CHAPTER 3

PRODUCTION OF SINGLE CHAIN VARIABLE FRAGMENT (scFv) ANTIBODY AGAINST sTIE2 AND ITS EFFECT ON ANGIPOIETIN INDUCED ANGIOGENESIS.
3. Introduction

Targeting tumor angiogenesis by blocking VEGF-A signaling pathways have been observed to be clinically ineffective due to the temporary responses by the patients, mainly as a result of drug resistance (Abdollahi and Folkman, 2010; Ebos et al., 2009; Bergers and Hanahan, 2008). Further, resistance can also be attributed to the existence of other angiogenic factors in the tumor along with presence of inflammatory and hematopoietic cells. Therefore, for a better anti-angiogenic therapy, ancillary targeting of multiple angiogenic factors along with metastatic and inflammatory pathways is necessary.

One such pathway that is feasible for therapeutic intervention is the angiopoietin pathway mediated by the Tie receptors, since this pathway is involved in both the angiogenic-vascular homeostasis and inflammatory pathways. Cancer therapeutics which target the angiopoietin signaling cascade are presently undergoing clinical trials exhibiting promising anti-tumor efficacy along with a better safety profile and pharmacokinetics compared to that of anti-VEGFA therapy (Herbst et al., 2009).

Cytokines of the angiopoietin signaling pathway, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) function through the signaling via vascular receptor tyrosine kinase, Tie2 (Eklund and Saharinen, 2013; Jones et al., 1999). Initially, Ang1 and Ang2 were considered to have antagonistic effects on each other, where Ang1 functions as a Tie2 agonist, responsible for vessel stabilization and maturation (Davis et al., 1996), and Ang2 being antagonist resulting in vessel destabilization (Falcon et al., 2009; Maisonpierre et al., 1997). On the contrary, recent investigations have shown that, akin to Ang1, Ang2 could also be an agonist of Tie2 inducing receptor phosphorylation and initiate functions like tube formation, cell migration, and sprouting of endothelial cells in the absence of Ang1 (Kim et al., 2000; Yuan et al., 2009). However, Ang2 is shown to be upregulated at sites of tumor angiogenesis and thereby promotes tumor growth in experimental cancer models (Oliner et al., 2004).

Ang1 and/or Ang2 ligand overexpression has been noted in patients with mammary, gastric, ovarian and non-small cell lung cancer and are also associated with the grade of cancer and bad prognosis (Li et al., 2015; Blank et al., 2015; Sallinen et al., 2014; Coelho et al., 2014; Naumnik et al., 2013).
Concurrently, Tie2 receptor upregulation is also seen in the nonvascular regions of several tumors that include leukemia as well as mammary, gastric, and thyroid cancers and glial cells of human gliomas (Kukk et al., 1997; Wang et al., 2005; Shirakawa et al., 2001; Mitsutake et al., 2002; Lee et al., 2006).

Accordingly, the angiopoietin-Tie2 signalling pathway is an excellent target for therapeutic intervention. Blocking the Tie2 signaling in ovarian, mammary and prostate cancers using Trebananib, a peptibody under active clinical trials, selectively bind to Ang1 and Ang2 and prevents Tie2 binding (Coxon et al., 2010).

Furthermore, from our previous studies, we have reported that sTie2 transfected Ehrlich ascites tumor (EAT) cells when transplanted into nude mice, reduced the tumor burden as well as reduced ascites secretion and peritoneal angiogenesis by sequestering the angiopoietin ligands (D'souza et al., 2010).

Moreover, various studies have shown that blocking the Tie2 receptor pathway using specific blocking agents like soluble dominant negative receptors (Stratmann et al., 2001), RNA interference (Niu et al., 2004), short synthetic peptides (Tournaire et al., 2004) and anti-Tie2 intrabody (Popkov et al., 2005) all efficiently inhibit tumor angiogenesis and growth.

Among all the different strategies available to selectively interfere with a single target molecule, antibodies are the first choice, by virtue of its high specificity. For treatment of cancers, about 30 different monoclonal antibody based therapeutics have been approved and still are the rapidly flourishing therapeutic molecules.

Further, conventional methods of antibody production by animal immunization and hybridoma technology may become obsolete since antibody fragments can be synthesized by the prokaryotic expression that can efficiently produce unlimited quantities of antibodies to virtually any antigen (Ahmad et al., 2012).

The single chain fragment variable (scFv) antibody fragment comprises the variable regions of the heavy (V\text{H}) and the light (V\text{L}) chains that are joined together in succession by a flexible linker peptide, which is crucial for the proper folding of the protein. For the formation of complete antigen binding site, the folding of V\text{H} and V\text{L} domains is critical and also dependent on the size of the linker, which should be \(\sim 35\AA\) (Huston et al., 1991).
Apart from length of the linker, the amino acid sequence of the linker is also important, wherein it should comprise of hydrophilic amino acids to prevent misfolding of the $V_H$ and $V_L$ chains (Argos, 1990). The linker is usually comprised of repeats of Glycine and Serine amino acids that impart flexibility along with certain charged amino acids like Glutamic acid and Lysine in between, which improve solubility (Whitlow et al., 1993).

This chapter discusses about the objective of production of single chain variable fragment (scFv) antibody against sTie2 protein and its effect on angiopoietin induced angiogenesis. Here, we have adopted an alternate strategy to block or target the Tie2 receptor by developing a scFv against the soluble, human extracellular domain of the Tie2 receptor protein (sTie2) that was expressed in Sf-21 insect cells using baculovirus expression system according to the methods followed by Saritha (2010). The scFv was cloned and expressed using the pCANTAB5E plasmid in E. coli, HB2151. Several transformed colonies were screened and evaluated for specificity and high level of scFv expression by a modified dot blot assay. The bacterial colony containing the specific scFv was induced, isolated from the periplasmic fraction and purified by affinity chromatography and its bioactivity was assessed in vitro and in vivo.
3.1. Materials

3.1.1. Animals, cell lines, and chemicals

BALB/c mice (6-8 weeks old) and Wistar rats (4-5 months old) were procured from the animal facility, Department of Studies in Zoology, University of Mysore, Karnataka, India. The animal experiments were approved by the institutional animal care and use committee (Reg.No.122/GO/ReBi/S/99/CPCSEA), University of Mysore, Karnataka, India. Sf-21 insect cells, MDA-MB-231, A-375, U-87MG and HEK293 were obtained from the National Center for Cell Sciences (Pune, India). The Sf-21 cells were maintained in Grace insect medium and the human tumor cells and the normal cells were maintained in DMEM complete media (Invitrogen), fetal bovine serum (FBS) was of South American origin and penicillin streptomycin mixture. Sf-900 II serum free media, phosphate buffered saline (PBS) without calcium and magnesium was purchased from Gibco, Life Technologies USA. pCEP4-sTie2 plasmid was a kind gift from Dr D. Marme (Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany). pFastBacHTA plasmid was obtained as a part of the Bac-Bac kit (Gibco-BRL, Gaithersburg, MD, USA). RNaseasy Midi Kit (Qiagen, USA) was used to isolate total RNA. For cDNA synthesis, the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used. pCANTAB 5 E expression vector was obtained from Amersham Biosciences, United kingdom and the scFv was prepared using the Recombinant Phage Antibody System (RPAS) (GE healthcare and life sciences, UK). Plasmid DNA was amplified and purified using plasmid isolation kit (Qiagen, Hilgenberg, Germany) in accordance with the manufacturer’s instructions. All other reagents were of highest grade that are available commercially.

3.2. Methods

3.2.1. Subcloning of sTie2 insert from pCEP4–sTie2 vector into pFastBac HTb

Following the methods mentioned by Saritha (2010), the 941bp sTie2 cDNA insert that code for the human extracellular domain of the Tie2 receptor present in the pCEP4-sTie2 vector was released by double digestion; first using BamHI restriction enzyme (37 °C for 90 min), followed by two rounds of phenol chloroform extraction and ethanol precipitation. The overhangs were endfilled using klenow polymerase to generate blunt ends (37 °C for 90 min) and subsequently digested with NotI restriction enzyme (37 °C for 2 h). Simultaneously the pFastBac-HTb donor plasmid was also
subject to double digestion using StuI and NotI restriction enzymes (37 °C for 90 min). Post digestion, the pFastBac-HTb linearized plasmid and 941bp sTie2 cDNA insert released were separated on 1% agarose gel and the respective bands were excised out and gel eluted using the QIAquick Gel extraction kit as per the instructions of manufacturer. Later the gel purified 941bp sTie2 insert and the linearized pFastBac-HTb donor plasmid were ligated using T4 DNA ligase (Pure Extreme, Fermentas) overnight at 16°C. Finally the ligated product was confirmed by agarose gel electrophoresis. The cloning strategy is as shown in Figure 3.1.

1. Digestion with Bam HI

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>12 µL</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>pCEP4-sTie2</td>
<td>10 µL</td>
</tr>
<tr>
<td>Bam H1</td>
<td>0.5 µL</td>
</tr>
<tr>
<td></td>
<td>25 µL</td>
</tr>
</tbody>
</table>

2. End filling with Klenow

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCEP4-sTie2</td>
<td>25 µL</td>
</tr>
<tr>
<td>Water</td>
<td>7.5 µL</td>
</tr>
<tr>
<td>Buffer</td>
<td>1 µL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2 µL</td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Klenow Polymerase</td>
<td>4 µL</td>
</tr>
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<td>40 µL</td>
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3. Digestion with NotI

Reaction mixture:

<table>
<thead>
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<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCEP4-sTie2</td>
<td>40 µL</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>5 µL</td>
</tr>
<tr>
<td>Water</td>
<td>4 µL</td>
</tr>
<tr>
<td>NotI</td>
<td>1 µL</td>
</tr>
<tr>
<td></td>
<td>50 µL</td>
</tr>
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4. Linearization of pFastBac HTb

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFastBac HTb</td>
<td>10 µL</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>3 µL</td>
</tr>
<tr>
<td>Water</td>
<td>15 µL</td>
</tr>
<tr>
<td>StuI</td>
<td>1 µL</td>
</tr>
<tr>
<td>Not I</td>
<td>1 µL</td>
</tr>
<tr>
<td></td>
<td>30 µL</td>
</tr>
</tbody>
</table>

5. Ligation of sTie2 into pFastBac HTb

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested plasmid (50ng/µL)</td>
<td>3 µL</td>
</tr>
<tr>
<td>5X buffer</td>
<td>6 µL</td>
</tr>
<tr>
<td>sTie2 cDNA (45.5ng/µL)</td>
<td>7 µL</td>
</tr>
<tr>
<td>Water</td>
<td>16 µL</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>3 µL</td>
</tr>
<tr>
<td></td>
<td>45 µL</td>
</tr>
</tbody>
</table>
3.2.2. Transposition and recombinant bacmid isolation

The recombinant pFastBac-HTa-sTie2 donor plasmid was initially transformed into DH5α E.coli competent cells by calcium chloride method (Sambrook and Russell, 2006) and the plasmid was purified using the Maxi plasmid purification kit (Qiagen), according to the manufacturer’s instructions. The presence of the sTie2 insert was confirmed by colony PCR using gene specific primers. Next, the recombinant plasmid was transformed into the DH10Bac E.coli [F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG/pMON14272/ pMON7124] competent cells for transposition of the sTie2 insert into the bacmid following the Bac-to-Bac® baculovirus expression system (Invitrogen) instructions. The DH10Bac cells harbor a baculovirus shuttle vector (bMON14272) and a helper plasmid (pMON7142) that are capable of supporting site-specific recombination between pFastBac-HTa and bMON14272 to generate high molecular weight bacmids.
Consequently, LB agar plates containing kanamycin, gentamicin, tetracycline, X-gal and IPTG were used for blue-white screening. The white colonies containing recombinant bacmid were cultured and the high molecular weight recombinant bacmid was isolated according to manufacturer’s instructions and presence of the insert was again confirmed by PCR using gene specific primers.

Forward primer 5'-GGAGAGAATTCGGGAAGCATGGACTCTTTAG-3'
Reverse primer 5'-CCGTAAAAACCAGGGTGCCAT-3'

**Colony and Bacmid PCR**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>PCR conditions: 40 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>Initial denaturation : 94 °C for 5 min</td>
</tr>
<tr>
<td>PCR buffer (10x)</td>
<td>Denaturation : 94 °C for 1 min</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM each)</td>
<td>Annealing : 58 °C for 2 min</td>
</tr>
<tr>
<td>Forward primer (10 pM)</td>
<td>Extension : 72 °C for 1 min</td>
</tr>
<tr>
<td>Reverse primer (10 pM)</td>
<td>Final extension : 72 °C for 10 min</td>
</tr>
<tr>
<td>White colony/Bacmid</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase (3U/µL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25µL</td>
</tr>
</tbody>
</table>

### 3.2.3. Transfection of Sf-21 cells with recombinant bacmid DNA

Sf-21 cells (9 × 10^5) were seeded into each well of a 6 well plate in 2 ml of Sf-900 II serum free media (SFM) with antibiotics and allowed to attach for 1 h at 27 °C. The cells were washed once with media without antibiotics. The media was removed and 1ml of Lipid-DNA transfection mixture was overlaid on the cells for the transfection of bacmid into the Sf-21 cells using the Lipofectamine LTX and PLUS reagent (Invitrogen). The cells were incubated for 5 h at 27 °C. Finally the transfection mixture was removed and fresh Sf-900 II SFM with antibiotics was added and incubated for 72 h. 72 h post transfection, the recombinant baculovirus particles in the spent medium was harvested by centrifugation (500 × g) for 5 min and the viral titer was further amplified by re-infection of fresh batch of Sf-21 cells for 48 h. Finally Sf-21 cells in log phase were infected with the recombinant baculovirus at a multiplicity of infection (MOI) 6 to express the recombinant protein. Cell viability and total cell number were monitored every 24 h. The graphical representation of the Bac-to-Bac baculovirus expression system is shown in Figure 3.2.
3.2.4. Ni-NTA purification and characterization of expressed sTie2 protein

72 h post incubation with baculovirus, Sf-21 cells were gently rinsed in PBS and centrifuged (1000 x g) for 5 mins. The pellet was lysed in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8 and 1% Nonidet P40) in the presence of protease inhibitor cocktail. The lysate containing sTie2-6xHis fusion protein was clarified by centrifugation (10,000 x g for 20 min at 4°C). To 4 mL of the clarified lysate, 200 µL of 50% Ni-NTA agarose slurry (Qiagen, Valencia, USA) was added and incubated at 4 °C for 2 h in a rotary shaker. The lysate-Ni-NTA mixture was then loaded into a column, washed using the native wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 6) and the bound protein was eluted using the native elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 6) following the instructions of The QIAexpressionist™ handbook. The protein fractions with our protein were pooled and dialysed against PBS overnight.

3.2.4.1. SDS PAGE and Western blotting

The protein concentration in dialysed protein was estimated by Lowry’s estimation and 50µg of protein was resolved in 12.5% SDS PAGE. Protein was visualized in gels by silver staining. For Western blotting, 80µg of protein was electroblotted onto an NC membrane by semidry western blotting unit V20-SDB, (SCIE-PLAS, Cambridge, UK), blocked in blocking buffer (5% w/v milk powder in 1×TBS, 0.05% v/v Tween 20) for 16 h at 4 °C. Probed with rabbit polyclonal anti Tie2 antibody, (sc-324, Santa Cruz Biotechnology, USA), washed with TBST and the
membrane was incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody in blocking buffer, followed by three washes in TBST. The membrane was finally visualized for immunoreactivity using an enhanced chemiluminescence system (Clarity Western, BioRad) and documented using the chemiluminescence imaging system (Fujifilm FLA 5000 imager).

3.2.5. **Studies on Tie2 receptor expression profile in tumor biopsies and cancer cells**

3.2.5.1. **Immunohistochemistry of Tie2 protein using human tumor biopsy sections**

Human breast cancer tumor biopsy sections were collected with informed consent from the Department of Pathology, J.S.S. Hospital, Mysuru, India. The sections were immunostained and developed using 3,3′-Diaminobenzidine (DAB) staining method (BioGenex, India) according to manufacturer’s instructions. Firstly, the sections were dewaxed in xylene thrice for 5 min each. The sections were rehydrated in descending concentrations of ethanol (100% ethanol for 5 min, 95% for 2 min and 70% for 2 min) and washed in distilled water. Retrieval of antigen was performed by steaming the sections at 95 °C for 15 min in a humidified atmosphere topped with 50mM sodium phosphate buffer. The sections were treated with 3% H₂O₂ in PBS to inactivate endogenous peroxidases and incubated in blocking serum for 30 min and later washed and incubated with rabbit polyclonal anti-Tie2 antibody (sc-324, Santa Cruz Biotechnology) for 2 hrs. Following PBS wash, incubated with goat anti-rabbit biotinylated secondary antibody for 1 hr at room temperature. The slides were washed in PBS for 5 min and the sections were covered with 1-3 drops of HRP-streptavidin complex for 30 min followed by 100 µL of substrate (DAB) for 5 min. The sections were washed thrice for 2 min in running water and twice in distilled water. Later on, the sections were counter stained using 2% hematoxylin for 30 sec and rinsed under running water thrice. Slides were then dehydrated sequentially in 50%, 80% and finally in absolute alcohol. After the xylene wash, slides were mounted and the sections were observed and photographed under a bright field microscope (AX10.Imager.A2, Carl Zeiss, Germany) at 400x magnification.
3.2.5.2. qPCR analysis for Tie2 gene expression profile in human tumor cells

Total RNA was extracted from a panel of three confluent cultures of malignant human tumor cell lines and a normal cell line namely MDA-MB-231, U87-MG, A-375 and HEK293, respectively using RNeasy Midi Kit (Qiagen, Valencia, USA) according to manufacturer’s protocol. The total RNA was eluted from the column using RNase free water and the quantitated using the nanodrop 2000c spectrophotometer (ThermoFischer, USA). The integrity of isolated RNA was ascertained by performing a denaturing formaldehyde agarose gel (Rio, 2015)

Equal amounts of total RNA (2 µg) from all the cell lines was used as template for the first-strand cDNA synthesis using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) by reverse transcriptase using random hexamers and oligo dT primers according to the manufacturer’s protocol. The reverse transcriptase was inactivated by heating to 85 °C for 5min.

The resulting first-strand cDNA was diluted and used as template (50ng) in the qPCR reaction comprising SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) (5µL), dNTPs, forward and reverse primers and nuclease free water using the Real Time thermocycler CFX-96 1000C (Bio-Rad) along with non-primer control (NPC) and non-template control (NTC). Custom primers were designed using primer3 software. The relative gene expression values were normalized to their respective vehicle treated control values using the comparative Ct method \(2^{-\Delta\Delta C_t}\). Using this method, an average fold-change in gene expression was obtained for each transcript. Reactions for each sample were performed in triplicate using equal amounts of template cDNA.

The sequences of the PCR primer pairs were as follows:
Forward primer 5’- CGCTACCTACTAATGAAG -3’
Reverse primer 5’- TGAATATGGCTACTGAGA -3’

<table>
<thead>
<tr>
<th>qPCR to assess Tie2 expression</th>
<th>qPCR conditions: 35 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction mixture:</td>
<td></td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7 µL</td>
</tr>
<tr>
<td>EvaGreen Supermix</td>
<td>10 µL</td>
</tr>
<tr>
<td>Forward primer (300nM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Reverse primer (300nM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>cDNA (50ng)</td>
<td>1 µL</td>
</tr>
<tr>
<td></td>
<td>20µL</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>94 °C for 3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C for 15 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C for 20 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C for 20 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C for 3 min</td>
</tr>
</tbody>
</table>
3.2.6. Construction of scFv against sTie2 protein

3.2.6.1. Immunization of BALB/c mice

Immunization of naive BALB/c mice was performed as described by (Leenaars and Hendriksen, 2005). Briefly, four mice (6-8 weeks old) were immunized (Figure 3.3) by sub-cutaneous (s.c.) injections containing 50 μg in 0.2 mL of sTie2 protein emulsified in complete Freund’s adjuvant (CFA) at five sites on the abdomen on day zero. The mice were given boosters two weeks later by s.c. injection with the same amount of sTie2 in incomplete Freund’s adjuvant (IFA). Boosters were given on days 21, 42 and 56, respectively, and the final booster of sTie2 in PBS was given seven days later. Post immunization, mice were bled by cutting the tail and sera was prepared. Also the pre-immune serum was prepared from the blood taken from naive mice.

![Figure 3.3 Scheme of immunization and booster doses of Balb/c mouse](image)

3.2.6.2. Isolation of mRNA from splenocytes and cDNA synthesis

The fully immunized mice were sacrificed, dissected and the spleens were excised, cleaned and stored in RNAlater solution (Qiagen) until use. Splenocytes were harvested by cutting open the spleen and filtered through 70 µM cell strainer (BD Falcon) by perfusion in DMEM medium (Invitrogen). The cells were centrifuged at 500 x g for 10 min at 4 °C. The RBCs present along with the cell pellet was lysed by resuspending the pellet in 5 mL of prechilled sterile PBS containing 0.17 M NH₄Cl followed by incubation on ice for 10 min and centrifugation at 500 x g for 10 min.

Total RNA was isolated from the splenocytes cell pellet using the illustra™ RNAspin Mini Isolation kit (GE healthcare lifesciences) according to the kit protocol. From the total RNA, mRNA was purified using the oligo(dT)-cellulose spin column chromatography from illustra™ QuickPrep mRNA purification kit (GE healthcare lifesciences). The entire methodology is illustrated in Figure 3.4.
Figure 3.4. Illustration of methodology of mRNA isolation from mouse spleen

The purified mRNA was quantified in the NanoDrop spectrophotometer, 2000c (ThermoFischer, USA). 200 ng of mRNA was reverse transcribed to cDNA using the M-MuLV based First-Strand cDNA Synthesis kit (GE healthcare lifesciences). After it was heated to 65 °C for 10 min and cooled immediately, the following reaction was set up in duplicate, one for the light chain and another for the heavy chain in RNase free 500 μL microcentrifuge tubes as per the manufacturer’s instructions.

3.2.6.3. Initial PCR amplification of the V<sub>H</sub> and V<sub>L</sub> cDNA

The entire cDNA was used as a template to amplify the variable heavy (V<sub>H</sub>) and light (V<sub>L</sub>) domains of the immunoglobulin genes by Polymerase Chain Reaction (PCR) using the Light primer mix and the Heavy primer mix (GE healthcare lifesciences) following the manufacturer’s instructions. The PCR amplification was performed for 30 cycles (94°C for 1 min; 55°C for 2 min; 72°C for 2 min). The amplified V<sub>H</sub> and V<sub>L</sub> fragments were gel extracted using MicroSpin columns, Sephaglas™ BandPrep Kit.

3.2.6.4. Linking the V<sub>H</sub> and V<sub>L</sub> cDNA

The gel purified V<sub>H</sub> and V<sub>L</sub> cDNAs were assembled together by a linker DNA in frame, that codes for GGGGSGGGGSGGGGS using the Linker primer mix (GE healthcare lifesciences). The assembly PCR was run for seven cycles (94°C for 1 min; 63°C for 4 min) according the manufacturer’s instructions.

3.2.6.4. Addition of restriction sites by PCR amplification

The assembled scFv fragment was amplified again using the RS primer mix to introduce in-frame SfiI and NolI restriction sites on the 5’ end of the V<sub>H</sub> gene and the 3’ end of the V<sub>L</sub> gene, respectively. PCR was performed as per the supplier’s protocol and run for 30 cycles of (94 °C for 1 min; 55 °C for 2 min; 72 °C for 2 min). The amplified PCR product was visualized in 1.5% agarose gel as an 800 bp band which was excised and gel extracted using the microspin column containing Sephacryl® S-400 HR resin (GE Healthcare,USA).
3.2.7. Cloning and expression of scFv

3.2.7.1. Cloning of the assembled scFv into the phagemid vector pCANTAB5E

The assembled ScFv cDNA containing the SfiI and NotI restriction sites was digested with the appropriate enzymes to yield a product suitable for cloning into a phagemid vector, pCANTAB5E was also digested with the same restriction enzymes. A 20 μL double digestion reaction was set up and the digested products of both the scFv cDNA and the linearized pCANTAB5E vector was separated on 1% agarose gel and the respective bands were gel extracted and purified using the QIAquick Gel Extraction Kit.

The digested scFv cDNA was then ligated into the linearized pCANTAB5E vector using different ratios of Vector: scFv (w/w) ratios of 1:1, 1:3 and 3:1 were set up in the ligation mixture and incubated overnight at 4 °C for the ligation reaction.

The ligation mixture was later transformed (as described in section 3.2.2.) into competent HB2151 cells, a suppressor-deficient cells E. coli strain [K-12 D(lac-pro) ara Nalr M15, thi/F9 proAB lacIq lacZD M15]. The transformed cells were selected by blue-white screening in presence of ampicillin in LB agar medium.

Recombinant white colonies were checked for presence of the pCANTAB5E-scFv construct by colony PCR using plasmid sequencing primers and also by restriction digestion analysis of the isolated plasmid using SfiI and NotI restriction enzymes.

pCANTAB5E forward 5’- GTAAATGAATTTTCTGTATGAGG -3’
pCANTAB5E reverse 5’- CAGGAAACAGCTATGAC-3’

3.2.7.2 Protein expression and downstream processing

The positive (white colonies) transformants were cultured in 2x-TY (16g tryptone, 10g yeast extract, 5g NaCl, pH to 7.0) medium with 2% glucose to maintain catabolite repression and 100μg/mL ampicillin at 30 °C to an OD 600 of ~ 0.8 to 1.0. The expression of the scFv protein was induced by addition of IPTG (final concentration of 1 mM) in 2x-TY media without ampicillin and incubated at 30 °C for 8 h in an orbital shaker at 250 rpm. The cells were pelleted by centrifuging at 6,000 x g for 10 min. The periplasmic (S1), osmotic shock (S2), and whole cell protein fractions (S3) were assayed to ascertain the expressed scFv.
For the periplasmic fraction, the *E. coli* cell pellet was resuspended in ice-cold 1x TES (0.2 M Tris HCl, 0.5 mM EDTA, 0.5M sucrose), incubated for 30 min on ice and centrifuged at 10,000 x g for 10 min. The harvested supernatant was the periplasmic fraction. The osmotic shock fraction was extracted from the residual cell pellet by adding of 0.5 M MgSO₄ and incubating on ice for another 20 min, followed by centrifugation at 10,000 x g for 10 min. The remaining cell pellet was resuspended in the lysis buffer (50 mM Tris HCl (pH 8.4) and 2 mM EDTA and 0.1% NP-40), sonicated and centrifuged at 15,000 x g for 10 min to obtain the whole cell extract. Finally, all the fractions from both un-induced and induced cultures were dialysed overnight against 1x PBS to obtain salt free refolded scFv. The dialyzed scFv protein was concentrated by reverse dialysis against sucrose and separated in 12.5% SDS PAGE.

### 3.2.7.3 Screening for expressed scFv against sTie2

For the screening of the specific sTie2 binding scFv, we adopted a modified dot blot assay as illustrated in Figure 3.5 wherein, ~60 random recombinant white colonies were picked from the petriplate used for blue-white screening, cultured in 6 mL, expression induced by IPTG and the periplasmic, osmotic and whole cell extracts were obtained as described in 3.2.7.2. The nitrocellulose membrane cut using a paper punch was placed into each well of a flat bottomed 96 well plate. The membranes were rinsed briefly using 50 µL of TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). All three extracts (20 µL) were blotted onto the NC membrane, dried for 1 h at room temperature, blocked in blocking buffer (5% skimmed milk powder in TBS, 0.05% tween-20), rinsed thrice for 5 min with TBS-T (TBS with 0.05% tween-20). sTie2 protein (20 µL of 1:500 dilution) was added and incubated for 1-2 h, rinsed with TBS-T (100 µL) thrice and incubated in primary antibody (20 µL), rabbit polyclonal ant-Tie2 (1:1000 dilution) for 1-2 h at 37 °C. The wells were rinsed with TBS-T (100 µL) thrice and incubated with secondary antibody (20 µL), goat anti-rabbit IgG-HRP conjugate (1:1000 dilution) for 1 h at 37 °C. Finally, the presence of sTie2 specific scFv was detected by enhanced chemiluminescence method after washing the wells with TBS-T thrice, using the Clarity Western ECL kit (Bio-rad, USA) and the chemiluminescence was detected and photographed using the the G: BOX imaging system (Syngene, Cambridge UK).
3.2.8. Validation and characterization of the scFv gene sequence

3.2.8.1. Sequencing of the recombinant pCANTAB5E-scFv-sTie2

The recombinant pCANTAB5E-scFv-sTie2 was isolated from the positive colony (HSN 6), identified from the previous screening experiment (section 3.2.7.2.) and the scFv-sTie2 insert was amplified by PCR by standardized conditions using sequencing primers as mentioned earlier (section 3.2.7.1.). The amplicon obtained was verified on 1% agarose gel and the recombinant plasmid was outsourced for sequencing using the same primers to Chromous Biotech Pvt. Ltd., Bangalore.

3.2.8.2. Characterization of the scFv sequence using bioinformatic tools

3.2.8.2.1. Nucleotide and amino acid sequence analysis

The nucleotide sequencing data obtained was analyzed using the Chromas chromatogram viewer software (ver.2.4.4) and the ~900bp forward and reverse sequences were aligned with the backbone sequence of pCANTAB5E plasmid using the SnapGene Viewer software (ver. 3.3.4) to check for overlapping sequences and to locate the sequence of the scFv-sTie2 insert. The scFv-sTie2 sequence was further translated to deduce the amino acid sequence and the open reading frame (ORF) using the online ExPASy translate tool (http://web.expasy.org/translate/). The nucleotide and the deduced amino acid sequence were subjected to BLAST (basic local alignment search tool) analysis at NCBI (National centre for biotechnology information) database to verify the identity of the scFv-sTie2 gene/protein sequence.
3.2.8.2.2. Homology modelling of scFv-sTie2 antibody and structure prediction

The nucleotide sequence obtained was subjected to the IgBLAST program (https://www.ncbi.nlm.nih.gov/igblast/) to analyze the immunoglobulin variable domain sequences. Using the online antibody design platform, SAbPred server’s (http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/WelcomeSAbPred.php), modeling pipeline ABodybuilder application, the \textit{insilico} analysis and characterization of the scFv light and heavy chains was performed (Leem \textit{et al.}, 2016), considering both the conserved framework regions (FR1, FR2 and FR3) and the variable complementarity determining regions (CDR1, CDR2 and CDR3).

Further, the amino acid sequence was submitted to an online platform for structural and functional prediction, Iterative-Threading Assembly Refinement (I-TASSER) for the generation of 3D structure based on the deduced amino acid sequence.

Structure validation of the final model was performed using the web based Structure Analysis and Verification Server (SAVES ver.4) using six different programs (https://services.mbi.ucla.edu/SAVES/), the distributions of $\Phi$-$\Psi$ dihedral angles of the backbone were also checked by the Procheck program.

3.2.8.2.3. Molecular Docking of the studies of scFv-sTie2 and sTie2.

An orientational docking of scFv-Tie-2 and Tie-2 receptor (RCSB PDF-2GY5) epitopes was carried out by ZDOCK docking server (http://zdock.umassmed.edu/) which generated 10 models. Since ZDOCK is a rigid body docking program, we chose the model with the highest ZDOCK score and refined it further using the RosettaDock molecular modeling algorithm (http://rosie.rosettacommons.org/) that implements a flexible docking method to remove possible clashes between the component proteins. Out of the best 10 models ranked based on the scores, one with best score was chosen.

3.2.9. Purification of the scFv-sTie2 from the positive colonies

3.2.9.1. sTie2 immobilization onto CNBr-activated sepharose 4B for scFv purification

Cyanogen bromide-activated Sepharose 4 B (C5338, Sigma Aldrich) freeze-dried powder was first swelled by incubating with cold 1 mM HCl solution for 30 min. The resin was then washed with distilled water to remove additives. The resin was mixed with sTie2 protein (2 mg protein per mL of resin) dissolved in the coupling buffer (0.1 M NaHCO$_3$, pH 8.3, with 0.5 M NaCl) and stirred gently for 16 h at 4 °C.
The unbound protein was washed off using the coupling buffer. The unreacted sites on the resin were blocked using the blocking buffer (0.2 M glycine, pH 8.0) for 16 h at 4 °C. The blocking buffer was then removed by 5 cycles of washes first with basic coupling buffer, pH 8.5 and then with 0.1 M acetate buffer, pH 4, with 0.5 M NaCl.

This sTie2 conjugated resin was washed with 1x PBS and then mixed with filter sterilized scFv containing S1 and S2 fractions and mixed in a rotary mixer at 4 °C overnight. This mixture was then packed into a column, washed with PBS to remove unbound proteins and finally eluted using elution buffer (0.17M glycine-Hcl, pH 2.5). The acidic eluate was collected into fraction collection tubes containing 1M Tris-Hcl to neutralize the solution. The scFv containing fractions were pooled together, dialyzed against PBS and verified by SDS PAGE and silver staining.

3.2.10. Validation of protein-protein interaction and bioactivity of scFv

3.2.10.1. Rat corneal micro-pocket assay

The angiogenesis inhibiting potential of anti-Tie2-scFv antibodies was studied using the in vivo rat corneal micro-pocket assay using female Wistar rats. The methodology of this experimental model has been described previously in section 2.2.5.6. The positive control group was implanted with 1 μg/pellet of Ang2; one of the test groups was implanted with a combination of 1 μg/pellet of both Ang2 and the angiopoietin trap, sTie2. Another test group was implanted with 1 μg/pellet of Ang2 along with 1 μg/pellet of anti Tie2-scFv antibody. Yet another group was implanted with Ang2 along with both sTie2 and anti-Tie2-scFv antibody. The vehicle control group was implanted with a pellet having PBS. All groups were observed for 7 days and the corneas were photographed using a stereo binocular microscope with CCD camera attachment, Stereo Discovery V20, Carl Zeiss, Germany. The number of blood vessels per mm² from limbus was counted and quantified using image J1.49 u software (National Institutes of Health, USA).
3.3. Results

3.3.1. Construction of recombinant bacmid

The gel extracted sTie2 gene (941 bp) released from pCEP4-sTie2 vector (11.3 kb) was successfully cloned into the linearized pFastBac-HTb donor plasmid (4.8 kb) using a combination of blunt end and sticky end ligation, culminating in increase in the molecular size of recombinant pFastBac-HTb-sTie2 (5.7 kb) as shown in agarose gel pictures Figure 3.6a, b and c, respectively.

Next the recombinant pFastBacHTb-sTie2 plasmid was transformed into competent DH10Bac E coli cells where the sTie2 expression cassette was transposed into the bacmid (bMON14272) with the help of the helper plasmid (pMON7124). The transformants harbouring positive recombinant bacmids were selected by blue-white screening (Figure 3.7a and b) and the high molecular weight bacmid DNA from the recombinant white colonies was isolated and presence of sTie2 insert was confirmed by colony and bacmid PCR, which showed up as a single prominent band at ~ 950 bp (Figure 3.7b).

3.3.2. sTie2 protein expressed in insect cells

Post transfection of Sf-21 cells with recombinant bacmid using Lipofectamine, the transfected cells had slightly altered morphology with increased size and shape (Figure 3.8a). The spent media containing the recombinant virus was enriched by repeated re-infection of fresh batch of Sf-21 cells. The cells were lysed and the lysate containing the recombinant 6xHis tagged sTie2 fusion protein was purified to homogeneity using Ni-NTA column chromatography. The homogeneity of the purified pooled protein fractions was confirmed by SDS-PAGE with silver staining analysis (Figure 3.8b), which revealed a polypeptide band corresponding to 116 kDa. Immunoblotting of the purified protein, using rabbit polyclonal anti-Tie2 antibody demonstrated cross-reactivity at 116 kDa, (Figure 3.8c) illustrating that the expressed protein was indeed sTie-2. The purified protein yield obtained was ~1mg/mL.
3.3.3. Tie2 receptor is overexpressed in highly metastatic tumor cells

The overexpression profile of Tie2 receptor in human breast cancer tumor biopsies was ascertained by immunohistochemistry and in human cancer cell lines such as MDA-MB-231, U87-MG, A-375 and HEK293 (IHC) by qPCR, respectively.

IHC staining of the paraffin sections of various grades of human ductal breast carcinoma tumor biopsies revealed upregulation in the expression of Tie2 receptors as the grade of cancer progressed, indicating that the Tie2 receptor overexpression is an event in the early stages of carcinogenesis (Figure 3.9a).

Further, the Tie2 receptor expression profile was examined by qPCR analysis in human tumor cell lines. The melt curve and melt peak analysis of the amplicons obtained at the end of qPCR are shown in Figure 3.9b. The qPCR results showed overexpression of Tie2 mRNA in MDA-MB-231 and U-87MG cell lines by 12.5-fold and 4.7-fold, whereas in A-375 cell line, the fold increase was negligible by 0.6-fold compared to the normal cell line HEK293 as shown in Figure 3.9c.

3.3.4. Preparation and cloning of complete scFv fragment into phagemid vector

The complete flowchart of construction and cloning of the scFv cDNA into the phagemid vector is schematically represented in Figure 3.10a. The yield of total RNA (133 ng/µL) isolated from the splenocytes was further purified to obtain mRNA. After the first-strand cDNA synthesis by reverse transcription of 200 ng of mRNA, the cDNA was used to amplify the V_H and V_L by PCR as shown in Figure 3.10b, with a 340 bp (V_H) and 325 bp (V_L) bands visualized on the agarose gel (lanes 2 and 3). The resultant V_H and V_L cDNA fragments were purified and assembled into a single chain fragment using a peptide linker (Gly4Ser)3 using overlap extension PCR resulting in a ~800 bp amplicon (Figure 3.10c). This ~800bp cDNA was gel extracted, purified and further amplified using primers containing SfiI and NotI sites to introduce these restriction sites. The cDNA was digested with SfiI and NotI restriction enzymes and ligated to the analogous sites in the linearized pCANTAB5E phagemid vector as shown in the recombinant plasmid map (Figure 3.10d). The purified scFv fragment (800 bp) and the digested vector (4.5 Kb) (Figure 3.10e) were successfully ligated as shown in Figure 3.10f.
3.3.5. Expression of scFv in pCANTAB5E-scFv transformed HB2151 cells

After the ligated pCANTAB5E-scFv was transformed into HB2151 E. coli, the positive transformed colonies were identified by blue-white screening (Figure 3.11a) and the presence of insert confirmed by colony PCR, plasmid PCR and plasmid restriction digestion. The recombinant plasmid had a size of 5.2 Kb and the PCR amplified scFv amplicon had the expected size of 800 bp (Figure 3.11b).

The transformed White colony cultured in 2x-TY medium was expressed in E. coli HB2151 cells after induction with IPTG. The SDS PAGE of all the respective uninduced and induced S1 S2 and S3 fractions showed a prominent scFv protein band corresponding to ~28 kDa in the IPTG induced periplasmic fraction (S1) (Figure 3.11c). The expressed scFv was also present in the induced osmotic (S2) as well as in the whole cell lysate fractions (S3), but at a lower concentration.

3.3.6. Identification of the sTie2 specific scFv by transformed colony screening

Among the ~60 pCANTAB5E-scFv transformed colonies that were screened for the expression of scFv that specifically bind to sTie2 antigen by the modified dot blot assay in the 96 wells plate format, the S1, S2 and S3 fractions from several colonies showed positive binding, some showed strong affinity and some showed weaker affinity. However, we ultimately culminated in two colonies namely HSN6 and HSN14 (Figure 3.12) that exhibited the strongest binding with potential scFv binding sites on the sTie2 antigen.

3.3.7. Homology modeling of scFv-sTie2 structure

The nucleotide sequence obtained after sequencing of scFv-sTie2 insert had 823 bp nucleotides encoding 274 amino acids that included a flexible amino acid linker of (Gly4Ser)3. The nucleotide or the amino acid sequences have not been disclosed here since they are patent eligible subject matter and have been deposited in the NCBI database.

The nucleotide BLAST analysis revealed ORF identity to some of the Mus musculus and synthetic immunoglobulin coding sequences (Figure 3.13a) and protein BLAST analysis revealed sequence similarity to other scFv synthetic constructs with conserved domains of IgVH and IgVL kappa with significant similarities of 80%
with anti-TNF single-chain Fv antibody (AJF21026.1), 79% with another anti-TNF single-chain Fv antibody (AJF20988.1) and 76% with Chain B, Cd47-diabody Complex (PDB: 5IWL_B) as shown in Figure 3.13b in the NCBI database.

The conserved framework regions (FR1, FR2 and FR3) and complementarity determining regions (CDR1, CDR2 and CDR3) of the V\text{H} and V\text{L} domains were determined according to the IMGT numbering system and Kabat by IgBLAST. Based on the amino acid sequence, considering the framework regions and the CDRs, a homology based 3D model of the V\text{H} and V\text{L} chains was obtained from the antibody design platform, SABPred (Figure 3.14a), which showed homology to the monoclonal antibody targeting CD20 (PDB code: 3PP4), with the CDRs in their characteristic loop structures, that form the antigen binding pockets which was built using the.

The complete 3D model of scFv-sTie2 including the VH-Linker-VL chains was predicted using i-TASSER. The best predicted structural model (Figure 3.14b) had a C-score (C-confidence) of 0.57, TM-score (TM-template modelling) of 0.79±0.09 and an estimated RMSD (root mean square distance) of 4.6±3.0Å.

The structural validation of the predicted model was first evaluated by inspecting the conformation of its backbone by the distributions of the \(\Phi, \Psi\) dihedral angles in Ramachandran plot obtained. The plot (Figure 3.14c) showed that 90.3% of the residues fell into the most favoured regions, 8.7% of the residues into the allowed regions, none of the residues fell into the generously allowed regions and 1.0% of the residues into the disallowed regions. The two serine residues in the disallowed regions were from the linker segment. Therefore, dihedral angles of the structure of the model were reasonable. For 99.60% of the residues, the 3D–1D compatibility score was greater than or equal to 0.2 by Verify-3D (Figure 3.14d), which is a method that determines the compatibility of a 3D protein model with its own amino acid sequence (1D).

3.3.8. Molecular docking of scFv-sTie2 to Tie2 protein

The extracellular domain of the Tie2 receptor comprises of two unique immunoglobulin (Ig)-like domains, three epidermal growth factor (EGF)-like motifs, a third immunoglobulin (Ig)-like domain, and 3 fibronectin type III (FNIII) domains. The crystal structure (Figure 3.15a) shows an arrowhead-shaped molecule made up of the
Ig-like domains (Ig1–Ig3) and EGF-like domains (EGF1–EGF3). The Tie2 arrowhead terminal bearing the Ig2 loops constitute the Ang2 binding site (Barton et al., 2006).

To further perceive the binding mechanism, we performed an orientational docking of the models of Tie2 protein and scFv-sTie2 using ZDOCK program and the resulting models were further refined and re-ranked using the RosettaDock program, which resulted in top ten refined models.

Furthermore, only the complexes interacting with Ig2 loops of Tie2 arrow head with the CDRs of scFv-sTie2 were retained and the complexes not interacting with CDRs of the scFv were removed. The best probable complex with a total score of -545.069 and an RMSD of 19.269 was chosen on the basis of maximum buried surface area (Figure 3.15b). It was found that Ig2 domain of Tie2 receptor interacts with CDRs of both the light and heavy chains of scFv.

3.3.9. scFv-sTie2 purification by immunoaffinity chromatography

Purified sTie2 antigen was coupled to CNBr activated sepharose 4B beads and used to purify scFv-sTie2 protein from the periplasmic fraction. A total of 2 mg/mL of periplasmic fraction was loaded onto the column from which 0.36 mg/mL of scFv-sTie2 was purified. SDS-PAGE analysis showed a single band corresponding to 28kDa, while this band was not seen in the uninduced fraction (Figure 3.16).

3.3.10. Inhibition of Ang2 mediated corneal neovascularization by scFv-sTie2

The inhibition of Ang2 mediated in vivo corneal neovascularization was evident in the presence of scFv-sTie2 antibody in comparison to the extensive neovascularization in Ang2 treated positive control cornea. Although the Ang2 trap, sTie2 could bring about inhibiton of Ang2 mediated neoangiogenesis by 0.5-fold, the Tie2 receptor neutralizing scFv-sTie2 antibody was slightly better in angiogenesis inhibition by 0.4-fold. However, their combined effect of sTie2 and scFv-sTie2 reduced the Ang2 mediated neoangiogenesis by 1-fold. Thus the quantification of angiogenesis clearly showed that anti-Tie2-scFv treatment could significantly reduced angiogenesis mediated by Ang2 in a non tumor context (Figure 3.17).
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Figure: 3.6. Subcloning of sTie2 gene from pCEP4-sTie2 vector into pFastBac HTb. (a) Agarose gel showing DNA ladder (Lane 1), BamHI digested, end filled and NotI digested pCEP4-sTie2 plasmid showing the 941 bp insert released (lanes 1 and 2) and undigested pCEP4-sTie2 plasmid (Lane 3). (b) Agarose gel showing DNA ladder (Lane 1), StuI and NotI double digested pFastBac-HTb plasmid (lanes 1 and 2) and undigested pFastBac-HTb plasmid (Lane 3). (c) Agarose gel showing DNA ladder (Lane 1) and the ligated pFastBac-HTb-sTie2 recombinant plasmid (5.7 kb) along with unligated insert (941 bp) (Lanes 2 and 3).

Figure: 3.7. Screening of recombinant DH10Bac cells with pFastBacHtb-sTie2 plasmid. The pFastBacHtb-sTie2 plasmid was transformed into competent DH10Bac E coli cells by calcium chloride method and the positive colonies were selected by blue white screening. (a) Competent control (b) Blue-white selection, white colonies marked by arrows and (c) 0.8% agarose gel showing the presence of sTie2 insert; Lane1: 1Kb DNA ladder, Lane2: PCR of a blue colony, Lane3: PCR of a white colony showing Tie2 insert and Lane4: PCR of bacmid isolated from white colony showing Tie2 amplicon (941bp)
Figure: 3.8. Expression of sTie2 protein in Sf-21 insect cells and purification. The recombinant bacmid isolated from white colonies was transfected into Sf-21 cells using lipofectamine for sTie2 protein expression. (a) Untransfected Sf-21 cells in culture, (b) Bacmid transfected Sf-21 cells after 72 h showing altered morphology. (c) 12% SDS PAGE of expressed sTie2 protein, Lane 1: Protein marker, Lane 2: Unpurified Sf-21 cell lysate, Lanes 2-4: Ni-NTA column purified sTie2 protein (116 kDa). (d) Western blotting of purified sTie2 protein (116 kDa).

Figure: 3.9. Tie2 receptor expression profile in human tumor biopsies and cell lines. (a) immunohistochemistry of sections of different breast tumors showing progressive increase in Tie2 expression as the grade of cancer progressed. (b) qPCR analysis results showing melt curve analysis, (c) melt peak analysis and Tie2 mRNA expression profile in MDA-MB-231, U87-MG and A-375 compared to HEK293 normal cells.
Figure: 3.10. Preparation and cloning of assembled scFv fragment into pCANTAB5E phagemid vector. (a) Schematic representation of the flowchart of construction and cloning of the scFv cDNA into the phagemid vector pCANTAB5E. (b) 1% agarose gel showing the reverse transcribed cDNA amplification of the V_H (340 bp) and V_L (325 bp) fragments by PCR. (c) Purified V_H and V_L cDNA fragments joined together using a peptide linker (Gly4Ser)3 by PCR resulting in a ~800 bp amplicon. (d) Recombinant pCANTAB5E-scFv plasmid map (5.2 kb) after PCR based restriction cloning of scFv fragment with 5’ SfiI and 3’ NotI restriction sites. (e) Agarose gel showing the gel purified, SfiI and NotI digested scFv fragment (800 bp) and pCANTAB5E vector (4.5 kb) (f) Agarose gel showing the ligated product of the scFv fragment and the linearized vector in three different ratios, with a size of 5.2 kb.
Figure: 3.11. IPTG induction of scFv in pCANTAB5E-scFv transformed HB2151 cells. (a) Blue white screening of pCANTAB5E-scFv transformed HB2151 colonies. (b) Agarose gel showing the presence of scFv gene insert; Lane 1: protein marker, Lane 2: recombinant pCANTAB5E-scFv plasmid (5.2 kb), Lane 3: colony PCR of a white colony showing expected band at 1500 bp, Lane 4: PCR product (1500 bp) of purified pCANTAB5E-scFv plasmid, Lane 5: Sfi-I and NotI double digestion of pCANTAB5E-scFv plasmid showing the 800 bp scFv insert released. (c) 12.5% SDS PAGE showing prokaryotic expression of scFv protein; Lanes 1 and 8: protein marker, Lanes 2, 4 and 6: uninduced S1, S2 and S3 fractions, Lanes 3, 5 and 7: Induced S1, S2 and S3 fractions, respectively.

Figure: 3.12. Representative 31 micro titer wells out of 60 wells screened for sTie2 specific scFv. Approximately 60 recombinant white colonies were induced by IPTG to express the scFv and all the three fractions, S1, S2 and S3 were screened for the presence of sTie2 specific scFv using a modified dot blot assay in a 96 well ELISA plate. The colonies HSN6 and HSN14 were positive for expression of scFv-sTie2.
Figure: 3.13. Screen shot of results of nucleotide and protein blast analysis of scFv nucleotide and amino acid sequence. (a) Nucleotide BLAST analysis showing ORF identity to some of the *Mus musculus* and synthetic immunoglobulin coding sequences. (b) Protein BLAST analysis showing presence of conserved domains of IgVH and IgVk kappa and sequence similarity to other scFv synthetic constructs.
Figure: 3.14. Structure prediction and *in silico* analysis of the scFv-sTie2 protein.

(a) The homology based 3D model showing the $V_H$ and $V_L$ domains and the looped CDRs. (b) 3D model of the complete $V_H$-linker-$V_L$ scFv molecule was *predicted by iTASSER* showing the $V_H$ and $V_L$ chains along with linker. (c) Ramachandran plot showing the $\Phi$-$\Psi$ dihedral angles. (d) The Verify-3D plot of scFv-sTie2 model.
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Figure: 3.15. Molecular docking of scFv-sTie2 to Tie2 protein. (a) The Crystal structures of the Ang2/Tie2 complex showing Ang2 ligand in complex with Tie2 ligand-binding domain comprising the Ig2 loop (illustration source Saharinen et al., 2015). (b) 3D models of Tie2 receptor (PDB: 2GY5) and the scFv-sTie2 model before docking. (c) 3D structure of the complex of Tie2 receptor and scFv-sTie2 after docking by ZDOCK and refinement by RosettaDock showing interaction of CDRs of scFv-sTie2 with the Ig2 domain of Tie2.

Figure: 3.16. Purification of scFv-sTie2 by immunoaffinity chromatography. (a) Schematic representation of conjugation of sTie2 protein to the CNBr activated sepharose 4B beads. (b) Silver stained SDS PAGE (12%) depicting purified scFv-sTie2 protein. Lane 1: protein marker, Lane 2: uninduced lysate, Lane 3: IPTG induced periplasmic fraction, Lane 4: IPTG induced periplasmic fraction, Lane 5: induced osmotic fraction, Lane 6: induced whole cell fraction, Lane 7: scFv-sTie2 purified by immunoaffinity chromatography.
Figure: 3.17. Ang2 mediated corneal neovascularization inhibition by scFv-sTie2. Representative photographs of rat corneal micropocket assay performed to study the anti-angiogenic potential of scFv-sTie2. Ang2, sTie2 and scFv-sTie2 or a combination of these (1μg/hydron polymer pellet) were surgically implanted into the micro-pocket in the cornea of one eye. On day 7, the extent of neovascularization or inhibition was visualized and photographed under a dissection microscope and a histogram showing quantitative comparison of the number of neovascular vessels per mm².
3.4. Discussion

Currently antibodies and antibody fragments can be genetically modified according to the requirement; employing advanced recombinant antibody technologies (Boss et al., 1984; Kontermann and Müller, 1999), which has in turn improved our perceptions of immunoglobulin structure and its functional constitution. Thus various antibodies and antibody fragments have been engineered that are beneficial for research, diagnostic, and therapeutic purposes.

Initial studies of production of recombinant antibodies by prokaryotic expression in E. coli were unsuccessful due to problems of cytosolic protein aggregation and improper folding (Boss et al., 1984; Cabilly et al., 1984). Thus, as an improvement of this technology, only the cDNA sequences coding for certain portions of the antibody molecule (Fab or Fv fragments) were expressed (Skerra and Pluckthun, 1988). Further refinement in prokaryotic expression of antibody fragments was accomplished by using phagemid vectors capable of expressing antibody fragments in a soluble form into the periplasmic space resulting in proper folding and formation of disulphide bridges (Skerra and Pluckthun, 1988; Better et al., 1988).

Thus, expression of small recombinant antibody fragments with the intact paratope (antigen binding site) is now feasible not only in prokaryotic system like E. coli (Skerra and Pluckthun, 1988), but also in yeast (Ho et al., 2006), insect cells (Choo et al., 2002) or mammalian cells (Ho et al., 2006) and also in plants (Galeffi et al., 2006).

The single chain fragment variable (scFv) is pharmaceutically superior compared to the monoclonal antibodies in several aspects such as minimal immunogenicity, rapid clearance from blood, decreased retention in non target tissue and improved tumor penetration. The ease with which the microbial systems can be exploited for the efficient production of such therapeutically and diagnostically important protein in fairly good quantities is an added advantage (Colcher et al., 1998).

In this objective of the study, we performed the cloning, expression, screening, and the assessment of biological activity of the purified scFv-sTie2 antibody against the Tie2 receptor using in silico and in vivo models.

Initially we expressed and purified the 116kDa soluble Tie2 protein using the baculovirus expression system and insect cells which yielded a protein concentration of ~1µg/mL. We further studied the expression profile of the Tie2 receptors in aggressive
cancers using human tumor cell lines and human tumor biopsies. Since the results revealed evident upregulation of the Tie2 receptors in certain cancers like the triple negative breast cancer, we strategized to exploit the overexpressed Tie2 receptors for the targeted drug delivery by means of nanomedicine by employing miniature antibody fragments such as scFv. Accordingly, we engineered the anti-Tie2-scFv antibody using the recombinant antibody technology, employing a phagemid vector and prokaryotic expression system wherein the soluble scFv was secreted into the periplasmic space.

However, instead of the elaborate conventional biopanning method of screening by phage display library of recombinant phages carrying sTie2-specific scFv, we adopted a simple method of screening to pick the sTie2 specific scFvs from a library of bacterial cell lysates by a modified dot-blot assay (Feng et al., 2013). The sTie2 specific scFv (scFv-sTie2) cDNA had an 823bp gene sequence that had conserved regions homologus to IgVH and IgVL regions of an antibody. The 274 amino acid had sequence similarity to other reported scFv molecules. Using the *in silico* approach we were able to predict the best possible model of our scFv-sTie2 protein, which was later used to perform the *insilico* molecular docking with the Tie2 receptor to find the potential binding sites. The docking studies revealed that the scFv-sTie2 was able to perfectly bind to the Ig2 loops in the extracellular domain of the Tie2 receptor with their CDRs.