anti-KDR1.3 and pDream2.1/MCS-anti-KDR1.3 vectors were checked for target gene insertion. According to colony PCR results, the colonies chosen were positive (Figure 2.16).

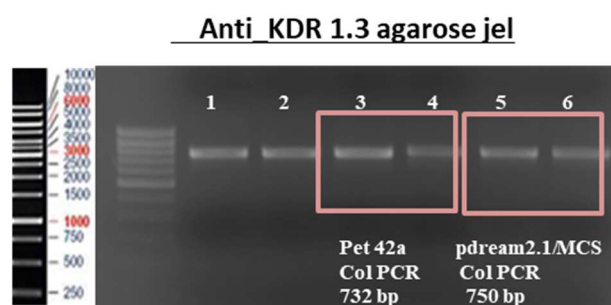


Figure 2.16 : Colony PCR reactions for anti-KDR 1.3. Samples from left; Marker, 1-2. positive control, 3-4. pET42a vector and 5-6. pDream 2.1 /MCS.

2.4.2 scFv production and purification in bacteria

For the analysis of the proteins, protein production studies were performed. One colony was picked from the culture plates for anti-KDR 1.3 and growth overnight at 37°C at 240 rpm. The culture was then increased by placing it to a higher mass amount of 2XTY containing antibiotics and 2% glucose and cultivated for 1-4 hours, or till culture intensity approached 0.50.6 OD600. As soon as the culture attained 0.5-0.6 OD, expression was stimulated by introducing IPTG (0.5 mM). The induction procedure was carried out for 20 hours at 30°C and 240 rpm. Cells were then at 8000 g for 10 min. Cells were resuspended in PBS and recentrifuged in a 50 mL tube. Supernatant was kept in another tube and pellet was kept at -20°C for later processing. Following overexpression control, first Ni-NTA purification procedure was carried out to isolate anti-KDR1.3 protein from the total protein lysate. After consecutive purification steps, fractions were monitored in SDS-PAGE gel electrophoresis and western blot analysis. Purified anti-KDR 1.3 (28 kDa) was represented in Figure 2.17.

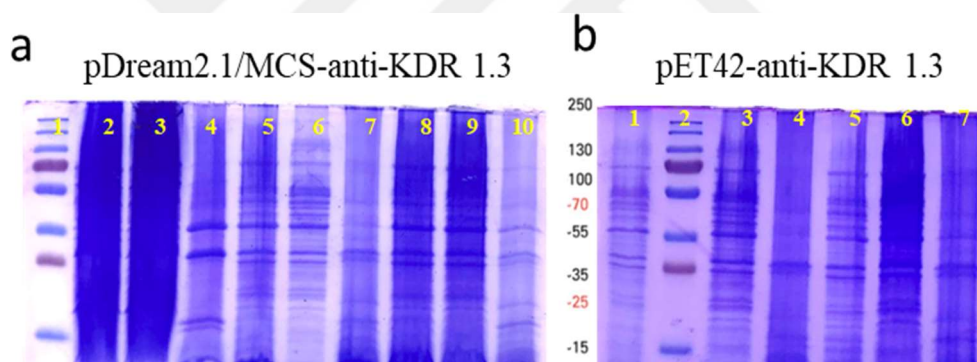


Figure 2.17 : SDS-PAGE results of anti-KDR 1.3 (28 kDa) pDream2.1/MCS and pET42a constructs. Samples from left; **(a)** 1. Precision plus Marker, 2. total, 3. soluble, 4. insoluble, 5. pDream 2.1/MCS total K6, 6. pDream 2.1/MCS soluble K6, 7. pDream 2.1/MCS insoluble K6, 8. pDream 2.1/MCS total K7, 9. pDream 2.1/MCS soluble K7, 10. pDream 2.1/MCS insoluble K7. **(b)** 1. pET42a total K6, 2. Precision plus Marker, 3. pET42a soluble K6, 4. pET42a insoluble K6, 5. pET42a total K7, 6. pET42a soluble K7, 7. pET42a insoluble K7.

The target band with the correspondance molecular weight (28kDa) for anti-KDR 1.3 protein has not seem to be observed in the induction process from pET42a and pDream2.1/MCS constructs. However, wild type anti-KDR 1.3 in pDUCK vector has been induced and purified. Protein detection analysis showed presence of His tagged target protein (Figure 2.18). The expressed protein was expressed in inclusion bodies.

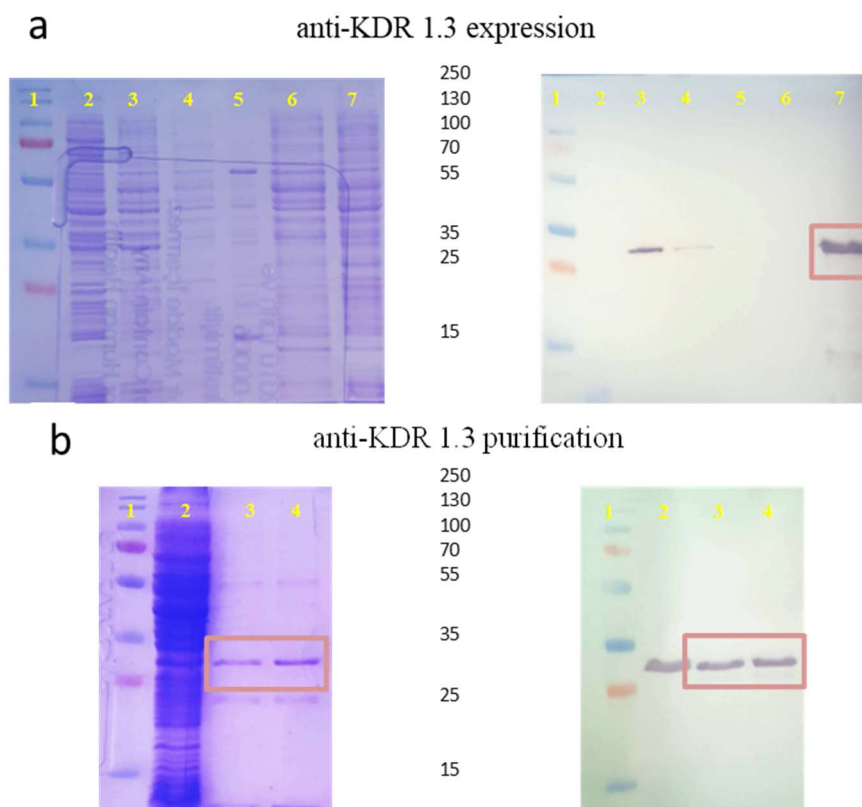


Figure 2.18 : Expression analysis of target protein anti-KDR 1.3 (28 kDa) and histidine purified anti-KDR1.3. Samples from left; **(a)** 1. Precision plus Marker, 2. t_0 , 3. t_{20} , 4. Supernatant, 5. Periplasmic extract, 6. Cytosolic extract, 7. Inclusion body. **(b)** 1. Precision plus Marker, 2. Flow through, 3. Elution 1, 4. Elution 2.

Activity of the proteins were evaluated by ELISA experiment (Figure 2.19) and concentration of lysates was evaluated by Bradford Assay (Bio-Rad, CA, USA). Anti-KDR 1.3 concentration was determined as 170 $\mu\text{g/mL}$ (6.07 μM).

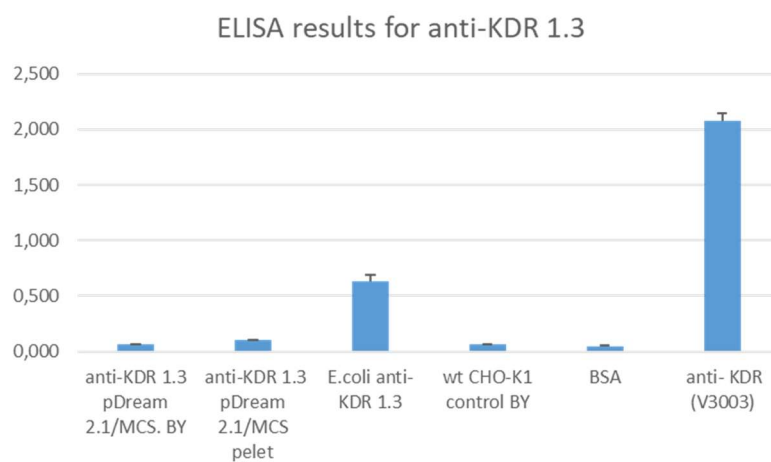


Figure 2.19 : ELISA results for mammalian and bacterial anti-KDR1.3 expression.

2.4.3 Transient transfection

When the results were analyzed, the highest transfection efficiency was observed with Lipofectamine 2000. It was determined that 175 ng of insert DNA and 0.3 μ l of the chemical gave the most optimum results. Lipofectamine 3000 and turbofect gave similar results. However, iN-fect has a lower transfection rate. Transfection efficiency of empty pIRES2-AcGFP1 vector with Lipo2000 was 92% (Figure 2.20).

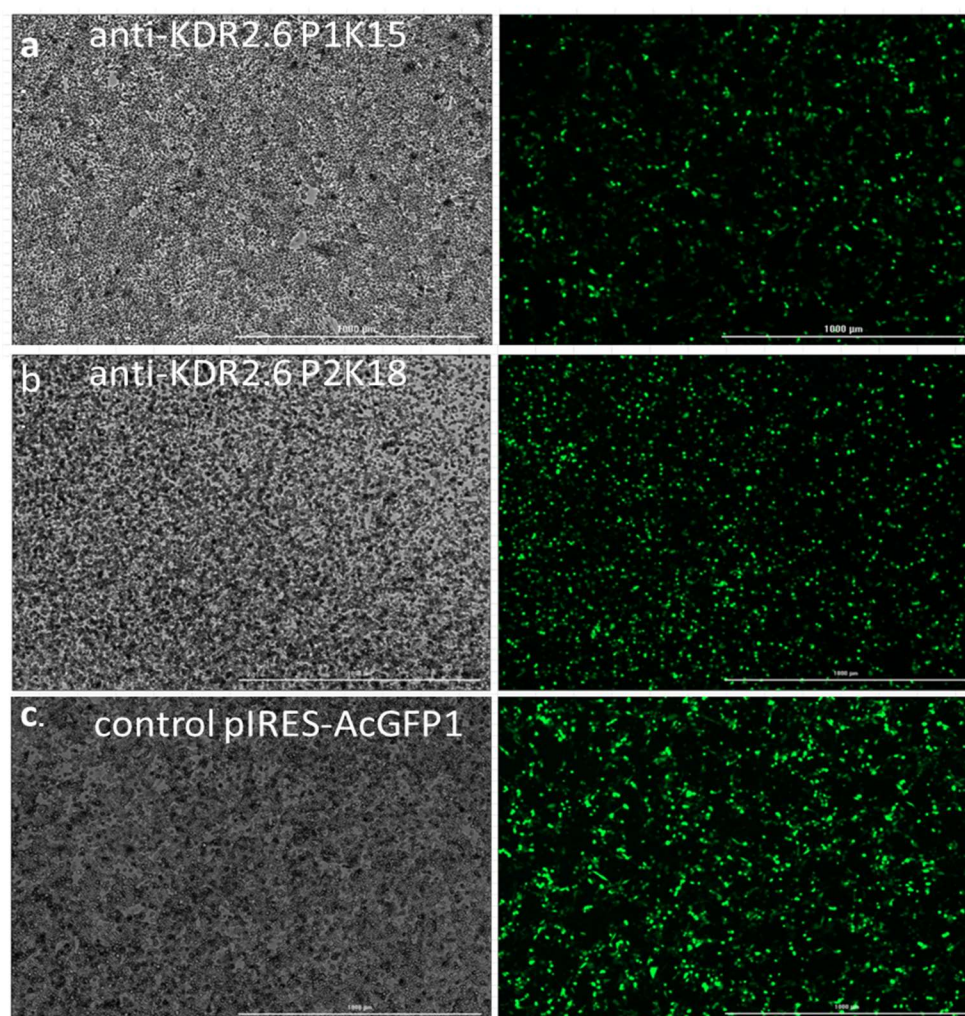


Figure 2.20 : Bright field (4X) and fluorescent image (4X) of cells on day 2 post-transfection;(a) anti-KDR2.6 P1K15, (b) anti-KDR2.6 P2K18, (c) pIRES-AcGFP1.

All the constructs (7 constructs explained in detail in the past reports) were transfected to the Hek293 cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). Hek 293 cells were maintained in Dulbecco's modified Eagles/F12 medium supplemented with 10% fetal bovine serum. Cells were incubated in 5% CO₂ humidified air at 37°C incubator. Cells were split upon reaching 80% (2-3 days) confluency using 0.25% Trypsin-EDTA. Cells were seeded 0.7×10^6 cells into 6 well plates. The next day, cells that were

confluent were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) chemicals.

2.4.4 Stable transfection

Based on the measurements (Figure 2.21) observed on the density graph and growth rates of each well taken at each time intervals; 0, 24, 48, 72, 168, 192, 216 and 240 hr, 400-600 $\mu\text{g/mL}$ antibiotic concentration was determined to be suitable for the establishment of a stable cell line. Similar experiments were done for the Hygromycin antibiotic as well, both for the CHO-K1 and Hek 293 cell lines and determined as 500, 300 $\mu\text{g/mL}$ respectively .

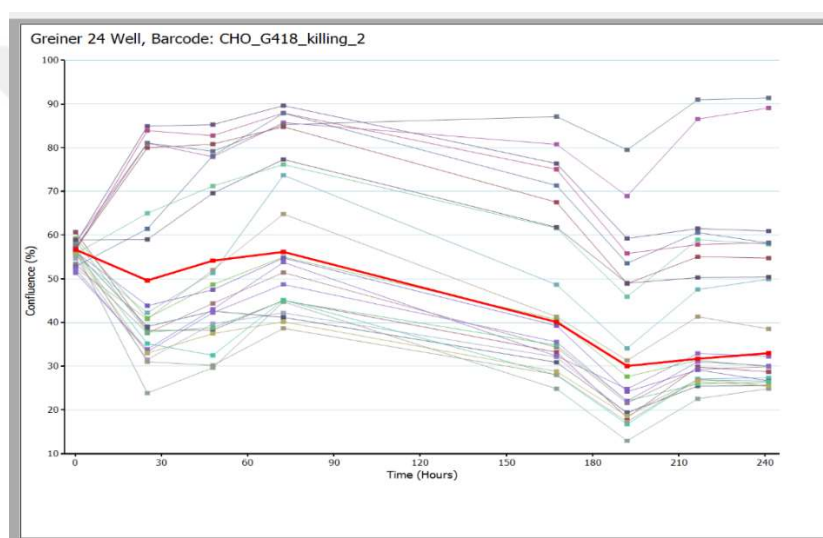


Figure 2.21 : G418 killing curve on CHO-K1 cells.

After the transfection, on the 3rd day, the cells were selected with antibiotics at the determined concentration. Control cells were observed to be dead within 5-6 days and the transfected cells were selected under antibiotic till 9-10th day to make sure that only transfected cells remain.

After the transfection, on the 3rd day, the cells were selected with antibiotics at the determined concentration (400 $\mu\text{g/mL}$). Control cells were observed to be dead within 5-6 days and the transfected cells were selected under antibiotic till 9-10th day to make sure that only transfected cells remain. The cell culture media was then collected for protein determination with SDS-PAGE analysis and ELISA. The media was centrifuged at 4000 g for 5 min at 4°C. To determine the binding activity of the proteins ELISA was carried out. VEGFR-2 (Merck, 676490) antigens were coated on a 96 well microplate directly in a coating solution at 4°C for overnight. After washing six times

with TPBS, the microplate was blocked with TPBS containing 1% BSA solution and incubated at room temperature for 2 hr. The wells were washed 6 times with TPBS. The microplates were then coated with culture media collected at room temperature for 1 hr. After washing six times with TPBS, the microplate was blocked with antibodies (Sigma, A7058 anti-his and A9544 anti human IgG) and incubated at room temperature for 1 hr. The substrates were added and 30 min incubation at room temperature. Plates were measured with a spectrophotometer at the specific nanometers. According to SDS-PAGE, western and ELISA results, any positive results could not be obtained for the target protein production in the mammalian cells (Figure 2.22).

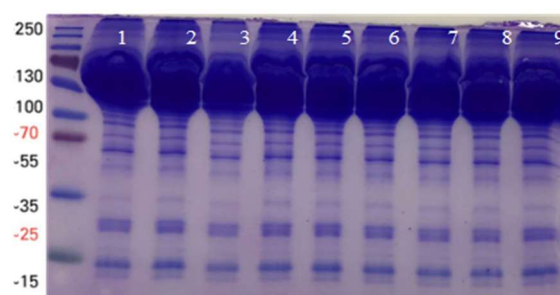


Figure 2.22 : SDS-PAGE analysis of stable transfection results of the target protein anti-KDR 1.3 & 2.6 (28 kDa). Samples from left; **1.** Precision plus Marker, **2.** Anti-KDR 1.3_pDream2.1_K7, **3.** Anti-KDR 2.6_optI_K2, **4.** Anti-KDR 2.6_optI_K3, **5.** Anti-KDR 2.6_optI_K3_Nucleofector, **6.** Anti-KDR 2.6_optI_K3_Lipofectamine 2000, **7.** Anti-KDR 1.3_pDream2.1_K6, **8.** Anti-KDR 2.6_optI_Fc_K11, **9.** Anti-KDR 2.6_optI_Fc_K15, **10.** Anti-KDR 1.3_pires_AcGFPI.

The expression signal for OPTI constructs cell pellet display 1,3 times higher than the signal for the wild-type CHO-K1S media (Figure 2.23). The remaining results were not significant in terms of expression levels.

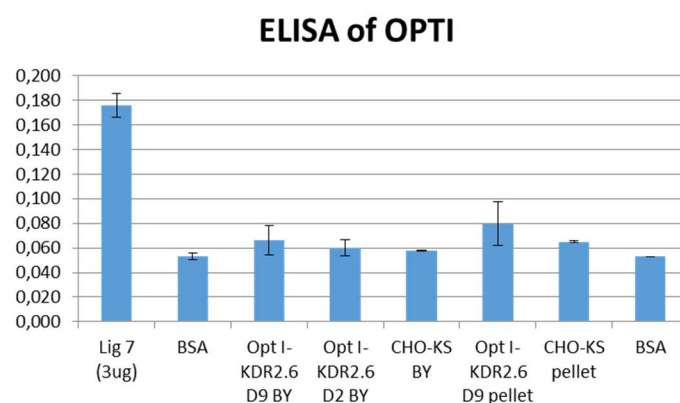


Figure 2.23 : ELISA results for supernatant and pellets of OPTI constructs in CHO-K1S cells.

2.4.5 Serum free adaptation and suspension growth

Cells became completely suspended during serum free adaptation. Below is an image of (Figure 2.24) serum free adapted suspension GFP-producing CHO-K1S cells observed with the Cytation 5 device.

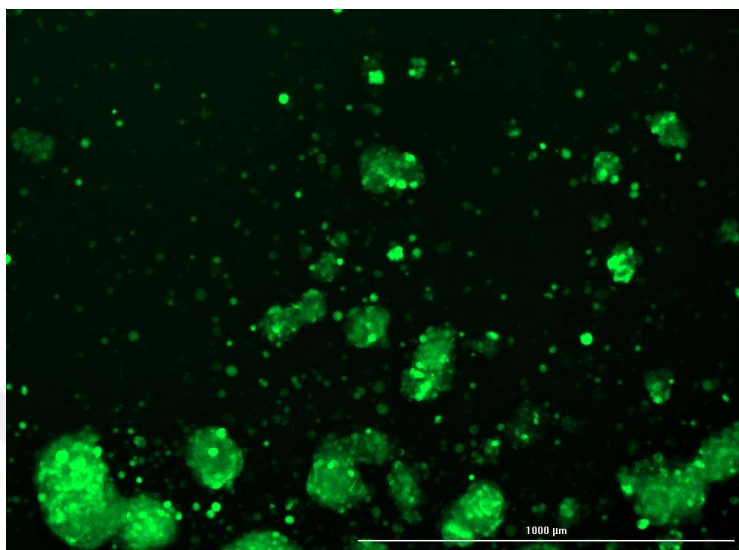


Figure 2.24 : Fluorescent image of suspension CHO-K1 cells.

pIRES-AcGFPI-anti-KDR 2.6 constructs P1K15 and P2K18 colonies were serum free adapted and suspended. Expression of the target protein was investigated by SDS-PAGE and western blot analysis (Figure 2.25). According to the results of the expression analysis, target protein bands could not be observed in the SDS-PAGE or western blot results.

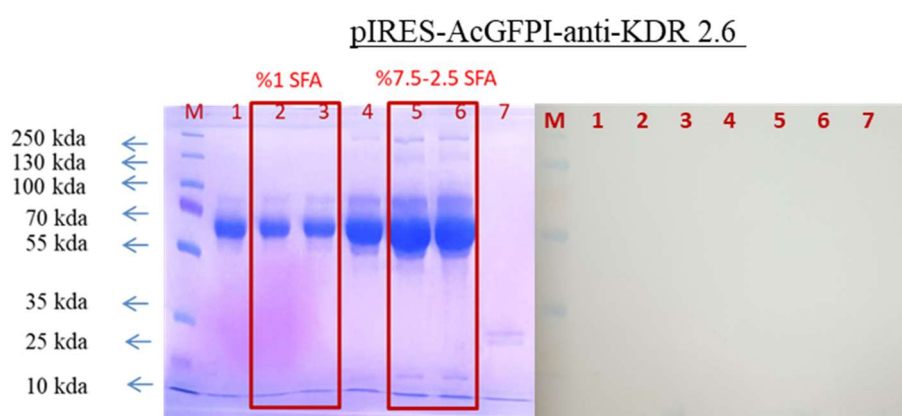


Figure 2.25 : SDS-PAGE and Western Blot analysis P1K15 and P2K18. Samples from left; Marker, 1. %1 SFA supernatant CHO-K1, 2. %1 SFA supernatant P1K15, 3. %1 SFA supernatant P2K18, 4. SFA supernatant CHO-K1, 5. SFA supernatant P1K15, 6. SFA supernatant P2K18, 7. Lig7 ScFV.

2.4.6 Feeding strategy, temperature shift, fed batch

Initially (Set 1), cells seeded in T175 plates were observed for their survival without supplementation. When the experiment was completed, the viability of the samples was respectively: 70% confluency for anti-KDR 1.3 (P6B9), 35% confluency for anti-KDR 2.6 (P1E3).

Protein expression levels were analyzed for both supernatant and cell pellet by SDS-PAGE and Western blot studies. A band was observed in the pellet in the same region as the control scFv. According to the western blot results, target protein expression could not be observed. Only the control lig7 band was observed (Figure 2.26).

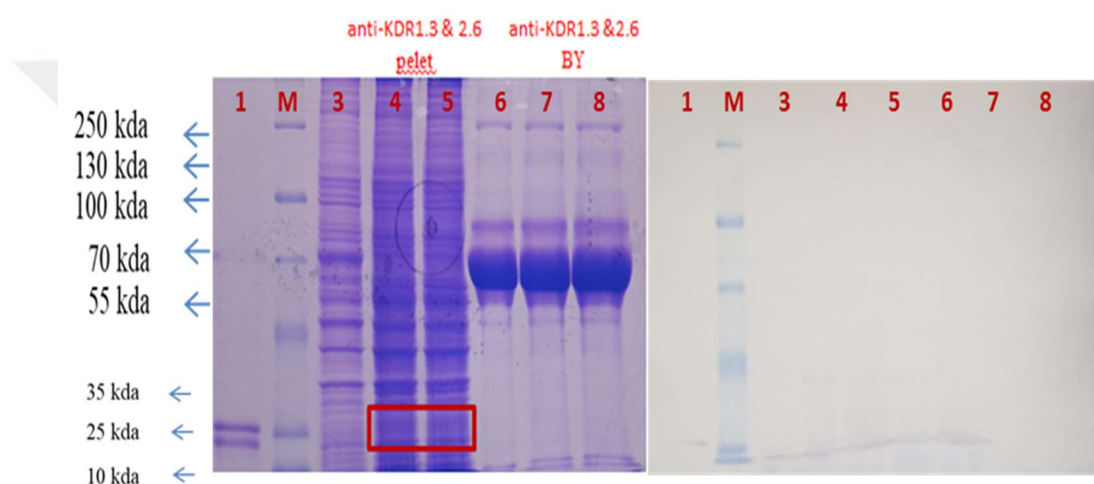


Figure 2.26 : SDS-PAGE & Western Blot results of expressed anti-KDR1.3 protein. Samples from left; Protein L, HRP antibody with 1/600 dilution. **1.** Lig7 control, **2.** Marker, **3.** CHO-K1 pellet **4.** anti-KDR 1.3 P6B9 pellet **5.** anti-KDR 2.6 PIE3 pellet **6.** %5 FBS CHO_K1 supernatant **7.** anti-KDR 1.3 P6B9 supernatant **8.** anti-KDR 2.6 PIE3 supernatant.

For determining the feeding strategy, different concentration of cell boost 7a & 7b supplements were added to the cells (Set 2) grown in 6 well plates. The condition of the cells was determined by examining MetaFLEX Bioanalyte Analyzer (Beckman) results. When the experiment was completed, the viability rates of the samples were observed for 31°C viability as: 85-90%, for 37°C viability as: 50-90%, respectively.

According to the obtained results, 1.5% Cell boost 7a and 0.15% 7b concentration were determined for the best viability (Table 2.9).

Table 2.9 : Feed media and cell numbers on Cytation 5 device.

7A &B (%)	Day3	Day4	Day5	Day8	Day9	Day10	Day11	Day12
PLATE 1	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C
0	3294	3689	5126	4242	3694			
0,5	2886	4257	3968	4847	6181	5735	5676	5841
1	2941	4389	4033	4602	6028	5706	5868	6006
1,5	3496	5267	5237	5476	6642	6337	6129	6540
2	2994	4812	5044	5382	6476	5869	5670	8195
3	2960	4531	4390	5292	6302	6203	6602	6597
PLATE 2	37°C	37°C	31°C	31°C	31°C	31°C	31°C	31°C
0	3094	3188	3767	3526	2275	2248		
0,5	4086	4387	4678	3922	5088	4430	4603	5218
1	3941	4049	5020	4486	4771	4717	4476	4475
1,5	4096	5426	5808	4720	5163	4717	5275	5140
2	3994	4991	5550	5335	5804	5300	5735	5560
3	3860	4747	5039	5214	5687	5278	5654	5483

After the feeding strategy was determined (Set 3), 2×10^6 cells were seeded in T175 cell growth plates. The experiment was terminated on the 15th day because the glucose ratio was high in the measurements. Key biochemical parameters were also monitored through the process (Table 2.10). During the cell culture process, cell growth, and protein expression are all affected by pH, dissolved oxygen levels, glucose and lactate levels. Fed-batch cell culture conditions can be monitored and adjusted according to the parameters obtained.

When the experiment was completed, the viability of the samples was respectively Anti-KDR 1.3 (P6B9): 58 %, Anti-KDR 2.6 (P1E3): 75 %. When the results were examined, 1.5%, 2% Cell boost 7a and 0.15-0.2% 7b concentrations were determined as appropriate. Protein expression levels were analyzed for both supernatant and cell pellet by SDS-PAGE and western blot studies (Figure 2.27).

In the protein expression analysis, a band was observed in the pellet in the same region as the control scFv. Target band was observed in 37°C supernatant sample. According to the western blot results, weak bands were observed for the target protein in the pellet and control lig7.

Table 2.10 : Biochemical parameters monitored by MetaFLEX device.

	pH	pCO2 (mmHg)	pO2 (mmHg)	cGlu (g/L)	cLac (g/L)
P1E3 D2	7.694	9.2	217	1.49	0.25
P1E3 D3	7.553	8.4	143	1.02	0.63
P1E3 D4	7.137	10.1	233	2.93	0.84
P1E3 D5	7.033	14.2	213	2.81	0.87
P1E3 D6	6.966	22.2	192	5.56	0.63
P1E3 D8	7.310	10.9	235	6.52	0.55
P1E3 D9	7.287	12.5	233	7.16	0.48
P1E3 D10	7.334	11	232	8.11	0.43
P1E3 D11	7.248	13.1	234	9.19	0.39
P1E3 D12	7.087	21.2	233	9.96	0.36
P1E3 D15	7.125	24.2	217	10.13	0.32
P6B9 D2	7.639	8.9	227	1.46	0.26
P6B9 D3	7.589	-	143	0.72	0.24
P6B9 D4	7.097	10	226	2.73	0.88
P6B9 D5	7.017	14.1	215	2.65	0.9
P6B9 D6	7.046	18.8	202	4.75	0.67
P6B9 D8	7.340	8.7	235	6.31	0.57
P6B9 D9	7.305	11.5	238	6.98	0.51
P6B9 D10	7.300	12	236	7.74	0.44
P6B9 D11	7.243	13.7	232	8.79	0.41
P6B9 D12	7.09	21.6	232	9.52	0.37
P6B9 D15	7.097	25.7	214	10.24	0.33

Protein expression levels were analyzed for both supernatant and cell pellet by SDS-PAGE and western blot studies (Figure 2.27).

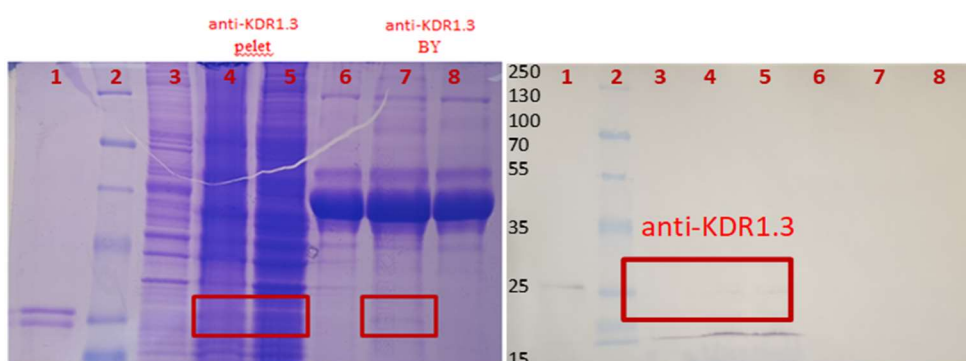


Figure 2.27 : SDS-PAGE & Western Blot results of expressed anti-KDR1.3 protein. Samples from left; Protein L, HRP antibody with 1/600 dilution. 1. Lig7 control, 2. Marker, 3. CHO-K1 pellet 4. anti-KDR 1.3 P6B9 pellet 37°C 5. anti-KDR 1.3 P6B9 pellet 31°C 6. %5 FBS CHO_K1 supernatant 7. anti-KDR 1.3 P6B9 supernatant 37°C 8. anti-KDR 1.3 P6B9 supernatant 31°C.

When the cells maintained at reduced temperature compared to the culture at 37°C, showed a highest viability on the harvest day. However, the results showed that a difference could not be observed between the protein expressions of the samples at 31°C and 37°C (Figure 2.27). When the experiment was completed, confluency results were as follows respectively Anti-KDR 1.3 (P6B9): 75 %, Anti-KDR 2.6 (P1E3): 58 %.

Another fed-batch experiment (Set4) was performed. The experiment was terminated on the 16th day. The measurements obtained by MetaFLEX Bioanalyte Analyzer (Beckman) was depicted in the Table 2.11.

Table 2.11 : Biochemical parameters for 37°C cell culture monitored by MetaFLEX. Device.

37°C	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)	cGlu (g/L)	cLac (g/L)
P1E3 D2	7.694	9.2	217	1.49	0.25
P1E3 D3	7.321	12.2	238	1.19	0.49
P1E3 D4	7.183	12	237	1.71	0.73
P1E3 D5	7.127	14.8	220	2.75	0.8
P1E3 D8	7.164	20.17	213	2.96	0.65
P1E3 D10	7.181	19.1	228	3.83	0.64
P1E3 D11	7.165	19.7	227	4.04	0.66
P1E3 D12	7.110	23.4	187	4.17	0.65
P1E3 D15	7.009	24	180	4.2	0.66
P6B9 D2	7.639	8.9	227	1.46	0.26
P6B9 D3	7.258	11.9	229	2.11	0.53
P6B9 D4	7.153	11.7	234	1.74	0.8
P6B9 D5	7.119	14.1	224	2.58	0.82
P6B9 D8	7.229	18	217	2.5	0.64
P6B9 D10	7.167	18.7	229	3.14	0.78
P6B9 D11	7.088	22.6	214	3.26	0.83
P6B9 D12	7.042	26.1	179	3.50	0.86
P6B9 D15	7.05	27	170	3.6	0.9

The measurements for 35°C parameter obtained by MetaFLEX Bioanalyte Analyzer (Beckman) was depicted in the Table 2.12.

Table 2.12 : Biochemical parameters for 35°C cell culture monitored by MetaFLEX device.

35°C	pH	pCO2 (mmHg)	pO2 (mmHg)	cGlu (g/L)	cLac (g/L)
P1E3 D2	7.694	9.2	217	1.49	0.25
P1E3 D3	7.364	11.2	234	1.21	0.47
P1E3 D4	7.246	10.4	228	1.80	0.76
P1E3 D5	7.171	14.1	223	2.5	0.77
P1E3 D8	7.209	17.9	211	2.71	0.64
P1E3 D10	7.134	18.8	238	3.56	0.69
P1E3 D11	7.108	20.4	216	3.79	0.69
P1E3 D12	7.076	23.2	184	3.98	0.68
P1E3 D15	7.009	24	180	4.2	0.66
P6B9 D2	7.639	8.9	227	1.46	0.26
P6B9 D3	7.258	11.9	229	2.11	0.53
P6B9 D4	7.171	11.3	226	1.79	0.79
P6B9 D5	7.138	14.3	225	2.55	0.81
P6B9 D8	7.201	17.4	214	3.12	0.68
P6B9 D10	7.132	20.2	233	3.72	0.8
P6B9 D11	7.072	22.7	214	4.07	0.85
P6B9 D12	7.027	26.5	179	4.35	0.88
P6B9 D15	7.05	27	170	3.6	0.9

Protein expression levels were analyzed for both supernatant and cell pellet by SDS-PAGE and Western blot studies. Target protein was observed in both supernatant and pellet samples. However, in the western blot results, target protein couldn't be observed in the supernatant. (Figure 2.28 and Figure 2.29).

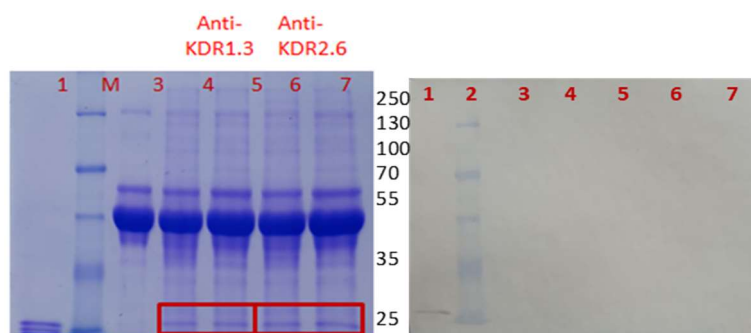


Figure 2.28 : SDS-PAGE & Western Blot results of expressed anti-KDR1.3 and anti-KDR2.6 protein in supernatant. Samples from left; Protein L, HRP antibody with 1/1000 dilution. 1. Lig7 control, 2. Marker, 3. %5 FBS CHO_K1 supernatant 4. anti-KDR 1.3 P6B9 supernatant 37°C 5. anti-KDR 1.3 P6B9 supernatant 35°C 6. anti-KDR 2.6 P1E3 supernatant 37°C, 7. anti-KDR 2.6 P1E3 supernatant 35°C.

In the Figure 2.28, weak bands were observed for the target protein in the pellet but as non-specific bands were also present, target protein expression could not be confirmed.

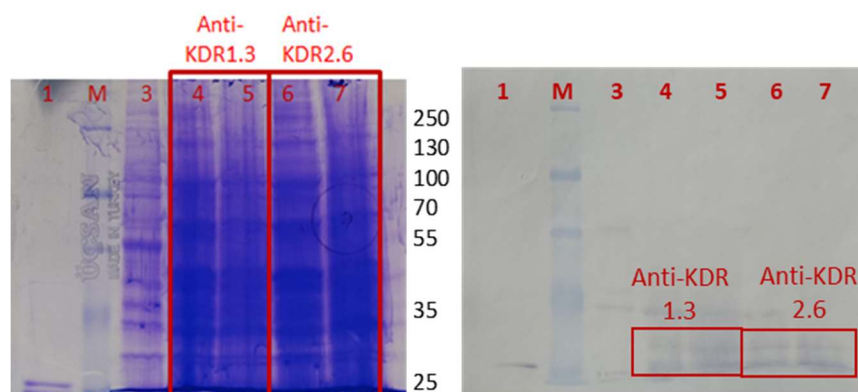


Figure 2.29 : SDS-PAGE & Western Blot results of expressed anti-KDR1.3 and anti-KDR2.6 protein in cell pellet. Samples from left; Protein L, HRP antibody with 1/1000 dilution. **1.** Lig7 control, **2.** Marker, **3.** pellet CHO_K1 **4.** anti-KDR 1.3 P6B9 pellet 37°C **5.** anti-KDR 1.3 P6B9 pellet 35°C **6.** anti-KDR 2.6 P1E3 pellet 37°C, **7.** anti-KDR 2.6 P1E3 pellet 35°C.

Expression results were analyzed by ELISA results, control samples worked properly as expected (Figure 2.30).

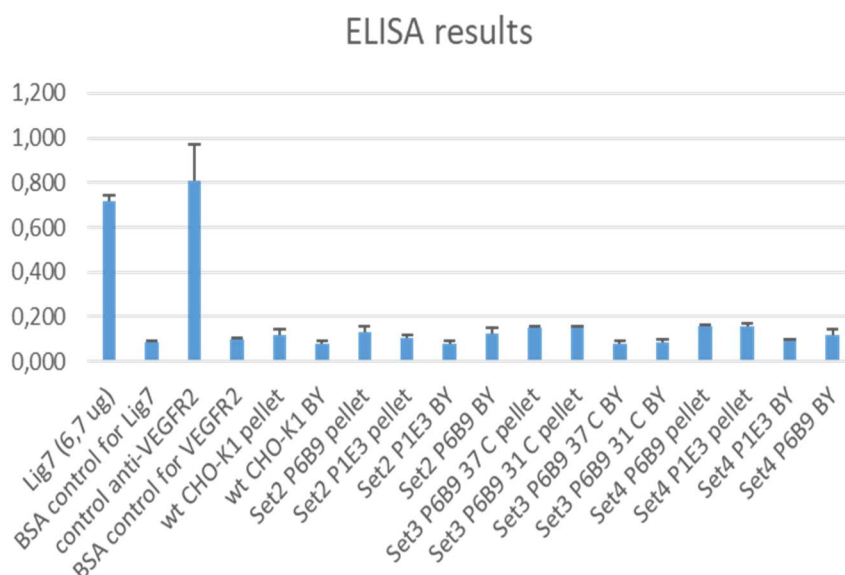


Figure 2.30 : ELISA results for supernatant and pellets of fed-batch cultures in CHO-K1 cells.

However, the expression results of the fed batch cultures for both the supernatant and pellets did not reflect any significant values.

2.4.7 HPLC analysis

Large peak of around 13 was obtained. It could not be analyzed because very low signal was obtained from cells blasted with RIPA. Lig7 gave a weak signal, but anti-KDR2.6 samples also signaled in the same place (Figure 2.31 and Figure 2.32).

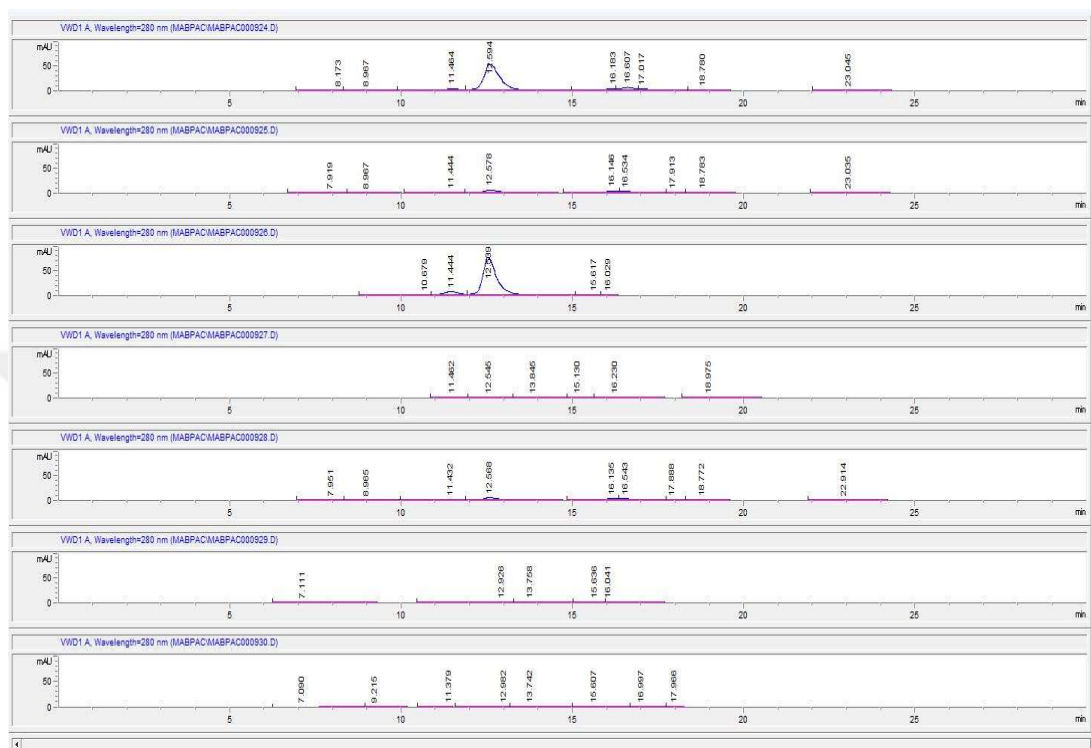


Figure 2.31 : SEC Analysis. Examples from top to bottom; P2K18 supernatant, P2K18 1% FBS supernatant, BSA, Lig 7 bacterial isolate, P1K15 1% FBS supernatant, RIPA P1K15, RIPA P2K18.

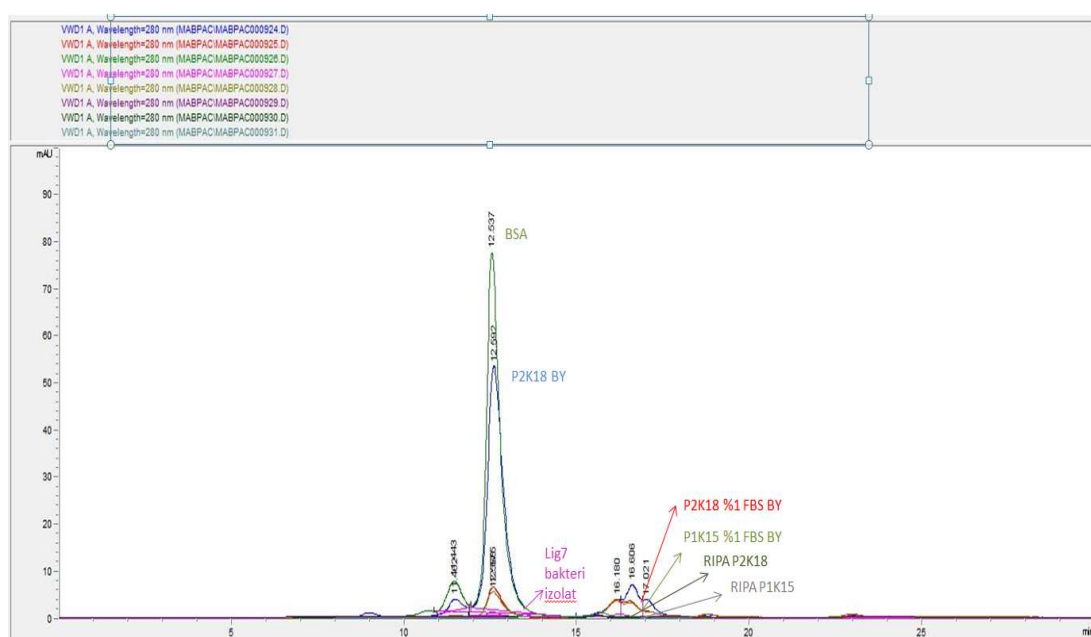


Figure 2.32 : SEC Analysis.

2.4.8 Monoclonal cell development

24 clones were obtained from the monoclonality study. The following figure represents an image of monoclonal detection of cell clones (Figure 2.33).

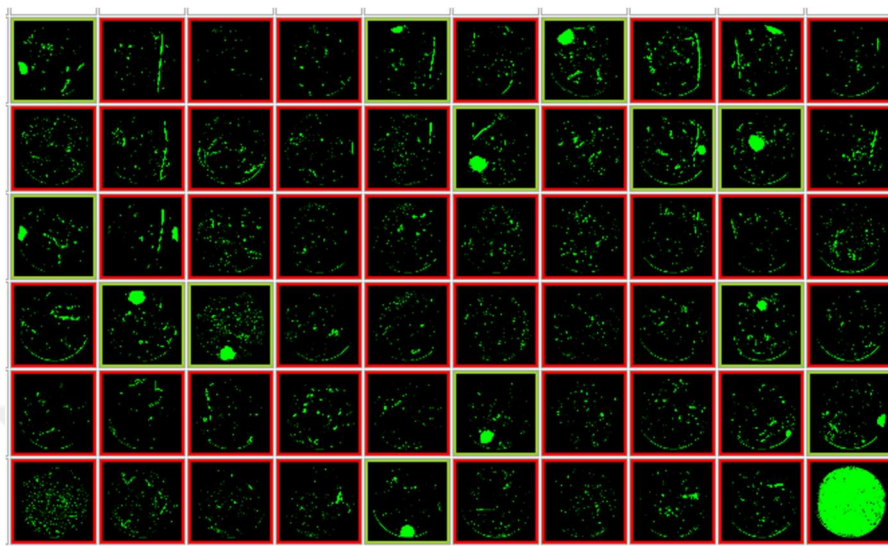


Figure 2.33 : Colony formation image of the 24 well plate on 10th day.

In the figures below, the image of culture at on seeding day, the image after 5 days and the image of colony formation on the 10th day are shown. Names of 24 detected monoclonal cells for anti-KDR 2.6 antibody; P1B2, P1B6, P1B8, P1C10, P1D2, P1E3, P1E4, P1E10, P1F7, P1C7, P1C9, P3B11, anti-KDR 1.3 antibody; P4B6, P4D4, P4E2, P4E7, P4E8, P4E10, P4F5, P6B9, P6E8, P6F7, P6 G10. The example images of two monoclonal cell clones were below in Figure 2.34 and Figure 2.35.

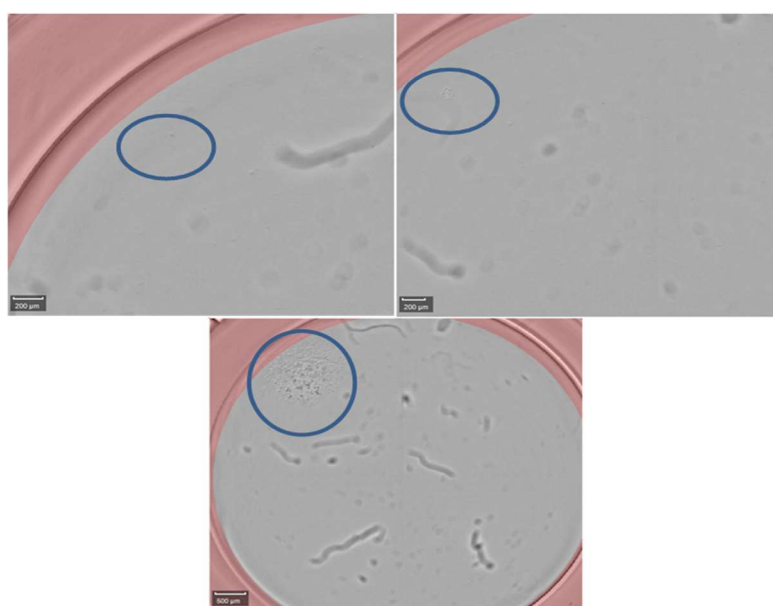


Figure 2.34 : The image of the monoclonality analysis result of clone P1B8.

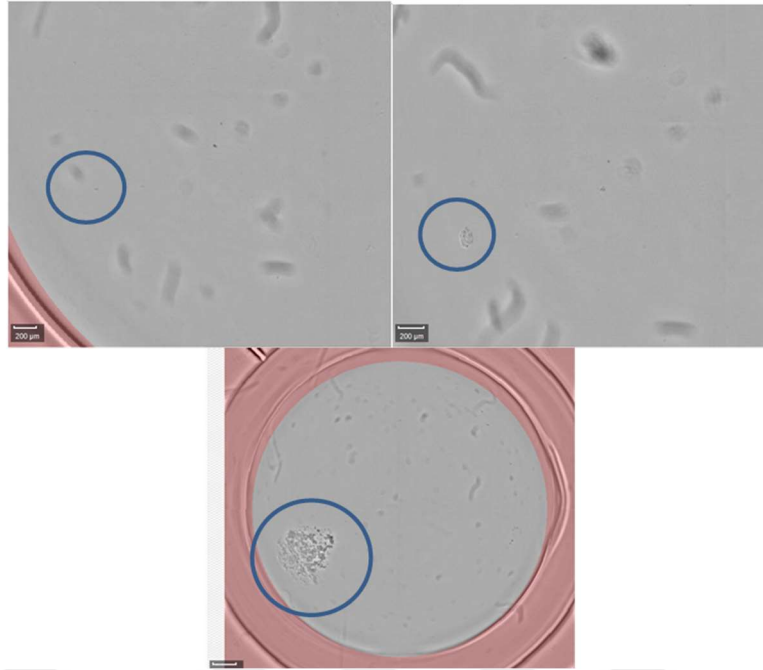


Figure 2.35 : The image of the monoclonality analysis result of clone P4E18.

13 colonies were selected by analyzing the fluorescent microscope images; P1E3, P4E8, P6B9, P4E7, P6E8, P1B8, P1C10, P1D2, P1E4, P1E10, P1C7, P4B6, P4D4. SDS-PAGE and western blot studies were done to observe protein production (Figure 2.36). According to the western blot results, a low signal for the target proteins were detected.

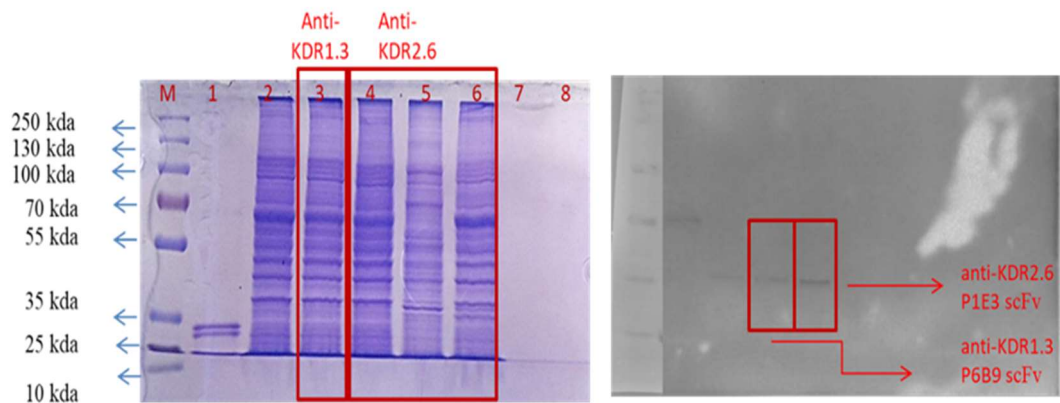


Figure 2.36 : SDS-PAGE and western blot results of monoclonal P6B9, P1K15, and P2K18. Samples from left; **1.** Lig7 Control, **2.** Lig7 pellet, **3.** anti-KDR 1.3 P6B9 pellet, **4.** anti-KDR 2.6 P1E3 pellet, **5.** P1K15 pellet, **6.** P2K18 pellet, **7.** P1K15 cytosolic protein, **8.** P2K18 cytosolic protein.

After obtaining positive results from the western blot results, ELISA studies were performed with a histidine tag, however any significant result could not be obtained (Figure 2.37 and Figure 2.38).

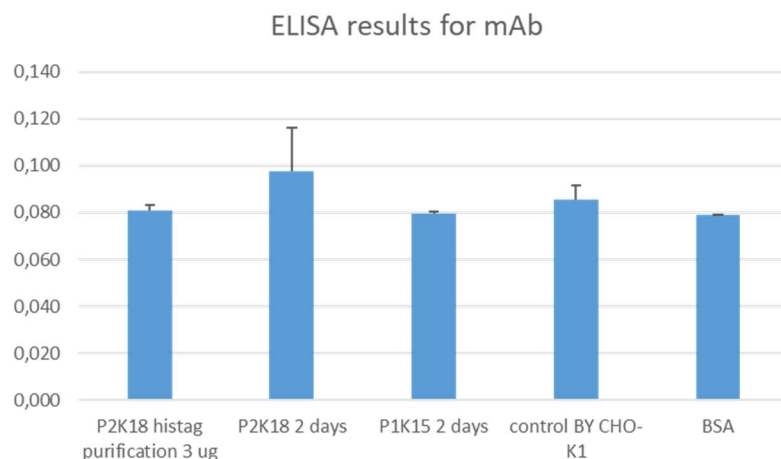


Figure 2.37 : ELISA results for monoclonal Ab expression of P1K15 and P2K18.

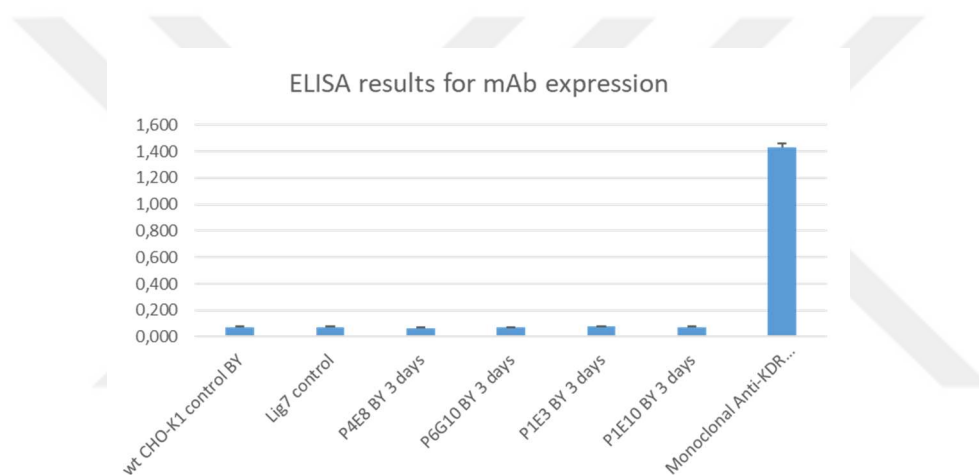


Figure 2.38 : ELISA results for monoclonal Ab expression.

2.4.9 Real time PCR studies

Primers suitable for the use of RT-qPCR synthesized for cells containing the gene construct have been indicated in Table 2.6. Primer pairs of the GAPDH gene were used as housekeeping gene usage in order to make relative quantification.

All primer pairs were tested by standard PCR reactions before used in the real time per assay. CHO-K1 was precipitated as 1×10^6 cells from control cells and transfected cells. Total RNA was isolated with the “Zymo Direct-zol RNA” isolation kit. The isolation protocol provided by the manufacturer was performed and cDNA was obtained.

cDNA synthesis was performed with the Bioline Sensifast cDNA synthesis kit. 1 μ g RNA was translated into cDNA by following the cDNA synthesis according to manufacturer’s instructions.

Real time PCR studies were carried out with cDNA for 50 ng by determining the concentrations of the cDNA samples obtained. PCR reaction was performed using iTaq Universal SYBR Green Supermix. Accordingly, the reaction protocol and thermal program used are given in the previous report. GAPDH and HPRT genes were used as housekeeping genes. dH₂O was employed as negative control.

All samples were run in 3 replicates. The Ct values obtained as a result of the RT-qPCR study are given below graphically according to the gene expression differences. As can be seen in the graphs containing the Ct values; A higher rate of reproduction was observed in the samples containing the construct compared to the control samples without the construct. Slight amplification was also observed in negative control samples defined as NTC (Figures 2.39).

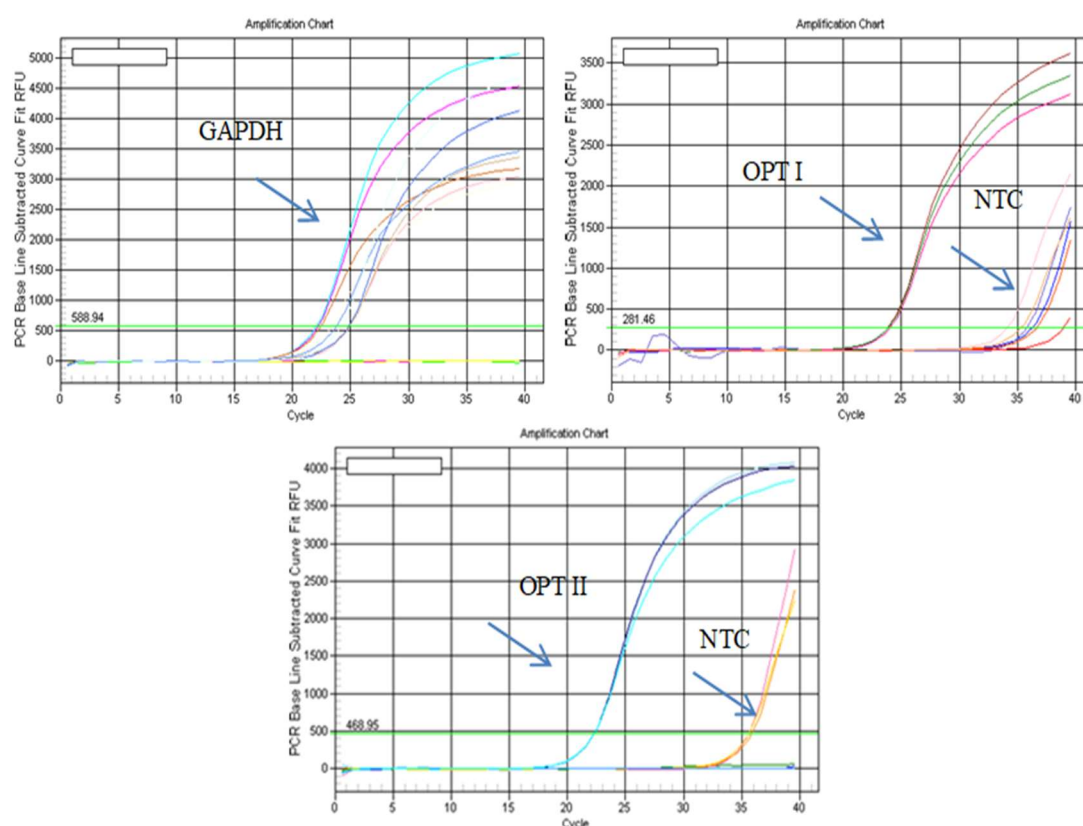


Figure 2.39 : RT-PCR results with control and transfected cells.

When the melting curve is examined, the peaks for housekeeping genes overlapped for all samples. Melting curves of the primers prepared for the samples containing the construct are different from the control samples (Figure 2.40). The results were obtained as expected.

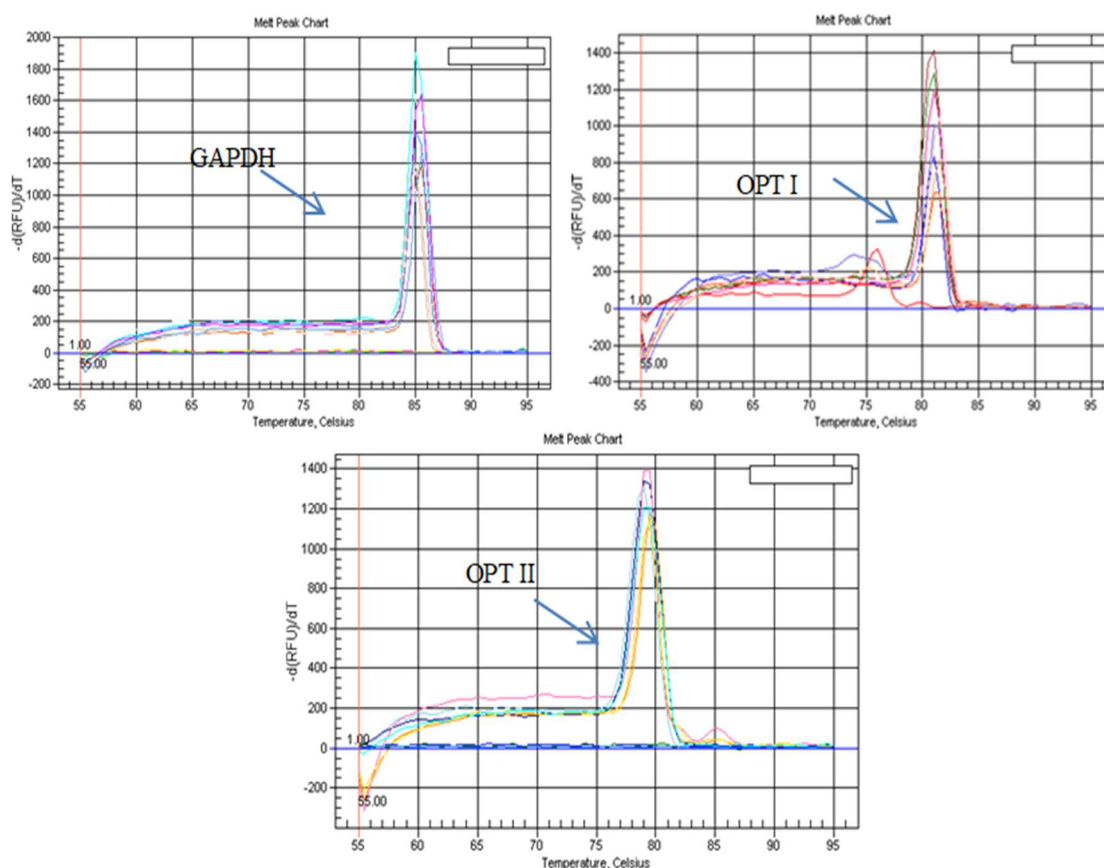


Figure 2.40 : RT-PCR melting curve results with control and transfected cells.

When the graphs are examined (Figure 2.41), the expression level of the cells containing the anti-KDR 2.6 P1K15 vector shows that the expression level of the cells with the GAPDH gene is 2916 times higher for 37°C, 2452 times higher for 31°C, 4608 for 37°C for 31°C, 5499 times higher than the HPRT gene.

The expression level of the cells containing the anti-KDR 2.6 P2K18 vector shows that the expression level of the cells is 5330 times higher at 37°C, 11113 times higher at 31°C, 6295 at 37°C, 6794 times at 31°C compared to the HPRT gene, relative to the GAPDH gene.

The gene expression level containing both vectors is quite high compared to housekeeping genes. P1K15 samples have higher 31°C expression level than HPRT gene, 37°C HPRT expression level higher than GAPDH gene. P2K18 samples have higher 37°C expression level than HPRT gene, 31°C expression level higher than GAPDH gene.

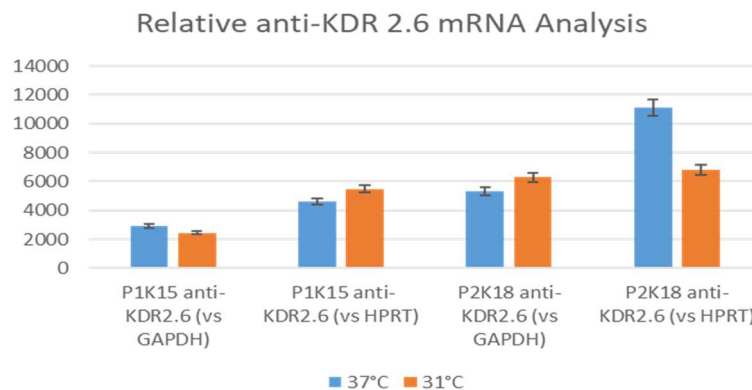


Figure 2.41 : Relative anti-KDR 2.6 mRNA expression of control and transfected cells.

When the graphs are examined (Figure 2.42), the expression level of the cells containing the anti-KDR 2.6 OPT I vector shows that it is expressed 9476 times more than the GAPDH gene. The expression level of cells containing the anti-KDR 2.6 OPT II vector indicates that it is overexpressed 3875 times relative to the GAPDH gene. Although the gene expression level containing both vectors is quite high compared to housekeeping genes, no results can be obtained from SDS-PAGE, western or ELISA.

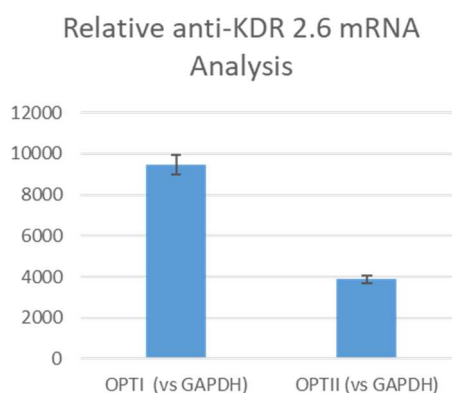


Figure 2.42 : Relative anti-KDR 2.6 expression results of control and transfected cells.

2.4.10 Flow cytometry

Flow Cytometry studies were carried out with the pIRES-AcGFP1-anti-KDR 2.6 (P6B9 & P1E3) constructs, which express the target gene together with the fluorescent protein AcGFP1. The fluorescence wavelength of the cells was controlled by CytoFLEX Flow Cytometer (Beckman Coulter) without any treatment. Following the initial testing, it was discovered that the AcGFP1 protein expressed in the cells emitted a signal at a wavelength close to FITC, indicating that this dye could not be utilized.

Therefore, the Rat anti-Ms IgG (Kappa light chain) antibody was conjugated with R-phycoerythrin. According to the results, significant binding to Rat anti-Ms IgG (Kappa light chain) could not be obtained. When the 3 day and 20 day expression data were compared, 20 day samples had a stronger signal than the 3 day expression samples. (Figure 2.43 and Figure 2.44).

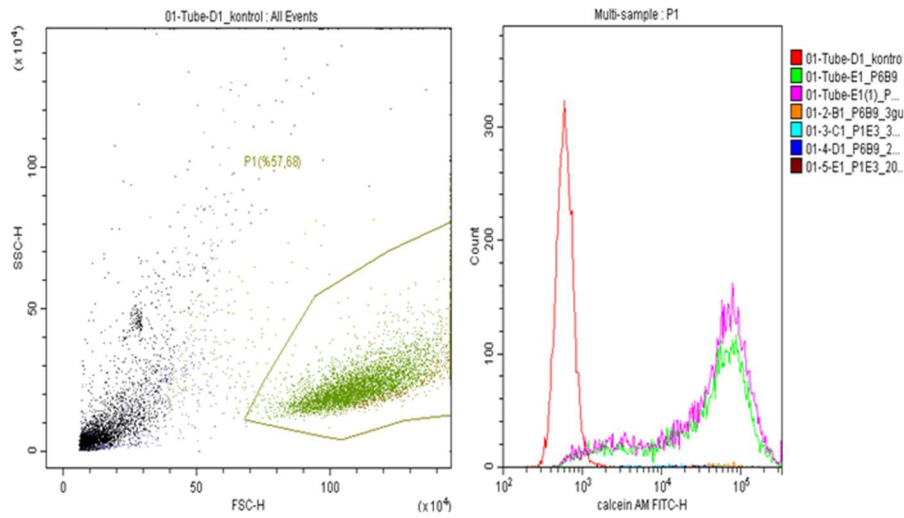


Figure 2.43 : Fluorescence wavelength analysis with Beckman Flow Cytometry device.

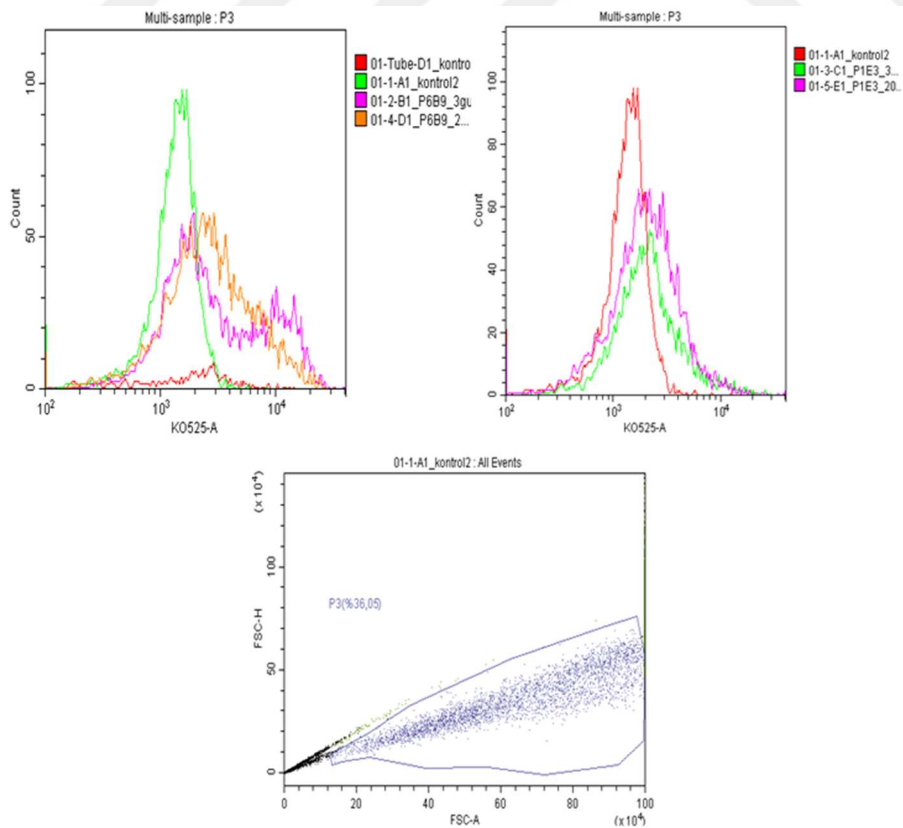


Figure 2.44 : Image of cells treated with IgG (Kappa light chain).

However, since the cells were permabilized without fixation, it was thought that some samples were lost at this stage and therefore no significant response could be obtained.

2.4.11 HDX-MS studies

The first HDX-MS experiment revealed the identification of 33 peptides within anti-KDR 1.3 with an 82.7 % sequence coverage with regions 1-2, 30-39, 53, 66, 81-85, 116-117, 145, 166-168, 187, 218, 239- 266 undetected (Figure 2.45). Time-course measurements of H/D exchange showed the overall solvent accessibility (Figure 2.46).

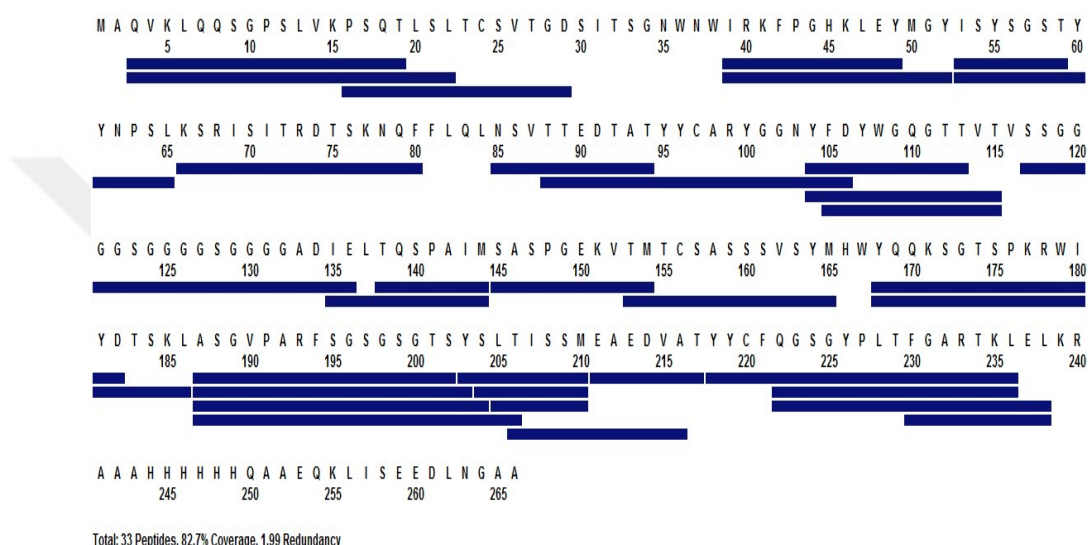


Figure 2.45 : Sequence coverage of anti-KDR 1.3 identified from four technical replicates. Peptides are represented as blue bars and aligned to their relative position along the primary sequence.

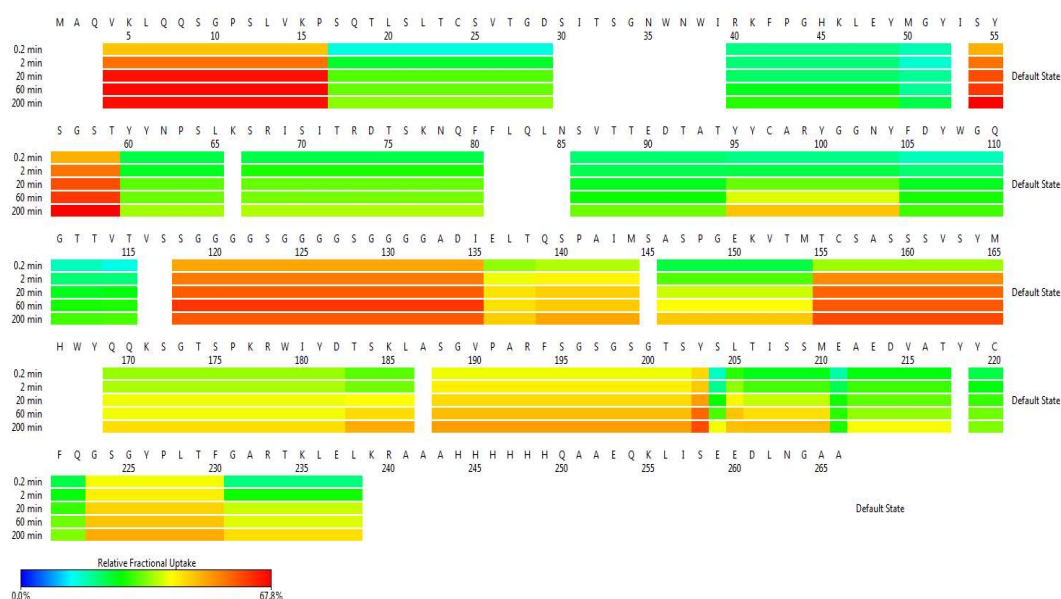


Figure 2.46 : Heat-map indicating time-course H/D exchange measurements of anti-KDR 1.3.

The level of deuteration was monitored at 0.2, 2, 20, 60, 200 min and 12 hr time intervals. The 12 hr deuteration data was removed as it was resulted in a high back exchange. Time-course measurements of H/D exchange of anti-KDR 1.3 showed the overall solvent accessibility information.

HDX-MS results on anti-KDR 1.3 revealed fast incorporation of deuterium at early time points in the most of peptides, whereas local exchange is evident beyond a specific time point (Figure 2.46).

Deuterium integration was evaluated on the overall structure and peptide specific deuterium absorption values were predicted onto the simulated structure of anti-KDR 1.3 (Figure 2.47 and Figure 2.48).

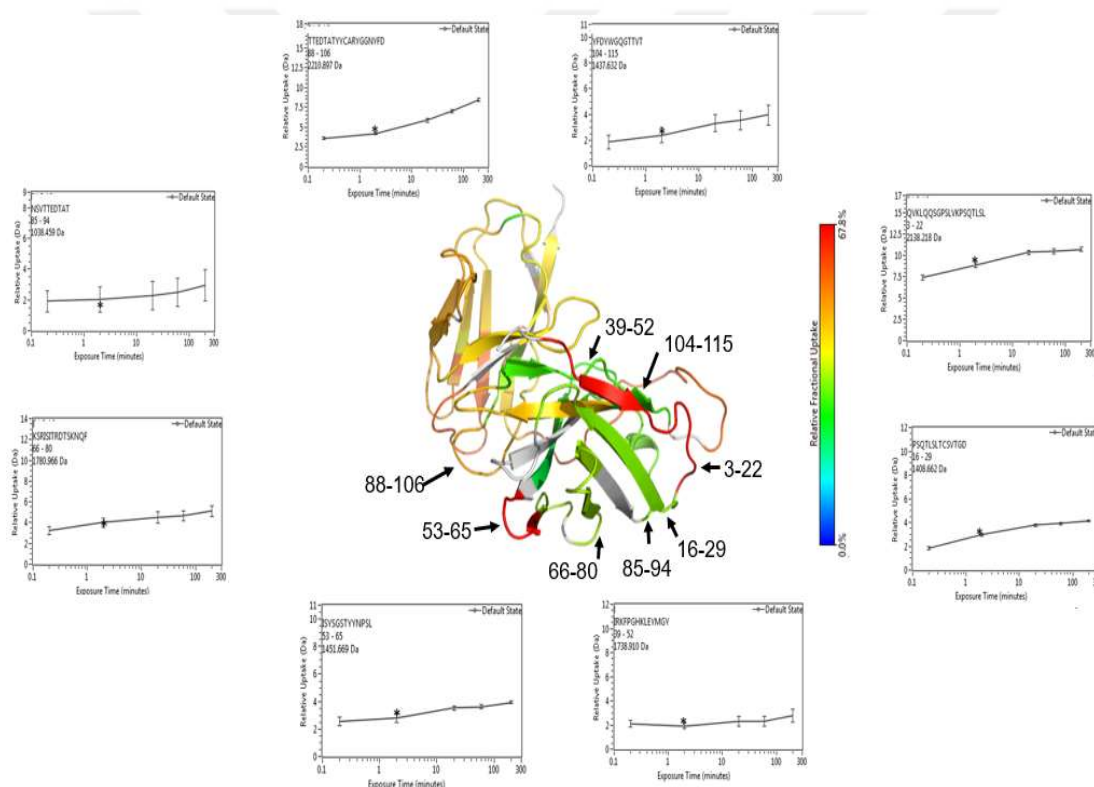


Figure 2.47 : Peptides with different deuterium uptake profiles at specified time intervals.

The highest degree of relative protection was observed in the CDR1 of VH domain. Similarly, linker region is more labile, stayed unexchanged during the entire monitored time. To assess the stability of the protein, samples were incubated for 3 hr and 20 min, the highest time point, and then labeled with 2 min of deuterium. The stability values at 2 min are very close meaningfully the protein is stable for 3 hr at room temperature (Figure 2.49).

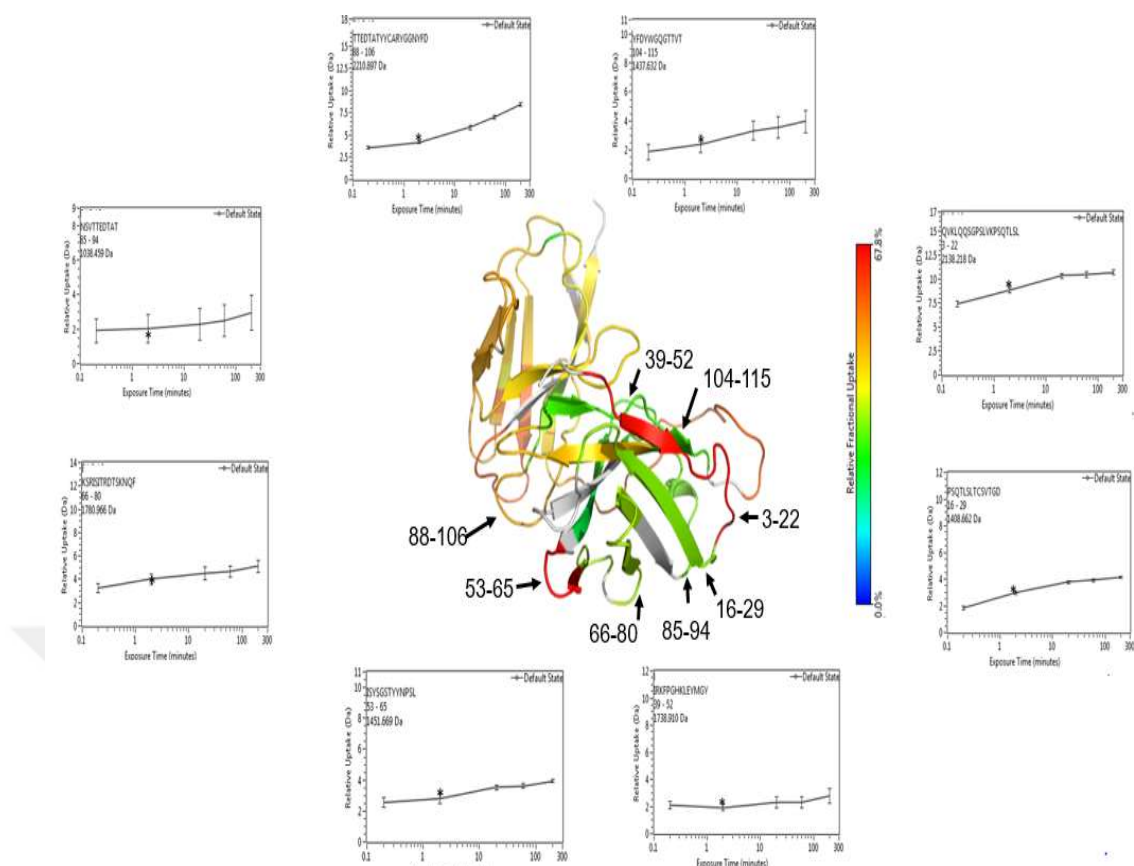


Figure 2.48 : Peptides with different deuterium uptake profiles at specified time intervals.

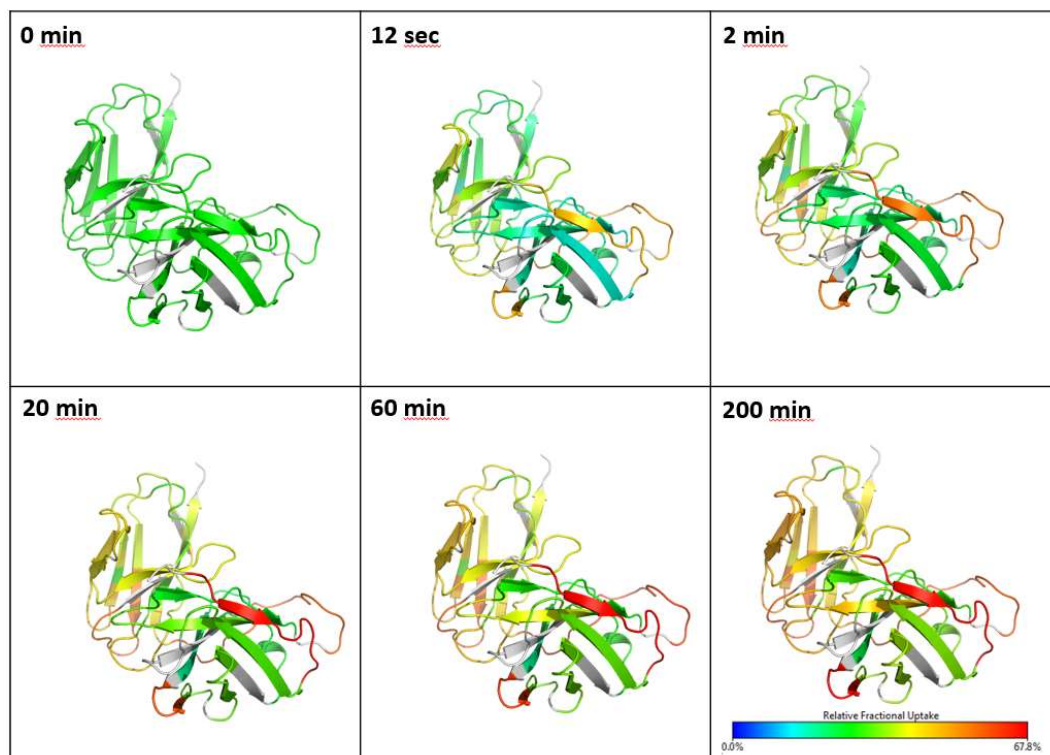


Figure 2.49 : Deuterium uptake kinetics of representative peptides of anti-KDR 1.3.

The data suggested that for both VH and VL all three complementarity determining regions (CDR) domains exhibited more solvent-exposed structure correlating well with the flexible and dynamic structure as expected (Figure 2.49).

In the first part of the thesis, target scFv production in mammalian cell culture were explored. The positive results of the real time PCR studies, indicate higher expression levels of the target proteins compared to housekeeping genes. However, higher expression efficiency for the target anti-KDR compounds in mammalian cells could not be observed as high protein levels. Monoclonal cell studies, fed-batch trials, codon optimizations, and signal peptide alternatives all confirmed the overall results of low or no expression.

The selection of high-product-generating clones was time-consuming and labor-intensive processes in the production of recombinant proteins. As a result, the second half of this thesis concentrated on creating a novel approach for rapidly increasing the solubility and synthesis of the target proteins.

3. CHAPTER II - SOLUBLE PROTEIN PRODUCTION VIA CDR GRAFTING

3.1 Purpose of the Chapter

The effect of the CDR grafting technique on the solubility and efficacy of the expressed protein was investigated in this chapter. The scFv structure has low biophysical features including a higher aggregation tendency and lower thermodynamic stability, leading low performance and application. To improve the biophysical properties of these scFv molecules, I used CDR grafting method to modify and upgrade the structure of the existing scFv with a more producible pre-existing scFv. I used computational methods to model our antibody structure and physicochemical properties, followed by experimental confirmation.

The newly developed and synthesized grafted scFv molecules' solubility will be compared to the original scFv molecule. SDS-PAGE and Western blot will be performed for the control of the increase in the solubility of the expressed protein. Also, SPR studies will be performed for the comparison of grafted molecule and the original molecules binding affinities to the VEGFR-2 receptor. *In vitro* activity assays, including HUVEC migration assays, HUVEC proliferation and wound healing assays, will be explored for investigation of the angiogenic properties of the newly synthesized grafted scFv.

3.2 Literature Review

3.2.1 *E. Coli* protein production advantages and disadvantages

The main factors contribute to *E. coli*'s potential as a host for producing recombinant antibody fragments are as follow; very well characterized; grows considerably quicker, achieves greater cell densities, and uses much less expensive growth medium than mammalian cells; although it lacks post translational modifications, it can produce disulphide bonds (Kasli et al, 2019).

Even after proper plasmid and host selection, it is impossible to predict whether the molecules will be produced in large quantities and soluble active state. Number of challenges that hinder progress toward that aim may arise, which occurs frequently. There are various problems that can be encountered throughout the production process in *E. coli* (Rosano and Ceccarelli, 2014).

No or low expression levels can be observed during the process. Low expression levels may arise from toxic proteins or codon bias.

Inclusion body formation is another big obstacle encountered during production. Inclusion body formation is observed when there is a problem in disulfide bond formation, improper folding, poor solubility, or a lack of post-translational modification.

Obtaining inactive proteins at the end of the production is another big problem. Inactive proteins may arise from incomplete folding or mutations in cDNA.

3.2.2 Solubility strategies

Protein folding is a critical step in the conversion of a polypeptide from a linear sequence to its 3D form. The oligomerization and/or polymerization of unfolded peptides and proteins is known as protein aggregation. A variety of techniques for recovering proteins from inclusion bodies are described in the literature. Overall, they may be split into treatments that refold protein from inclusion bodies and techniques that modify the expression strategy to achieve soluble expression. Recovering from inclusion bodies is often regarded as undesirable, although it is occasionally the preferred procedure.

The main challenges include low recovery yields, the need to optimize refolding parameters for the target protein, and the risk of losing the stability and activity of the target protein. Furthermore, abundantly expressed soluble protein purification has low cost and save time compared to the refolding and purification from inclusion bodies technique (Middelberg, 2002). Optimizing the development of recombinant proteins in soluble form is thus a more appealing option compared to the refolding technique. There are a variety of strategies to express a protein without engineering of the protein (Figure 3.1).

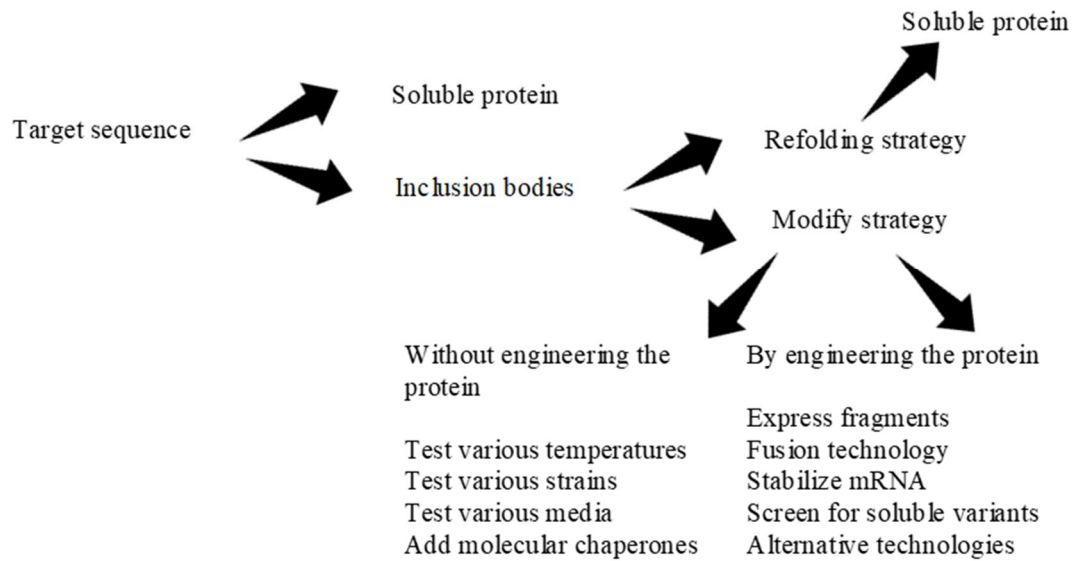


Figure 3.1 : Solubility strategies for protein production.

In principle, producing recombinant proteins causes a "metabolic burden" for the cell (Sorensen and Mortensen, 2005). Inclusion bodies form as a result of the excess concentration of denatured protein. They are not directly related, yet they are both critical elements in determining a cell's capacity to make soluble recombinant protein. As, they can be regulated by a variety of environmental conditions, production of soluble protein is probable.

Cultivation at low temperatures is a well-known strategy for reducing recombinant protein aggregation. This strategy has been shown to enhance the solubility of a variety of several problematic proteins (Schein, 1989; Vasina and Baneyx, 1997). Bacterial growth at low temperatures is expected to minimize protein aggregation because it reduces production and folding, minimizes the hydrophobic relations critical for aggregation (Schumann and Ferreira, 2004). Bacterial growth, on the other hand, is also limited, resulting in a reduction in biomass (Weickert et al, 1996).

To minimize the metabolic burden associated with high levels of protein production, a variety of host strains have been produced. Several methods have been employed for engineering the bacterial strains including protein production, breakdown, export in cell, and folding (Makino et al, 2011). These modified organisms include Rosetta (Invitrogen), SHuffle strain (New England Biolabs), Origami strains (Novagen), C41 and C43 strains (Avidis), and BL21 (DE3) strain (Novagen) which increases the synthesis of proteins (Makino et al, 2011).

Media selection and optimization of cultivation parameters are critical factors in soluble recombinant protein production. Several proteins require the presence of prosthetic groups and certain cofactors to fold properly. The addition of polyols, sucrose, ethanol, thiols and disulfides to the culture media might stabilize the target protein structure (Weickert et al, 1999; Yang et al, 2003). Overall, the content of the cell culture media, pH, inducing time and amount, are all critical in preventing protein aggregation, needs to be precisely controlled (Jana and Deb, 2005).

Coexpression of molecular chaperones is one potential method of avoiding inclusion body development. Molecular chaperones aid protein folding by preventing partly folded, misfolded polypeptides, protein aggregation, and transporting molecules to their intracellular destinations (Haacke et al, 2009). The chaperones mediating protein folding in diverse ways include Hsp70 and Hsp60 families (hsHsp proteins), DnaK, GroEL, and ClpB (Nishihara et al, 1998; Deuerling et al, 2003; Kolaj et al, 2009; Costa et al, 2014).

For the proteins that can not be solubilized by the strategies above, there are a variety of methods involving engineering of the proteins. Fusion protein strategy have been used for a long time. They have been shown to boost protein production, reduce proteolysis, and enhance solubility, as well. The most frequently used fusion proteins are; MBP, small ubiquitin modified (SUMO) and GST (Smith and Johnson, 1988; di Guan et al, 1988; Marblestone et al, 2006).

If, despite all efforts, the desired protein persists in inclusion bodies, the best solution is to denature and refold. However, neither of these approaches guarantees that recombinant protein solubility will increase and solve the aggregation problems.

3.2.3 Computational approach

Several computational techniques focusing on investigation of physicochemical characteristics of proteins and possible connection to form and activity have been developed during the last several decades. Many of these computational researches have resulted in significant breakthroughs in protein structure analysis, stability, folding, aggregation, solubility, PTMs and function (Hwang and Park, 2008).

A variety of techniques based on amino acid sequences have been developed in recent years to estimate protein solubility (Amador, 2015). Some of the available protein

solubility predictive models which have an accuracy range of 51 – 64% include PROSOII, ccSOL, SOLpro, PROSO, RPSP, SoluProt (Ghomi et al, 2020).

Molecular dynamic simulations analyze millions of particle interactions across billions of time steps, which might necessitate massive quantities of processing power and time. Simulations involve leap-frog Verlet, velocity Verlet, Brownian and stochastic dynamics, as well as energy reduction, normal-mode analysis. Simulations can be atomistic or coarse-grained, utilize explicit or implicit solvents, and apply a variety of geometric restrictions. There are a number of commonly used molecular dynamic simulations including; GROMACS, AMBER, NAMD, CHARMM, LAMMPS, and Desmond (Abraham et al, 2015).

One of the most common issues in the protein expression is the functional characterisation of a protein sequence. This effort is frequently aided by a precise 3D form of the target protein. Comparative homology modeling estimates the three-dimensional structure of a particular protein based mostly on its alignment to existing ones (Eswar et al, 2006). Homology modeling is predicated on observation when 2 sequences have a significant level of similarity, their structures are likewise comparable. MODELLER (Webb and Sali, 2014), MaxMod, PyMod, and PRIMO are popular computational methods to estimate 3D forms by homology modeling (Gromiha et al, 2019).

3.2.4 CDR grafting

Despite the apparent advantages, one notable disadvantage has been the potential of non-human-derived antibodies to induce human immunogenic response. In general, a considerable immune response is generated against foreign sequences of murine antibodies, resulting in a significant human-anti-mouse antibody (HAMA) response (Apgar et al, 2016). The HAMA response shortens the half-life of proteins by rapidly removing them from body; limiting their application. Because of these constraints, many antibody engineering approaches have been developed for decreasing the HAMA effect, causing production of chimeric and humanized antibodies (Mirick et al, 2004).

Complementarity determining regions (CDR) grafting technology was invented and patented by Sir Greg Winter and colleagues for humanization of mAbs to reduce their immunogenicity (Riechmann et al 1988, Jones et al 2004). Six CDRs are commonly

found on the antigen-binding surface of mAbs, which are distributed between the heavy chain and light chain variable domains. The most significant information on antigen-specificity in a target protein is found in the CDRs and adjoint regions. This understanding has led to the invention of CDR-grafting, one of the most widely used methods of antibody humanization, which incorporates these regions into the structure of a human antibody (Paul, 2012). As a result, the patient's immune system is less likely to identify the target protein as a foreign molecule. For antibody humanization, CDR-grafting remains the most favored approach. A traditional technique for humanizing antibodies includes identifying antigen-binding domains in the main antibody and selecting FR to give physical integrity for grafted CDRs linked to human antibody. Furthermore, it entails adjusting amino acids beyond grafted CDRs in order to preserve/increase the affinity of humanised antibody in Figure 3.2 (Almagro and Fransson, 2008).

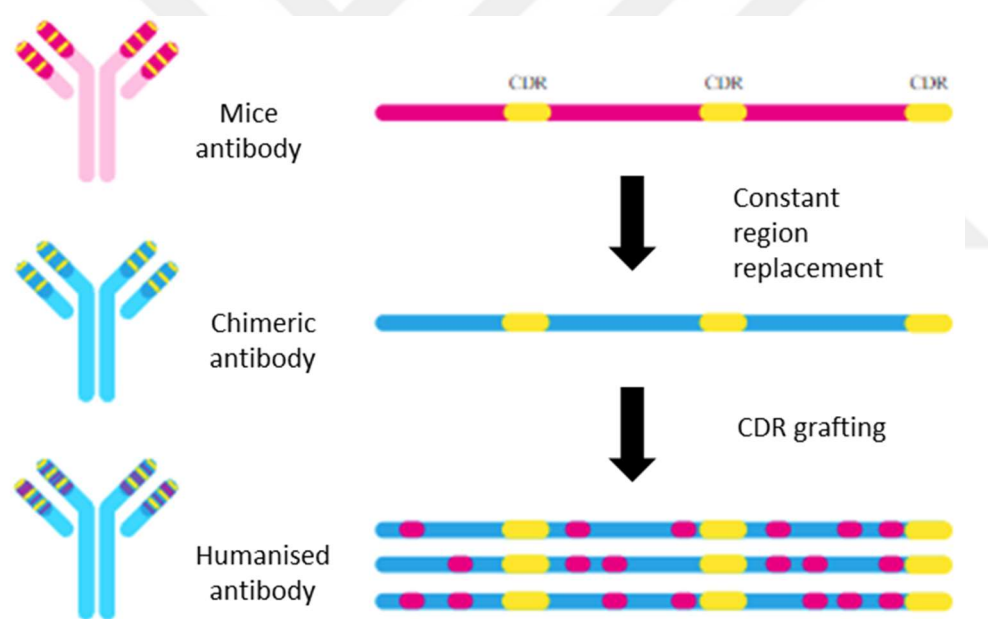


Figure 3.2 : Generation of chimeric and humanized antibodies.

The protein's affinity for its initial target is frequently decreased after CDR grafting. This is because, although not actively engaged in antigen binding, some residues are critical for stabilizing configuration of the CDR loops and sustaining the interaction VH and VL regions. To avoid challenges presented by CDR grafting approach, computer modeling and sequence optimization techniques are commonly preferred (Lonberg, 2005). Human FWs with largest similarity to the FRs of other antibodies are often selected as acceptors for CDR grafting (Safdari et al, 2013).

3.3 Materials and Methods

3.3.1 Sequence analysis and CDR grafting

The IMGT numbering method was used to calculate FR and CDR of anti-KDR 1.3 and Lig7 molecules using the Abyss web server (Table 3.1). The grafted scFv molecule was constructed by grafting the six anti-KDR 1.3 CDR loop sequences onto the Lig7 framework. The original linker sequence (GGGSGGGSGGGGS) was kept for covalently attachment of VH and VL chains.

Table 3.1 : IMGT numbered FR and CDR sequences of anti-KDR 1.3 and Lig7 molecules.

Region	anti-KDR 1.3	Lig7
HFR1	QVKLQQSGPSLVKPSQTLSTCSVT	QVQLQESGGALVKPGGSLKLSAAS
HFR2	WNWIRKFPGHKLEYMGY	MSWVRQTPEKRLEWVAA
HFR3	YYNPSLKSISRITRDTSKNQFFLQLNSV TTEDTATYYC	YYPDTVKDRFTISRDNVKNTLYLQMRSLR SEDATVYYC
HFR4	WGQGTTVTVSS	WGQGTTVTVSS
HCDR1	GDSITSGN	GFTFSNYA
HCDR2	ISYSGST	INNNGGST
HCDR3	ARYGGNYFDY	VRQWSWADY
LFR1	DIELTQSPAISASPGKVTMTCSAS	DIELTQSPASLSVSVGETVTITCRAS
LFR2	MHWYQQKSGTSPKRWIY	LAWYQQKQGKSPQLLVY
LFR3	SKLASGVPARFSGSGSGTSYSLTISSM EAEDVATYYC	TNLADGVSSRFSGSGSGTQFSLKIHSLSQSG DFGTYC
LFR4	FGARTKLELKRA	FGGGTKLEIKRA
LCDR1	SSVS	ENIFRS
LCDR2	DT	DA
LCDR3	FQGSYPLT	QHFWGTPWT

HFR: Heavy chain framework, LFR: Light chain framework, LCDR: Light chain complementary determining region, HCDR: Heavy chain complementary determining region.

The grafted scFv molecule was constructed by grafting the six anti-KDR 1.3 CDR loop sequences onto the Lig7 framework. The original linker sequence (GGGGSGGGSGGGGS) was kept for covalently attachment of VH and VL chains.

For homology modeling, the MODELLER software tool was utilized to identify a three - dimensional structure for all three scFv molecules. The entire sequence (VH Linker VL) of each scFv molecule was compared to MODELLER's PDB database to detect homologous sequences (Webb and Sali, 2016). The sequences were aligned against built-in-PDB database of MODELLER to determine homolog sequences with a cut off value; coverage: 75%, sequence identity: 60%. For each scFv molecule, several PDB structures with high homology to the query sequence were employed as templates for homology modeling (Table 3.2).

Table 3.2 : Template PDB structures used for homology modelling of scFv molecules.

scFv molecule	Template Pdb Code (Pdb Chain)
anti-KDR 1.3	1F3R (B), 1QOK (A), 6TOU (A), 5JYM (B), 4LAQ (H), 3GKZ (A)
Lig7	5B3N (A), 6EK2 (H), 5LX9 (H), 6G8R (A), 6EHX (B), 5DFW (H), 6DSI (A)
anti-KDR 1.3G	5B3N (A), 2GHW (B), 6EK2 (H), 6KN9 (D), 6G8R (A), 6EHX (B), 5F72 (T), 6DSI (A)

Using the corresponding templates, 100 models were constructed for each scFv molecule, with each model being first optimized using the variable target function method (VTFM) and then improved by a molecular dynamic technique. The corresponding settings were reset to MODELLER defaults for the optimization protocol. There was no usage of loop modeling. To analyze and rank the model structures, Discrete Optimized Protein Energy (DOPE) scores were determined, and the highest 10 models were exposed to reduction using molecular dynamics simulations. For each scFv molecule, the structure with the lowest foldX energy score was selected for future molecular dynamics simulations (Schymkowitz et al, 2005).

GROMACS 2020.1 simulation program was used to run molecular dynamics simulations of scFv molecules. The molecule in aqueous phase was analyzed using the CHARMM36m force field (Huang et al, 2017) and the TIP3P (Jorgensen et al, 1983) water model. A dodecahedron simulation box with a size adjustment was employed to ensure that sequential pictures of molecules do not collide with one another. Due of the charged amino acids present in scFv molecules, a suitable amount of counterions (Na^+ and Cl^-) was injected to counteract each surface charge on scFv molecules. Each scFv molecule was initially reduced and afterwards allowed to equilibrate using a number of NPT simulations in which the scFv molecule's constraints were progressively removed. Following equilibration, a Berendsen barostat (Berendsen et al, 1984) was employed to maintain a constant pressure (1 bar) in all directions (with a time constant of 1 ps). To maintain the temperature at 298 K, V-rescaling was used (with a time constant of 0.1 ps). The equilibration time was sufficient to detect the box volume converging. The equilibrated scFv/solvent complex was next reconstructed for 200 ns, that provided sufficient time to see the scFv molecule's root mean square deviation (RMSD) convergence from its native form. The Berendsen barostat was substituted for the Parrinello-Rahman barostat in the simulations (Parrinello and Rahman, 1981) (with a time constant of 5 ps). Across whole MD simulations, the time interval was set to 2 fs and the Verlet cutoff technique was used with the suggested restrictions and cutoff values (for the CHARMM36m force field, see the CHARMM website).

The original models were revised using 200 ns molecular dynamics simulations, with convergence determined by RMSD of each FR and CDR unit relative to the original configuration. For every molecule, the first half of the 200-ns MD simulation was deleted for equilibration considerations, and the final 3D structure was obtained by time-averaging the remaining 100 ns trajectory.

Ultimately, for equilibration, the first part of the simulation duration (100 ns) was removed. For the second part, a mean structure was calculated first using GROMACS's "gmx rmsf" subroutine, and then optimized using a variety of energy minimizations. This procedure was performed for every scFv, and the mean structures arising from it were physically matched via the framework areas. For FW and CDR loops, the RMSD scores between the C (and backbone) atoms of anti-KDR 1.3, Lig7, and grafted scFv

molecules were calculated separately. VMD 1.9.3 visualization toolkit was used to generate all images (Humphrey et al, 1996).

3.3.2 Bacterial strain and plasmid design

For vector amplification and cloning, the *Escherichia coli* (*E. coli*) BL21 Star was employed. The protein sequence acquired through modelling was reverse-translated into the nucleotide sequence using the OPTIMIZER web service (<http://genomes.urv.es/OPTIMIZER/>). I produced an expression vector, pET-22b(+) (GenScript), which contains the nucleotide sequence together with a pelB leader sequence at the N terminus, c-myc tag, and a poly-histidine tag (His6) at the C terminus. The generated vector was transformed into *E. coli* BL21 Star cells.

3.3.3 ScFv expression and solubility assay

A single colony of transformed bacterial cells was seeded into 4 mL 2xYT medium with 100 g/mL ampicillin and 34 g/mL chloramphenicol, and grown overnight at 37°C. The culture was then expanded by adding the starting culture to a larger volume of 2XTY with antibiotic, % 2 Glucose, and incubating for 1-4 hr until the culture density reached 0.50.6 OD₆₀₀. Expression was stimulated by adding IPTG to a final concentration of 0.5 mM when the culture achieved an OD₆₀₀ of 0.5-0.6. After 20 hr of induction at 30°C at 240 rpm, the cells were harvested by centrifugation.

The level of expression and the location of the protein were investigated in total, soluble, and insoluble protein samples. SDS-PAGE and Western blot analyses were performed on samples taken from the expression culture. Samples were collected from cultures by centrifugation at 5000 g for 15 min at 4°C for total protein preparation. B-PER (Thermo Scientific, 90078, USA) was used to dissolve the pellets in bacterial protein extraction reagent (lysozyme, Dnase, protease inhibitor cocktail). Total protein samples should be centrifugated at max speed for 10 min at 4°C to prepare soluble and insoluble protein samples. The pellets contain insoluble proteins, whereas the supernatant contains soluble proteins.

The folding characteristics of recombinant proteins at 30°C were determined by examining the soluble and insoluble components. Another method for evaluating the folding characteristics was to determine the activity of protein expressed in bacteria.

SPR analysis was used to determine the binding of grafted molecule to the VEGFR-2 receptor.

3.3.4 SDS-PAGE and western blot analysis

SDS-PAGE procedures were carried out according to standard procedures. Similar amounts (15 L) were mixed with an equal volume of 2x loading buffer (100 mM Tris–HCl (pH 6.8), 4% SDS, 0.2 percent bromophenol blue, 20% glycerol, and 200 mM -mercaptoethanol), which was incubated at 95°C for 5 minutes before being loaded onto a 12% separating gel. Gels were stained using Coomassie Brilliant Blue at a concentration of 0.1%. The Trans-Blot Turbo Transfer System was used to transfer gels to nitrocellulose membranes in preparation for Western blot (Figure 1). (BioRad). A 2-hour blocking buffer (including 1% BSA in PBS and 0.1% Tween-20) was used to treat the nitrocellulose membrane after transfer. The presence of target bands was identified via anti-His antibody that had been labeled by HRP (Sigma). For a total of 5 minutes, three washing procedures were performed in TPBS. Adding the 4-Chloro-1-Naphthol (4C1N, Sigma) substrate solution to the antibody that had been tagged with the membrane allowed us to see the color creation.

3.3.5 scFv purification

The soluble scFv fragments were purified using affinity chromatography on AKTA Avant 25 chromatography system (GE Healthcare Life Sciences Chicago, IL, USA). After centrifuge, a binding buffer was used to dilute the soluble fractions (20 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 7.4). At a flow rate of 0.5 mL/min, a 25 mL diluted sample was applied to a prepacked 1 mL HisTrap FF crude column equilibrated with the same binding buffer. Following a wash cycle with binding buffer (W1), a second wash cycle was used to raise the imidazole percentage using a step gradient with 5% elution buffer. Target protein was obtained in elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4).

3.3.6 Surface plasmon resonance

Binding interactions were evaluated using a BIACORE T200 spectrometer Cytiva equipment. Using an amine coupling method, VEGFR-2D1-7 (Sigma-Aldrich, 676490) was immobilized on a CM5 Sensor Chip. A 1:1 solution of EDC/NHS [N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxy

succinimide] was used to activate the surface. VEGFR-2 (30 g/mL) was immobilized on the active surface at a flow rate of 10uL/min in a 10mM sodium acetate buffer, pH4.0. Final level of immobilization attained: 12900 response units (RUs). The matrix's residual active molecules were neutralized using 1 M ethanolamine HCl at a pH of 8.5. G Anti-KDR1.3Grafted scFv binding experiments were conducted in triplicate, whereas anti-KDR1.3 binding experiments could only be conducted once owing to poor transfection efficiency. Three cycles of start-up assay with the running HBS-EP+ buffer were used to conduct the study (10 mM HEPES pH 7.4, 0.15 M NaCl, 0.003 M EDTA with 0.05 % Surfactant P20). The analytes anti-KDR 1.3 (12.3 nM, 37 nM, 111 nM, 330 nM, and 1000 nM), as well as HBS-EP+ buffer alone, were then flowed over the flow cell immobilized with VEGFR-2 and the empty control flow cell. The Biacore T200 Evaluation Software 3.1 was used to analyze the double reference subtracted binding curves.

3.3.7 *In vitro* activity assays

3.3.7.1 Cell culture

Human umbilical vein endothelial cells (HUVECs, C2519A) were acquired from Lonza in Switzerland. HUVECs with lower than 8 passages were utilized. Cells were cultured in endothelial basal medium EBM-2 (CC-3156, Lonza) supplemented with 1 % penicillin (10,000 Units/mL) - streptomycin (10,000 Units/mL) (Gibco, Cat No 15140122) and EGM-2 SingleQuots (CC-4176, Lonza, Switzerland) containing growth factors (insulin-like growth factor, human fibroblast growth factor, human epidermal growth factor, vascular endothelial growth factor), and supplements (ascorbic acid, heparin, hydrocortisone, fetal bovine serum). Cell passage was performed three times each week, taking into account their population doubling period. In a humidified CO₂ incubator, cultures were maintained at 37°C. The HUVEC cells were used in all of the angiogenesis *in vitro* tests.

3.3.7.2 HUVEC migration assay

The real-time cell migration test was conducted with xCELLigence RTCA DP device. CIM-Plate-16 is a "Boyden chamber" system comprised of upper and lower chambers that are electrically connected for real-time and label-free evaluation of invasion and migration. The upper chamber is supported by a microporous polyethylene

terephthalate (PET) membrane, which allows cells to flow into the lower chemoattractant. The lower side of the membrane is covered with gold microelectrode sensors that detect a decrease in electrical current when cells adhere to it. This "impedance" signal enables quantitative kinetic analysis of cell movement between the upper and lower chambers.

To avoid variable cellular response, culture media was exchanged 16 hours before studies with starvation medium (EBM-2 with 2% serum and without growth factors). The xCELLigence equipment was maintained overnight in an incubator at 37°C. All through study, the equipment was maintained in incubator.

The membranes in the CIM-16 plate were coated with 0.1 percent gelatin to enhance cell adhesion. The lowest side of the CIM-16 plate was treated with starvation medium containing VEGF at a 5X concentration as a chemoattractant. To stabilize the membrane with the medium, 25 µl of starvation media was added to the upper side and incubated in the xCELLigence system for 1 hour. After incubation, the background was determined in the absence of cells.

In the upper side of the CIM-16 plate cells were cultured in starvation medium as 3×10^5 cells/well. The cells were cultured in 0.53 µM for anti-KDR 1.3 and 0.53, 1.32, 2.68 µM for anti-KDR 1.3G. After a half-hour incubation at RT, the CIM-16 plate was placed incubated in xCELLigence to allow the cells to disseminate and attach evenly on membrane. The migration assay was continually tracked for 24 hours at 15-minute intervals. The RTCA DP program was used to evaluate and display the cell index values.

Further studies were conducted with only grafted protein, as anti-KDR 1.3 could not have been expressed in sufficient quantities for activity assays.

3.3.7.3 HUVEC proliferation inhibition assay

The proliferation inhibition assay was carried out utilizing the xCELLigence RTCA DP real-time cell analysis tool. HUVEC cells were seeded in EGM-2 media and cultured at a total cell density of 5×10^3 cells per well into a disposable sterile 16-well E-plate of the xCELLigence RTCA DP device. Throughout the study, the device containing the cells was placed in a 37°C, 5% CO₂ humidified incubator. The cells were loaded on a 16-well E-Plate and cultured for cell adhesion using the xCELLigence RTCA DP device. Following incubation, EGM-2 media was exchanged

with starvation medium (EBM-2 with 2% serum and without growth factors) and cultivated for 16 hours. Following incubation, the starvation medium (EBM-2 with 2% serum and without growth factors) was aspirated and grafted molecules in VEGF-containing starvation medium were placed on E-Plate wells at different concentrations (1.32, 2.68, 5.36 M). Real-time measurements were recorded at 15-min intervals for 72 hr. At the end of the experiment, the data gathered were evaluated using RTCA DP software.

Every 15 minutes, the xCELLigence DP instrument translates electrical conductivity into the unitless cell index (CI) metric. A higher CI value indicates increased cell survival, whereas a lower value indicates cell death. Utilizing proliferation measurements, the doubling time of HUVEC cells was determined using the RTCA DP software. The IC₅₀ value for the grafted molecule was determined using sigmoidal dose-response curves utilizing the RTCA DP program.

3.3.7.4 HUVEC wound healing assay

The HUVEC cells (10×10^3 cells/well) were cultured in 96-well plate for the wound closure assay (Greiner CellStar, US). Following cell adhesion to the surface, the EGM-2 media was removed and the cells were cultured for 16 hours in a starvation media. After incubation, the plate was placed in the AutoScratch Wound Making Tool (BioTek) to produce uniformly divided wounds on cell monolayers. After that, the media were replenished with grafted molecules in a VEGF-containing starvation medium at various concentrations (0.05, 0.53, 1.32, 2.68, 5.36 M). For 24 hours, the cells were maintained in Cytation5 device at 37°C with 5% CO₂. Images were taken hourly to track cellular migration to the wound area. The Cytation5 device's Gen5 Software was used to measure and evaluate wound areas.

3.4 Results And Discussion

3.4.1 Molecular dynamic simulations

The formation of inclusion bodies, which is related with the low solubility of anti-KDR 1.3, is a significant setback that must be addressed in order for the scFv molecule to be further developed. It is aimed to increase the solubility of anti-KDR 1.3 by grafting its six CDR loops onto the framework of another scFv molecule, Lig7, which is known to have a higher solubility empirically. Before constructing the grafted scFv

structure, cutting-edge computational techniques were used to anticipate the influence of grafting on the biophysical characteristics of scFv molecules, such as solubility and stability, which are required for scFv activity. So for this purpose, it was proceeded by determining whether Lig7 is a viable contender to use as a framework platform. Its outstanding features, as demonstrated in earlier investigations, include high expression profile and a soluble active molecule (Sushma et al, 2011; Erdag et al, 2017).

I initially attempted to determine if variations in sequence and/or structure between anti-KDR 1.3 and Lig7 may explain their distinct expression patterns; inclusion body formation of anti-KDR 1.3 and soluble expression of Lig7. The VH/VL interfaces were focused on one of these two scFv molecules in particular to investigate if inclusion body formation of anti-KDR1.3 is caused by an impaired VH, VL pair. To accomplish this, homology modeling was used to predict the tertiary structures of anti-KDR 1.3 and Lig7 scFv molecules, and then used molecular dynamics simulations to improve the projected models and investigate the structural and thermodynamic properties of the underlying scFv molecules.

In terms of hydrogen bonding and solvent accessibility, Lig7 and anti-KDR 1.3 have distinct interfacial properties (see Figure 3.3). To identify residues between VH and VL, I first use (1) a distance-based criteria (VH residues near to VL (0.5 nm) and vice versa) and then (2) evaluate solvent accessibility (exposed surface area change > 15% following VH/VL pairing) of residues selected in step one.

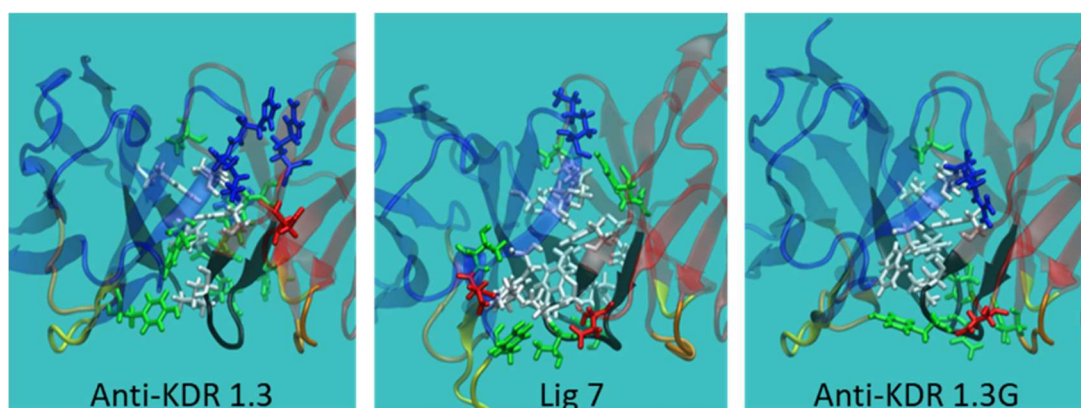


Figure 3.3 : VH/VL interface residues (shown as Licorice) of anti-KDR 1.3, Lig7 and Anti-KDR 1.3G scFv molecules colored according to their IMGT classes. Hydrophobic (white), polar (green), positively (blue) and negatively charged (red). Transparent cartoon representation is adopted for VH (blue) and VL (red) regions. CDR loops are color coded as follows: H1 and L1 (orange), H2 and L2 (yellow) and H3 and L3 (black). The interfaces are shown for trajectory-average structures (see Methods for details) of respective scFv molecules.

Anti-KDR 1.3 has an average of 2.5 hydrogen bonds per VH/VL contact, whereas Lig7 has 4.4 hydrogen bonds per VH/VL interface. Anti-KDR 1.3, interestingly, lacks a common interfacial hydrogen bond (L38Q-H39Q, according to Chothia numbering) (Kuroda and Gray, 2016) since H39 is occupied by lysine (rather than glutamine) in anti-KDR 1.3. The near proximity of H43H and L101R (0.25 nm) molecules in anti-KDR 1.3 seemed to be an abnormal point to consider for the VH/VL interface owing to a seldom formed hydrogen bond (created in percent 12 of the entire simulation period) (see Fig. 1). The arginine would be positively charged and the charge on the histidine molecule might be positively charged (pKa:6.22, PropKa) in low percentage. The histidine molecule would be in a hydrophobic area and will be affected by the surrounding amino acids, which can affect its protonation. So, overall, although in low percentage histidine protonation can be a destabilizing factor.

In terms of hydrophobic interactions, which are an important determinant of VH/VL interfaces, anti-KDR 1.3 has a total of 16.21 nm² of hydrophobic area (based on IMGT categorization; hydrophobic residues include Ala, Met, Cys, Phe, Ile, Leu, and Trp). For Lig7, this region expands to 25.6 nm². These results suggest that Lig7 has a more reliable VH/VL interface than anti-KDR 1.3. In terms of the grafted scFv molecule, where the CDR loops of anti-KDR 1.3 are grafted onto the Lig7 framework (see Methods for details), the buried hydrophobic area is calculated to be 20.4 nm², which appears to be greater than the one calculated for the original anti-KDR 1.3. Moreover, for the grafted scFv molecule, the average number of interfacial (VH/VL) hydrogen bonds is maximized (5.35), and the interfacial hydrogen bond between L38Q and H39Q is preserved.

The first step in grafting is to identify the framework and CDR sections of antibody molecules' variable domains. Although numerous antibody numbering schemes exist, including Kabat, Chothia, and AHO, the IMGT numbering scheme is the most widely used and recognized in immunogenetics.

Using the AByss web server (Swindells et al, 2017), the variable domains of anti-KDR 1.3 and Lig7 were IMGT numbered (see Methods for IMGT numbered anti-KDR 1.3 and Lig7 sequences). The solubility of scFv molecules was assessed using a standalone version of SoluProt, a machine-learning-based solubility prediction method. This sequence-based method outperforms previous solubility prediction algorithms in terms of accuracy (Hon et al, 2021). The solubility scores of anti-KDR 1.3, Lig7, and grafted

scFv molecules were evaluated as 0.48, 0.69, and 0.69, respectively, according to a notable solubility predictor, in which any value over 0.5 implies high solubility and molecules with a value lower than 0.5 implies insolubility.

Next, I explored at whether Lig7 grafting will affect structural characteristics important for scFv activity, such as VH/VL angularization and the conformation, as well as the arrangement of CDR loops. Anti-KDR 1.3 and Lig7 have 55.2 percent sequence similarity, which rises to 61.1 percent when just FR regions are examined. Furthermore, the lengths of specific framework sequences (e.g., HFR1, HFR2) of anti-KDR 1.3 and Lig7 are the identical (see Figure 3.4). Homology modeling was used to identify 3D structures for anti-KDR 1.3, Lig7, and grafted scFv molecules because neither of the underlying scFv molecules has a well-defined crystal structure (see Methods for details).

The RMSD analysis indicate that the framework and CDR scores of anti-KDR1.3 , Lig7 and anti-KDR 1.3G reaches convergence within the simulation time except for Lig7 CDR RMSD scores. . The RMSD plots of the Lig7 CDR region exhibit larger fluctuation values. However, because the anti-KDR1.3 and anti-KDR1.3G structures are the ones to be identified and compared, md simulations were continued.

Structures mounted on top of each other reveal that the β -sandwich structures that form the framework of variable domains have a significant degree of structural resemblance in the original and grafted molecules (RMSD = 0.23 nm). Furthermore, it is projected that the CDR loops of anti-KDR 1.3 and grafted molecule will fold into comparable conformations (RMSD = 0.26 nm) (see Figure 3.4).

A comprehensive examination of the VH/VL interfaces of projected anti-KDR 1.3 and Lig7 structures reveals that Lig7 is superior in terms of hidden hydrophobic area and the number of interfacial hydrogen bonds. Another significant change is the lack of the L38Q-H39Q hydrogen bond in anti-KDR1.3. Grafting anti-KDR 1.3 CDR loops onto the Lig7 framework improves the interfacial (VH/VL) characteristics compared to the original scFv molecule.

These findings imply that grafting onto the Lig7 framework will increase the stability of the VH/VL interface and reduce any (mis)pairing-based aggregation propensity of the original anti KDR1.3 molecule.

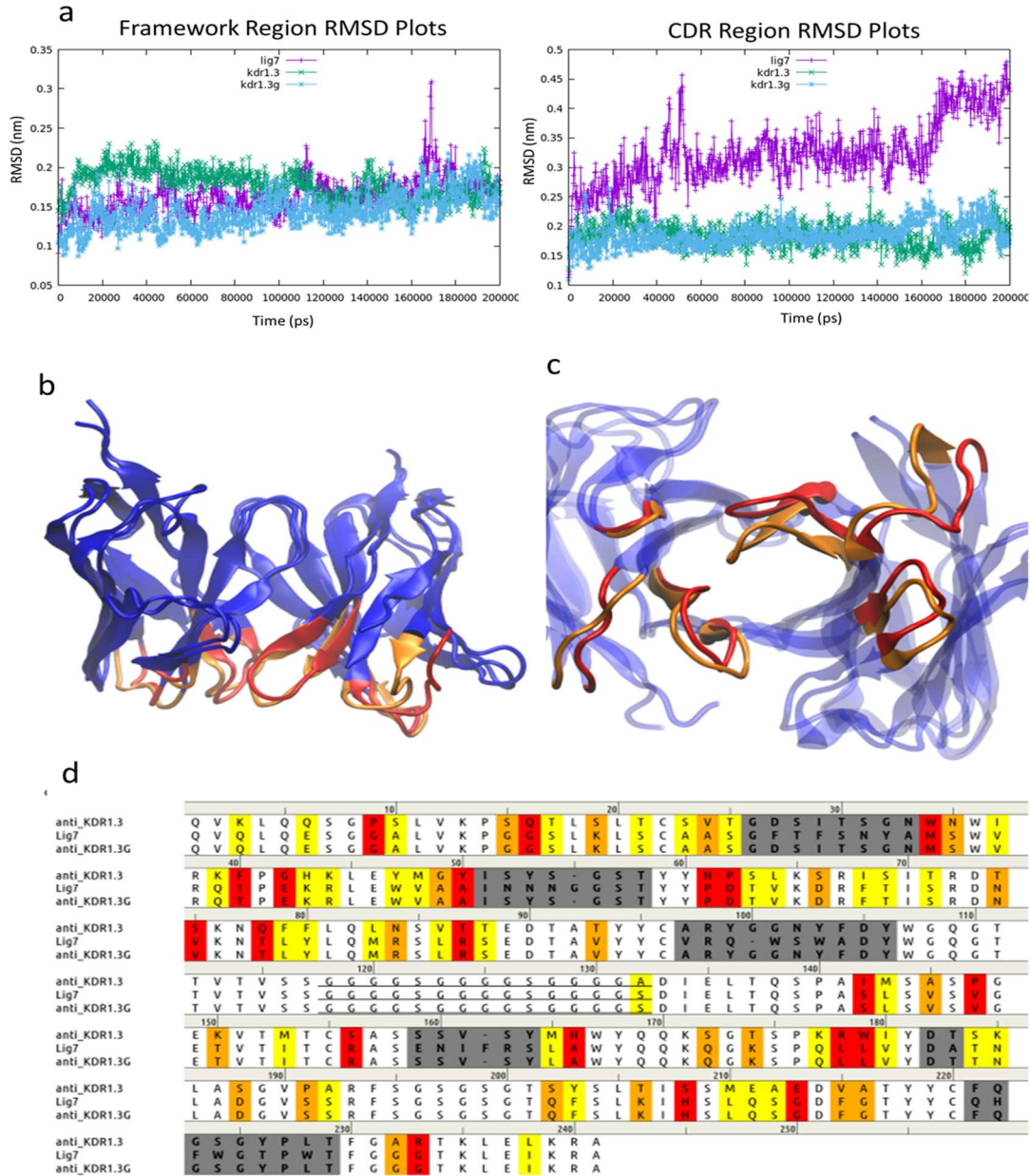


Figure 3.4 : RMSD plots of FW and CDR regions and comparison of superposed structures and aligned amino acid sequences of scFv molecules. (a) The RMSD scores between the C (and backbone) atoms of anti-KDR 1.3, Lig7, and grafted scFv molecules for FW and CDR loops colors representing green (anti-KDR 1.3), blue (anti-KDR1.3G), purple (Lig7). (b,c) Superposed structures of anti-KDR1.3 and grafted anti-KDR1.3 molecules. Frameworks are blue colored whereas CDR loops are red and orange colored for original and grafted anti-KDR 1.3, respectively (d) Aligned amino acid sequences of three scFv molecules. Amino acid similarities are represented by background coloring where white shows the identical residues.

Nonidentical residues are color coded based on the similarity of their physicochemical characters (e.g. aliphatic, aromatic, positively/negatively charged, etc.) where going from yellow to orange denotes a decrease in the degree of similarity amongst the residues that occupy the respective position. Red coloring represents the nonmatching residues. CDR loops (based on IMGT CDR definition) are gray shaded. The underlined amino acid sequence represents the linker region.

Regarding the data, an improvement in projected solubility scores after grafting onto the Lig7 framework suggests that the low solubility of anti-KDR 1.3 is mostly due to its framework, and that replacing it without changing antigen-specific CDR sequences can enhance solubility. The anticipated conformations of anti-KDR 1.3 superposed scFv structures and grafted scFv structures are quite similar.

The configurational variations in the CDR loops of anti-KDR 1.3 and grafted anti-KDR 1.3 scFv can be ascribed to the slightly different VH/VL orientations of these molecules. Overall, grafting scFvs, lig7, and anti-KDR 1.3 sequences are a perfect fit for frameworks, but a misalignment between CDRs and framework can lead to instability of the particular CDR-grafted domains (Honegger et al, 2009).

3.4.2 Soluble protein expression and purification

Solubility is a crucial property that is usually determined during the production of recombinant antibodies. Insufficient antibody solubility can cause self-aggregation which can cause loss of function, enhanced immunogenicity (Ratanji et al, 2013).

In *E. coli* high level production of scFv antibodies has largely resulted in the inclusion bodies, necessitating a comprehensive downstream process. To experimentally explore the expression of the designed grafted scFv, the designed vector was transformed and produced in *E. coli*. Then I further explored the *in vivo* folding behavior of induced scFv constructs. As indicated in the Methods section, samples were taken from the expression culture for SDS - PAGE and western blot analyses.

Total, soluble and insoluble protein samples were evaluated to determine the presence of expression and the location of the protein. *In vivo* folding analysis of grafted and original anti-KDR 1.3 molecules for soluble and insoluble fractions of periplasmic extracts by SDS_PAGE and Western blotting.

Figure 3.5 depicts soluble and insoluble scFv protein fractions that accumulated in the periplasm. When compared to the original anti-KDR 1.3, which has no solubility, the anti-KDR 1.3G molecule has increased in solubility (see Figure 3.5b).

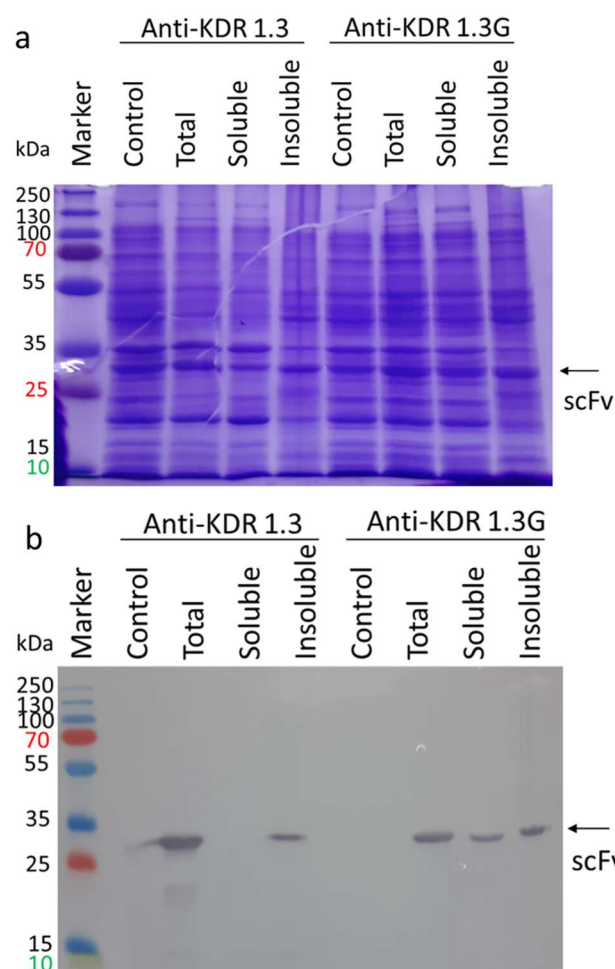


Figure 3.5 : *In vivo* folding analysis of grafted and original anti-KDR 1.3 molecules for soluble and insoluble fractions of periplasmic extracts by SDS_PAGE and Western blotting. **(a)** % 12 SDS-PAGE analysis **(b)** Western blot analysis. In the following order; Marker (Thermo PageRuler Plus Prestained Protein Ladder, 10-250 kDa) 1. No IPTG control for anti-KDR 1.3, 2. Total anti-KDR 1.3 t20 3. Soluble anti-KDR 1.3 t20 4. Insoluble anti-KDR 1.3 t20 5. No IPTG control for anti-KDR 1.3G, 6. Total anti-KDR 1.3G t20 7. Soluble anti-KDR 1.3G t20 8. Insoluble anti-KDR 1.3G t20.

An anti-KDR 1.3G scFv was purified from the total soluble protein fraction of a 25 mL cell culture at 25°C using a KTA Avant chromatography system. The second wash phase was done with a 5% elution buffer gradient after washing with binding buffer (20 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 7.4). (20 mM sodium phosphate, 300 mM NaCl, 500mM imidazole, pH 7.4). The quantity of contaminating protein in the eluted scFv fractions was significantly decreased after the second wash phase. Elution of target protein was observed by UV absorbance at 280 nm as a strong peak (see Figure 3.6 a). SDS-PAGE and western blot analyses were used to evaluate and semi-quantify the amounts of anti-KDR 1.3G scFv in the load, flow-through, wash, and elution fractions, revealing that unbound, non-specific proteins were mainly

eliminated in the flow-through and wash fractions (see Figure 3.6 a,b). The elution fractions contained a high concentration of anti-KDR 1.3G scFv. The eluted fraction was desalted to eliminate excess imidazole from the sample and stored at -20°C for long-term storage.

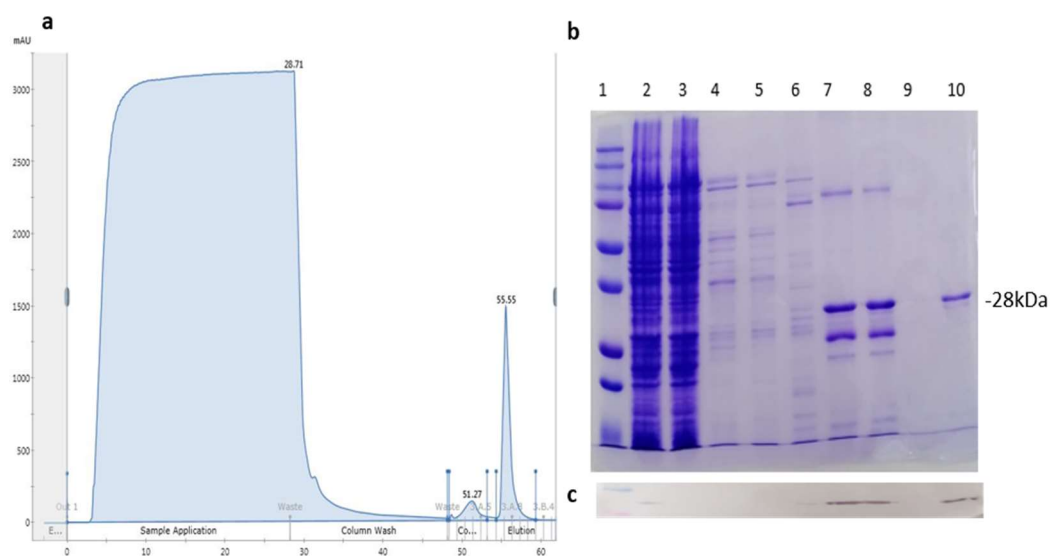


Figure 3.6 : Purification of soluble anti-KDR 1.3G protein.

After the completion of the computational and statistical methods, it was needed to transform and show the expression and analyze the solubility of the target protein. I have demonstrated that the expressed protein was mostly soluble by analyzing the SDS-PAGE and western blot results. It was shown that when compared to the original scFv, the grafted molecule had a significant effect on solubility, which validated the findings of the computer analysis.

3.4.3 SPR studies

First, I used amine coupling chemistry to immobilize the VEGFR-2 (RU 12900) and injected anti-KDR 1.3 and anti-KDR 1.3G scFvs onto the flow cell surface. When VEGFR-2 is saturated, peak signal (Rmax) of around 3225 RU of scFvs is predicted if the scFvs bind according to a 1:1 interaction model and VEGFR-2 is completely active for scFv binding. For 270 secs, the scFvs were injected on the flow cell immobilized with VEGFR-2 and the control flow cell surface. A dissociation time of 180 sec was left before regeneration.

Anti-KDR 1.3 has a maximum SPR signal of about 35 RU, whereas anti-KDR 1.3G has a maximum SPR signal of around 300 RU. Low binding signals indicate a low

immobilization rate, which might be caused by the ligand or the amine coupling process, which blocks the majority of active binding sites. Despite this, SPR signals revealed that anti-KDR 1.3 and anti-KDR 1.3G bind to VEGFR-2 in a concentration-dependent manner (see Figure 3.7). The affinity values from kinetic analysis (1:1 Binding model) were determined to be 86 nM for anti-KDR 1.3 binding to VEGFR-2 and 36 nM for anti-KDR 1.3G binding to VEGFR-2.

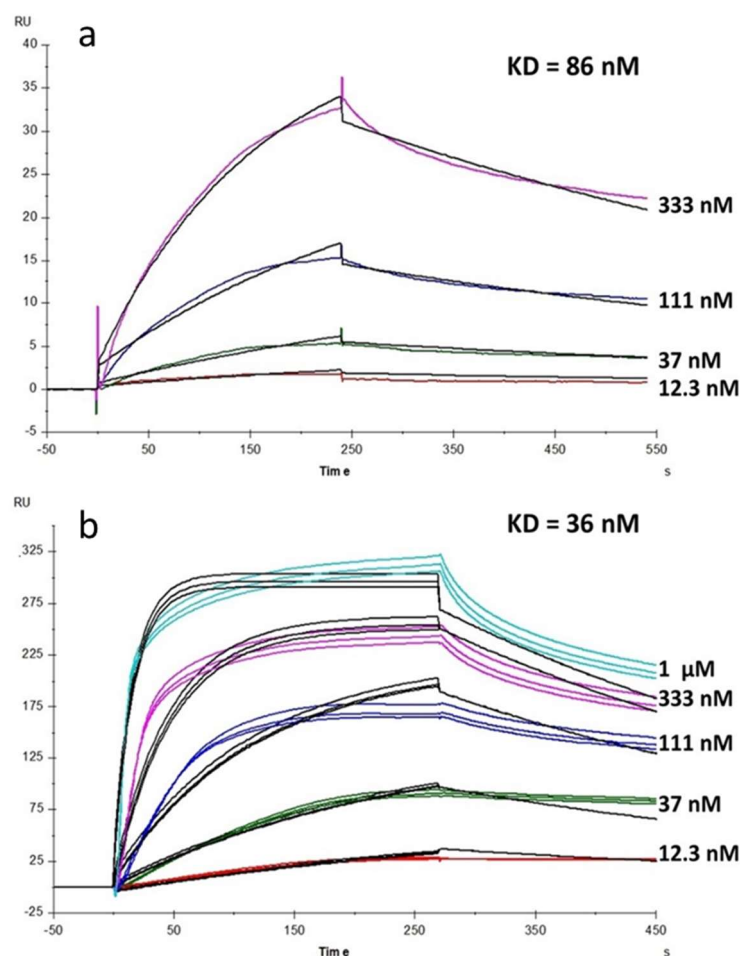


Figure 3.7 : Surface plasmon resonance analysis of the single chain variable fragment-vascular endothelial growth factor family receptors 2 interactions. scFv anti-KDR1.3. **(a)** at concentrations 0 nM, 12.3 nM, 37 nM, 111 nM and 333 nM and scFv anti-KDR 1.3G **(b)** at concentrations 0 nM, 12.3 nM, 37 nM, 111 nM, 333 nM and 1000 nM, were injected on a flow cell.

The Biacore T200 SPR device was employed to assess molecular interactions including antigen-antibody binding (Helmerhorst et al, 2012). When the anti-KDR 1.3G binding values are compared to the original anti-KDR 1.3 scFv binding to the VEGFR-2 receptor, the KD value of anti-KDR 1.3G is almost half of the original molecule, meaning better binding affinity for the target molecule. This can be

attributed to loss in catalytic activity due to aggregation and not proper folding of the anti-KDR 1.3 protein.

3.4.4 HUVEC migration

The capability of anti-KDR1.3 and anti-KDR 1.3G scFv molecules to inhibit cell migration of HUVECs in the presence of VEGF was evaluated using CIM-plates. The results show that VEGF-stimulated HUVEC cells moved in larger numbers through the bottom side of the compartment than non-stimulated controls. When cells were treated with 0.53 M anti-KDR 1.3 and anti-KDR 1.3G, the number of cells that migrated and invaded the lower chamber was comparable and lower than the control. Furthermore, as compared to untreated controls, cells treated with anti-KDR 1.3G substantially inhibited HUVEC cell migration in a concentration-dependent manner (see Figure 3.8).

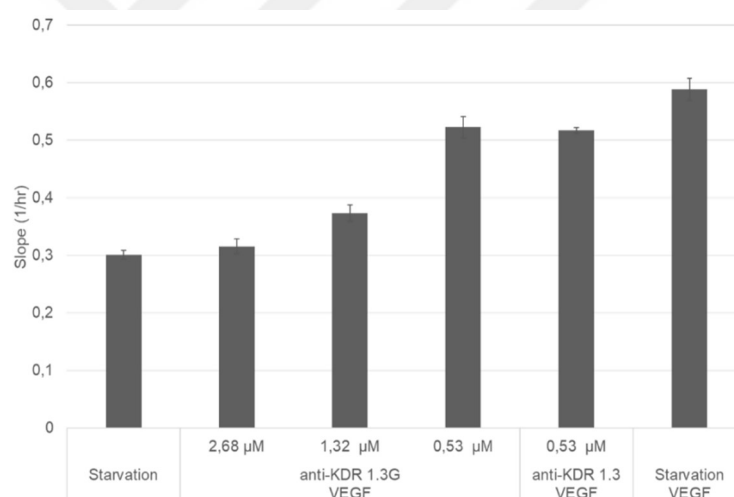


Figure 3.8 : The effects and comparison of anti-KDR 1.3G and anti-KDR 1.3 on endothelial growth factor-induced human umbilical vein endothelial cells migration.

3.4.5 HUVEC proliferation inhibition

HUVECs were grown alone with full media, with starvation media, starvation media + VEGF, and various doses of grafted anti-KDR 1.3G scFv molecules to assess their proliferative response to grafted anti-KDR 1.3 scFv molecules. The results in Figure 3.9 a were standardized by the time point at which anti-KDR 1.3 scFv proteins were introduced to the experiment. The samples in Figure 7.7a were labeled with their color codes. As a positive control, starvation medium (EBM-2 with 2% serum and no growth factors) was utilized, while VEGF-containing starvation medium (EBM-2 with 2% serum and no growth factors) was employed as a negative control. Figure 3.9 a,b

demonstrate the impact of 1.32, 2.68, and 5.36 M concentrations of anti-KDR 1.3G scFv protein on HUVEC cell proliferation in the presence of VEGF for 72 hr. The graphic shows that HUVECs activated with VEGF during starvation increase in cell number. Treatment with grafted anti-KDR 1.3 scFv molecules (2.68, 5.36 M) significantly reduced HUVEC proliferation compared to the control group, as shown in Figure 3.9. However, as compared to the negative control, the lowest concentration (1.32 M) of anti-KDR 1.3G was insufficient to suppress HUVEC cell proliferation.

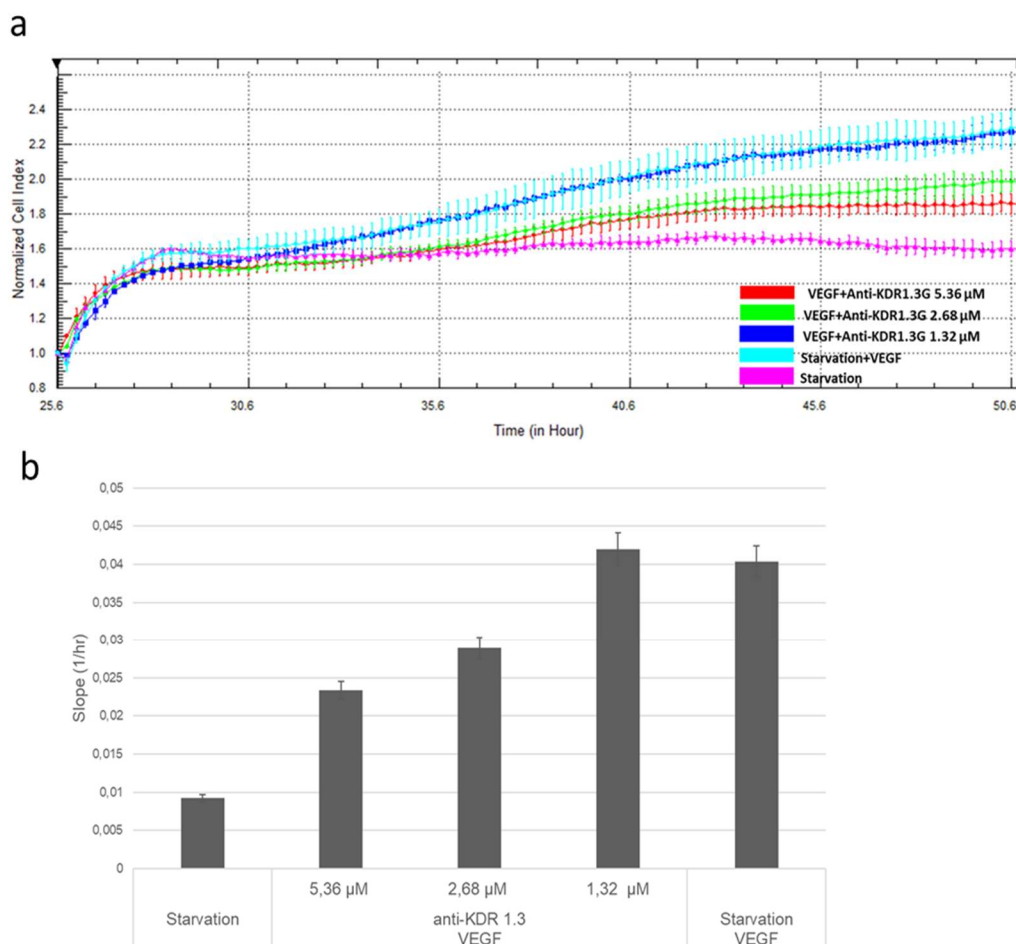


Figure 3.9 : The effects of anti-KDR 1.3G on vascular endothelial growth factor-induced human umbilical vein endothelial cells proliferation. **(a)** Effect of anti-KDR 1.3G scFv protein on proliferation of HUVEC cells in the presence of VEGF using a real-time cell analysis system. The red line in the graph belongs to the protein at 5.36 μ M, the green line at 2.68 μ M, and the dark blue line at 1.32 μ M. All values are normalized before the molecules are placed on the cell **(b)** HUVECs proliferation response to different concentrations of (1.32, 2.68, 5.36 μ M) grafted anti-KDR 1.3 scFv molecules in the presence of VEGF using the real-time cell analysis system.

HUVEC cells have VEGFR-2 receptors upon which anti-KDR 1.3G proteins can bind and block receptor activity. The xCELLigence device, which is used to monitor proliferation assays, continually detects and monitors changes in electrical impedance

caused by cellular activities, and then reports these changes as an arbitrary cell index (Abumaree et al, 2017; Alshabibi et al, 2017). As a consequence, it generates real-time data, giving it a considerable advantage over traditional end-point analysis approaches.

Our findings show that when the concentration of our grafted scFv molecule increases, the response of HUVECs to VEGF diminishes. As demonstrated in the results, the grafted molecule inhibited VEGF-induced proliferation of cells in a concentration dependent way. The lowest concentration of the grafted scFv molecule, however, was insufficient to inhibit endothelial cell growth.

3.4.6 HUVEC wound healing

The wound-healing test is a well-known *in vitro* method for investigating two-dimensional collective cell migration. Mechanical disruption creates a cell-free region (a wound area) in a confluent monolayer in this experiment. The experimental results are given in Figure 3.10 a, b as percentage values of the empty wound regions of HUVEC cells as wound closure versus concentration of anti-KDR1.3G at 24 h.

The time required for HUVEC cells to double was 38.7 ± 1.3 hr and in starvation with VEGF was 98 ± 9.5 hr. Considering the doubling time values at a 24-hour period, wound healing would be mediated by migration rather than cellular growth.

As a positive control, starvation medium (EBM-2 with 2% serum and without growth factors) was utilized, and as a negative control, a starvation medium containing VEGF was employed. After 24 hours of induction with VEGF (25 ng/mL), 60% of the wound was mostly healed. (Figure 3.10 a), as can be observed in live cell images.

Compared to the initial wound region, samples containing starvation medium media used as positive controls show a 54% wound opening closure at the end of the 24-hr cycle. The wound opening tends to be the same as tests of starvation medium media closure was very similar for the starvation media and for the cells treated withby using a $5.36 \mu\text{M}$ ($150 \mu\text{g/mL}$) concentration of anti-KDR 1.3G scFv protein. When compared to untreated controls, cells treated with anti-KDR 1.3G inhibited wound closure in a concentration-dependent way, as expected. As seen in both figures, the wound opening is decreasing in time when compared with the negative control. The wound-healing assay provided researchers to investigate cell migration (Jonkman et al, 2014). I used AutoScratch Wound Making Tool (Biotek) and the Scratch Assay App (Biotek) to improve the quality and reliability of the assay.

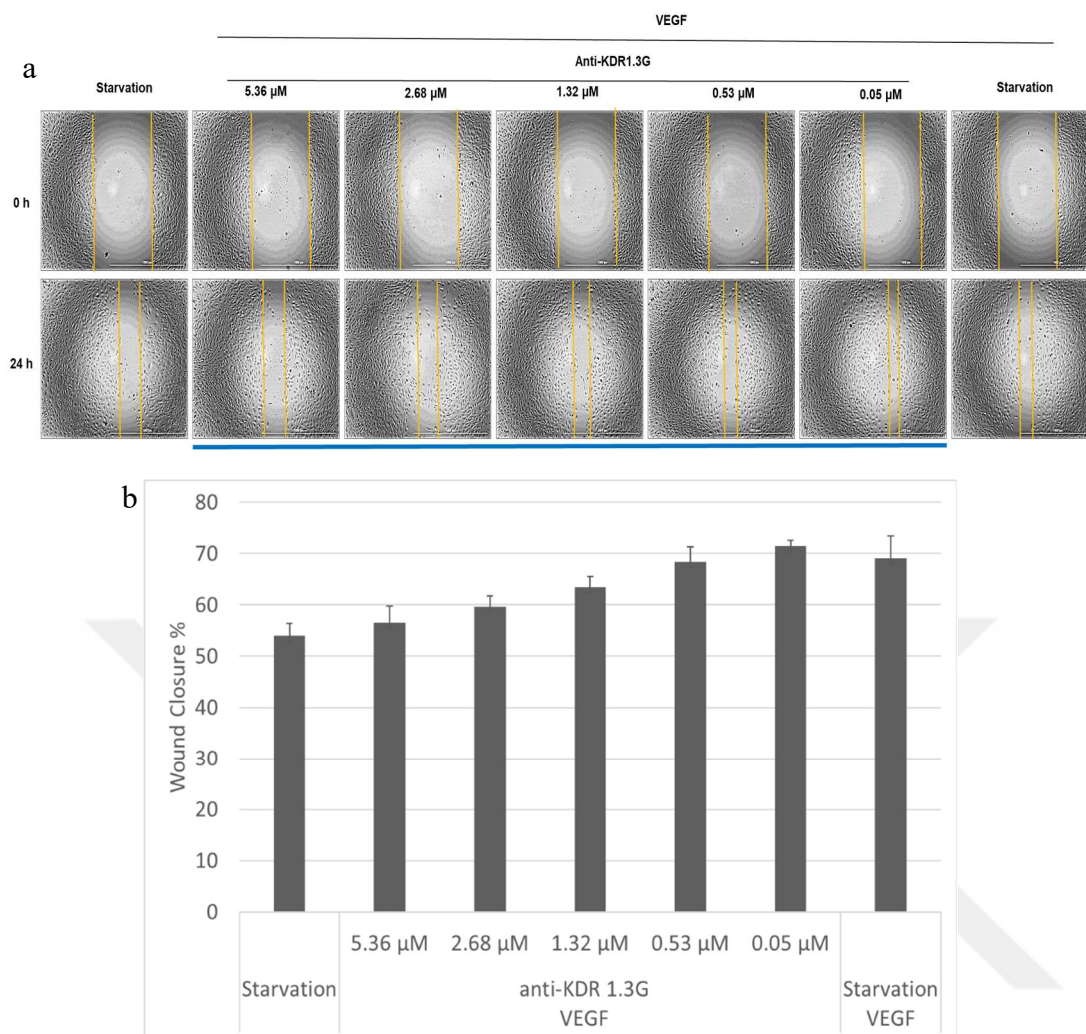


Figure 3.10 : The effects of anti-KDR 1.3G on vascular endothelial growth factor - induced human umbilical vein endothelial cells migration and wound healing. **(a)** Live cell images of HUVEC wound healing at 0, 24 hr taken by Cytation5 Device using a 4X objective of bright field filter **(b)** Wound closure % vs concentration graph of cells treated with starvation media (control) and VEGF (25ng/mL) in the presence or absence of anti-KDR 1.3G (0.05, 0.53, 1.32, 2.68, 5.36 μ M) at 24 hr.

The grafted scFv molecule's inhibition of VEGFR-2 is likely to interfere with endothelial angiogenic pathways and limit endothelial cell proliferation. The findings of this research revealed that our grafted molecule prevented the VEGF molecule's effect. Additionally, grafted molecule exhibits dose-dependent impact at lower concentrations. The anti-KDR 1.3G scFv protein does not seem to have an inhibitory effect on the VEGF molecule at the lowest concentration (0.05 μ M) used in this study. Those findings imply that grafted molecule inhibits HUVEC proliferation via inhibiting the VEGFR-2 pathway and angiogenesis.

Ultimately, my data show that the CDR grafting technique had no detrimental effects on the original molecule's binding kinetics or anti-angiogenic properties. As a result,

it supplied us with vital knowledge that antigen recognition for the anti-KDR 1.3 molecule happens largely via CDR loops and that FR may be replaced without a net decrease in inhibitory activity. I intend to use the modular structure of anti-KDR1.3 to further humanize it, which will hopefully improve the potential potency of this scFv molecule.





4. CONCLUSIONS

Antibodies are critical for treating angiogenesis-related disorders, although there are currently just a few alternatives on the market. It is vital to enhance the availability of these medications in order to enhance treatment efficacy. Numerous antibodies are being developed that have high drug potency but are unsuitable for industrial usage due to their low production levels. Elevated cytoplasmic production of proteins in *E. coli* mostly resulted in aggregation of inclusion bodies, necessitating comprehensive *in vitro* downstream processing to gain their biological functions. Bioactive proteins, on the other hand, are typically harvested with a very low yield. Hence, to overcome the lack of solubility, here I have employed a CDR grafting strategy for our poorly soluble anti-KDR 1.3 scFv molecule. The six CDR loops of anti-KDR 1.3 scFv were grafted onto the framework of another scFv molecule, Lig7, which is experimentally known to have a better solubility. Prior to production of the grafted scFv molecule, I used existing computational methods to estimate how grafting would affect the physicochemical characteristics of scFv molecules, such as solubility and stability, which are required for scFv activity.

I have shown that a fully expressed anti-angiogenic scFv can be resurrected by grafting its CDR regions to a soluble scFv. Those results demonstrate the efficacy and practical utility of *in silico* techniques for resolving the solubility issue of scFv proteins that exhibit therapeutic potency with scale-up production limitations.

This study demonstrates that by grafting CDR loops from an originally insoluble scFv onto framework sections derived from inherently soluble sequences, a soluble scFv could be produced without losing its binding affinity or activity. Here I have highly increased the drug potency of a low yield anti-angiogenic scFv (anti-KDR 1.3) with inclusion body formation tendency with a CDR grafting strategy.



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CURRICULUM VITAE

Name Surname : Melis DENİZCİ ÖNCÜ

EDUCATION

- **B.Sc.** : 2010, Bogazici University, Science Faculty, Environmental Sciences Department
- **M.Sc.** : 2006, Middle East Technical University, Science Faculty, Department of Biological Sciences

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2007-Still, TUBITAK MRC Biotechnology and Genetics Department, Senior Researcher.
- 2007, İleri Tıp Teknolojileri, Research Assistant.

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Melis Denizci Öncü** , Bertan Koray Balcıoğlu , Beytullah Özgür , Hasan Ümit Öztürk , Müge Serhatlı , Şeyma Işık , Berrin Erdağ , Gizem Dinler Doğanay , Aylin Özdemir Bahadır. 2021. Structure Based Engineering of an Anti-Angiogenic scFv Antibody for Soluble Production in *E. Coli* without Loss of Activity. Biotechnology and Applied Biochemistry. <https://doi.org/10.1002/bab.2273>.

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- Bertan Koray Balcıoğlu, **Melis Denizci Öncü**, Hasan Ümit Öztürk, Fatıma Yücel, Filiz Kaya, Müge Serhatlı, Hivda Ülbeği Polat, Şaban Tekin, Aylin Özdemir Bahadır. Turkish Journal of Biology SARS-CoV-2 neutralizing antibody development strategies, Turkish Journal of Biology, 44(203-214), 2020.

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