The inhibition of vascular endothelial growth factor receptor-3 (VEGFR-3) signaling has important implications in circumventing tumor angiogenesis and lymphangiogenesis. Monoclonal antibodies inhibiting ligand binding to its receptor are already in clinical trials, and a murine antibody targeted towards the dimerization domain of the receptor has recently been described. This paves the way for new targeting modalities for counteracting receptor dimerization and activation, but human antibodies have thus far not been reported.

We sought to generate novel human Fab antibody fragments targeted towards the dimerization domain of VEGFR-3, by surveying the artificial human Fab phage display library (size $\sim 3 \times 10^{10}$) through affinity selections with the antigens b-R3D45 (biotinylated domains 4 and 5 of VEGFR-3) and R3D17 (full length extracellular domain of VEGFR-3) in mobile and solid phase panning respectively.

After 4 to 5 rounds of affinity selection, significant enrichment of VEGFR-3-specific clones was achieved, and subsequent characterization revealed at least 11 promising novel human Fab candidates. These Fabs were expressed in bacterial expression systems and validated by immunoblotting. Future work may be directed towards their purification and assessment in functional assays, such as the inhibition of ligand-mediated cell survival.

In conclusion, this study has demonstrated the robustness of the antibody library in generating novel human Fabs against the VEGFR-3 dimerization interface that could be translated towards the clinical inhibition of pathological angiogenesis and lymphangiogenesis in tumors.

**Keywords**
- VEGFR-3, antibody phage display, tumor angiogenesis and lymphangiogenesis
Phage Display Selection of Antibody Fragments targeted to VEGFR-3 Dimerization Domain

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Master's Thesis

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<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>b-R3D45</td>
<td>Biotinylated VEGFR-3 domains 4 and 5</td>
</tr>
<tr>
<td>BEC</td>
<td>Blood vascular endothelial cell</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CDR-Hn (where $n = 1, 2$ or $3$)</td>
<td>Heavy chain CDR 1, 2 or 3</td>
</tr>
<tr>
<td>CDR-L3</td>
<td>Light chain CDR 3</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding antibody fragment</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human anti-mouse antibody</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LEC</td>
<td>Lymphatic endothelial cell</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS containing Tween-20</td>
</tr>
<tr>
<td>Phage</td>
<td>Bacteriophage</td>
</tr>
<tr>
<td>R3D17</td>
<td>VEGFR-3 full length extracellular domain</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>VEGF receptor</td>
</tr>
<tr>
<td>2E11</td>
<td>Murine monoclonal antibody against VEGFR-3 dimerization domain</td>
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1. INTRODUCTION and REVIEW OF THE LITERATURE

1.1 Phage Display

1.1.1 History of phage display and its development

Phage display is a methodology that was first described by George P. Smith in 1985 in the prestigious journal *Science*. Its humble beginnings transpired with the exogenous expression of a small peptide fragment of the *Eco*RI restriction endonuclease on the surface of virions – filamentous bacteriophages which were then regarded as novel expression vectors (Smith, 1985). Though confined to the expression of small peptides in the early years, the first antibody phage display was reported just 5 years after the technique was first introduced (McCafferty, Griffiths, Winter, & Chiswell, 1990). Since then, its use has burgeoned into the generation and selection of large peptide and antibody libraries, where such phages could be exploited to display an immense array of different peptides, to the order of $10^{11}$. Such advances make it plausible and attractive to generate artificial antibody repertoires and diversity beyond what our natural immune repertoire could achieve (Sidhu & Koide, 2007). In addition, phage display has proven to be a robust method of surveying binding interactions against a diverse array of selector antigens, and selected phage-displayed biomolecules assume numerous forms, including the likes of zinc finger proteins (Choo & Klug, 1994), Src-homology-3 (SH3) domains (Hiipakka, Poikonen, & Saksela, 1999), hormones (Bass, Greene, & Wells, 1990) and enzymes (Demartis et al., 1999).

Antibody phage display, which exploits the bacteria *Escherichia coli* (*E.coli*) in the production of recombinant antibodies, serves as an enticing alternative to the traditional and laborious hybridoma technology of monoclonal antibody production. Indeed, an added advantage of using phage display as a choice method for antibody generation is the isolation of human antibodies that circumvent problems with immunogenicity such as hypersensitivity reactions and the elicitation of human anti-mouse antibodies (HAMAs) (Miersch & Sidhu, 2012). One of the early successes of antibody phage display is exemplified by the drug Adalimumab (HUMIRA), the first fully human phage display-derived monoclonal antibody, which has been used in the clinics to inhibit the inflammatory cytokine tumor necrosis factor-α (TNF-α) in autoimmune disorders such as rheumatoid arthritis and Crohn’s disease (Lorenz, 2002).
1.1.2 Biology of the filamentous bacteriophage M13

The M13 filamentous bacteriophage belongs to a group of closely related phages known as the Ff phages, hence called because of their characteristic of being F pilus-specific, which also include the f1 and fd phages (Rakonjac, Bennett, Spagnuolo, Gagic, & Russel, 2011). The M13 is shaped like a cylinder, and carries a single-stranded DNA (ssDNA) genome of about 6400 base pairs encompassing 11 genes in total. It infects specifically male *E.coli* which possess the F-pilus, encoded by the F-plasmid, and this interaction, though not completely understood, is known to be mediated by pIII, the minor coat protein of the virus. pIII is made up of two N-terminal domains N1 and N2 and a C-terminal CT domain, and is also required for the release of newly assembled phage progeny. The critical primary step entails the binding of N2 to the F-pilus, resulting in pilus retraction that facilitates the ensuing binding between N1 and the host cell periplasmic receptor TolA. The viral genome is then injected into the host cytoplasm, and the host inner membrane is inserted with the viral coat proteins. The CT domain mediates the release of progeny virus from the host membrane (Carmen & Jermutus, 2002). Interestingly, minute quantities of the f1 phage pIII have been demonstrated to inhibit further viral entry due to disruption in the host membrane integrity and the F-pilus (Boeke, Model, & Zinder, 1982). These phages differ from bacteriophages such as λ and T7 which replicate and assemble progeny viruses in the host cell cytoplasm, releasing them as part of their lytic life cycle. On the contrary, filamentous bacteriophages do not lyse their host cell upon replication and assembly, therefore allowing the host and the ‘hijacker’ to thrive and propagate, consequently enabling high viral titers to be achieved.

1.1.3 Concept of phage display

The fundamental principle of phage display is the linkage of phenotype and genotype, where the displayed fusion protein (phenotype) is coupled to the encoding genetic information (genotype) within a single phage particle, and as such, the genetic information that confers a target-selected interaction can be easily decoded. Fig. 1 below illustrates the entire phage display process, where unique phages are coded in red, blue and yellow. In brief, the golden rule of phage display involves binding – washing – eluting – and amplification. First, a library of phages each expressing a different unique molecular signature is introduced to a selector antigen either immobilized onto a surface or present in solution phase. Next, antigen-captured phages are separated from nonspecific phages by washing to remove the unbound phages, followed by the elution of the bound phages which are then used to transduce *E.coli* to amplify the phage population, at which point would have been enriched with target-specific clones. The process is cycled over a few times until the desired enrichment is attained, at the end of which specific clones could be isolated for further analysis. The M13KO7 helper phage is used for the rescue of phage progeny and amplification of phagemid-bearing clones (refer to below, section 1.1.4).
Figure 1. Principle of phage display.

The entire cycle of phage display is illustrated. A library of phages each displaying a unique peptide (and bearing its corresponding unique genome) is introduced onto a selector antigen. Following binding to the antigen, nonspecific clones are removed by washing and specific clones are eluted for transduction of bacteria and amplification of the phage pool, aided by helper phage co-infection. The amplified population, now enriched with antigen-specific clones, is now subjected to the next round of selection. The process continues until desired enrichment is observed.

1.1.4 Phage display formats

The two different formats of phage display are phages and phagemids, as illustrated by Fig. 2 below. Phage formats are generated by direct manipulation of the phage genome (phage vector) such that gene of interest is fused to the genetic information encoding a phage coat protein, resulting in the expression of fusion protein. Because all copies of the phage coat protein are expressed as fusion proteins, this provides a multivalent display of the exogenous peptide (Lee, Iorno, Sierro, & Christ, 2007).

Phagemids, on the other hand, are phage-derived plasmid vectors of approximately 4.6 kilobases (kb) which contain key elements such as the replication origin of a plasmid, a selectable marker of antibiotic resistance, a phage coat protein gene upstream of which the foreign gene is cloned, a promoter region, a signal sequence, restriction enzyme sites and an intergenic region (IG). The IG region carries the packaging sequence. An obligate requirement of phagemid vectors in producing infectious progeny phage particles is the co-infection of helper phage such as M13KO7 and VCSM13. The helper phage contributes the necessary genetic information to direct phage assembly and replication, though having itself carry a modified IG region and hence subjected to less efficient replication and packaging as compared to the phagemid which bears the wild-type IG region (Carmen & Jermutus, 2002). Importantly, this upholds the integrity of the genotype-phenotype linkage, because the genetic blueprint (phagemid genome) that specifies the selected target behaviour (displayed peptide) is not lost upon amplification in subsequent rounds of selection.

The phagemid format is more common due to several reasons rendering it superior to the phage format. First, the smaller size of the phagemid genome allows it to harbor a larger exogenous gene of interest. Second, for the same reason of having a smaller size, it exhibits a higher transformation efficiency consequently allowing the generation of libraries with large diversities. Third, its genome is more stable even upon subsequent cycles of amplification. Also, DNA manipulation is facilitated through the presence of numerous restriction sites in the phagemid genome. Last, elements such as the LacZ promoter within the phagemid genome also offer tight control over the expression of recombinant proteins, which may for example inhibit superinfection (Qi, Lu, Qiu, Petrenko, & Liu, 2012).

Both phage display formats require the fusion of the peptide of interest to a phage coat protein, usually the gene VIII-encoded major coat protein pVIII or the gene III-encoded minor coat protein pIII. In the phagemid format, particularly with pIII-fusions, a monovalent (single-copy) display of the fusion protein is most prevalent, with the majority of the pIII proteins (approximately 5 copies in total) being wild type and contributed by the helper phage. Therefore, the pIII-fusion phagemid system is tailored for probing high-affinity selections while phage formats and even the pVIII-fusion phagemid system are more adapted towards probing interactions relating to weak to moderate affinities, due to multivalent display.
and the contribution of the avidity effect (there are about 2,700 copies of the pVIII major coat protein in a single phage particle) (Qi et al., 2012). Because of the limitations of pVIII fusions in expressing larger proteins (Iannolo, Minenkova, Petruzelli, & Cesareni, 1995), pIII fusions are more suited for display of antibody fragments, as in the case of the work described herein.

Figure 2. Phage and phagemid display formats.

Fusion of the antibody gene (shown in red) to the phage gene III (shown in green and yellow) encoding the minor coat protein pIII is used as the model for illustration. (A) A classical multivalent phage display format where the antibody gene is directly engineered into the phage genome. (B) A modified phage display format which contains an extra copy of the genetic information that encodes the pIII protein, therefore capable of encoding both wild type pIII and pIII fusion proteins. This allows for the display of the fusion protein over a range of valencies. (C) A typical phagemid display format that only encodes pIII fusion proteins. All other phage genes encoding structural elements required for phage assembly as well as the information encoding wild type pIII are provided by the helper phage. This results in predominantly monovalent display of the fusion protein, although multivalent display is possible. (D) A modified version of the phagemid display format where the phagemid encodes pIII fusion proteins while the helper phage confers the genetic information for all other phage genes except for that encoding the wild type pIII, therefore resulting in multivalent display. Source: (Miersch & Sidhu, 2012).
1.2 Antibodies and the human Fab antibody library F-Library

1.2.1 Antibody structure and domains

The antibody structure and its various domains are illustrated in Fig. 3 below, using an immunoglobulin G (IgG) molecule as a model. An antibody is constituted by 2 heavy chains (shown in blue) and 2 light chains (shown in green) and presents with 2 identical antigen-binding sites on its ‘arms’ (one on each arm), hence being regarded as a bivalent molecule. Fabs are antibody fragments comprised of the antibody heavy chain N-terminal domains $V_{H}-C_{H1}$ and the entire light chain $V_{L}-C_{L}$. These fragments can assemble independent of the whole IgG molecule and are known to exhibit higher stability than smaller antibody fragments such as the single-chain variable fragment (scFv) (Sidhu & Koide, 2007), which consists of the variable domains of the heavy and light chains joint together via a peptide linker. The utility of scFvs in phage display is compromised as these fragments are often plagued with problems with multimerization and aggregation (Ponsel, Neugebauer, Ladetzki-Baehs, & Tissot, 2011). Within the variable domains of the Fab (and also in scFv), there are in total 6 complementarity determining regions (CDRs), hypervariable loops that govern antigen recognition and specificity, 3 of which are present in the heavy chain and the other 3 present in the light chain (shown in red).
Figure 3. Antibody structure and domains.

The whole IgG molecule is represented, assembled from 2 heavy chains shown in blue and 2 light chains shown in green. The Fab is the fragment consisting of an entire light chain and the heavy chain V_{H}-C_{H1} domains; while the scFv presents with only the variable domains of the heavy and light chains connected via a peptide linker. The V_{H} represents a further minimalistic domain comprised only of the variable domain of a heavy chain. Source: (Miersch & Sidhu, 2012).

1.2.2 Description and design of the human Fab antibody library F-Library

The human Fab antibody library F-Library used in our selection experiments has previously been generated and reported in as Library D, which had the greatest randomization profile and largest diversity (complexity) (Fellouse et al., 2007). This library has been used successfully in isolating antibodies specific to a diverse range of antigens, including human vascular endothelial growth factor hVEGF, the extracellular domain of prolactin receptor and 13 other protein antigens from the Midwest Center for Structural Genomics (MCSG), demonstrating its robustness and repertoire. The library has been constructed with the humanized Fab 4D5 as a scaffold, in a progressive fashion based on a synthetic minimalistic design such that the heavy chain CDRs and the third CDR of the light chain (CDR-L3) are restricted to a binary amino acid code between the residues Tyr (Y) and Ser (S) (Fellouse et al., 2005), with each step introducing added diversity. Fig. 4 below shows the library design and CDR diversity. Non-paratope positions (shown in orange) are subjected to randomization with a limited array of amino acid choices (between 2 or 4 amino acids) commonly observed at such positions with natural-occurring antibodies; while positions that are shown in grey are not randomized (often buried residues). Positions highlighted in blue are randomized with loops containing varying number of residues, therefore providing chemical diversity that significantly augments the library repertoire.
Figure 4. Library Design and CDR diversity.

Randomization at the complementarity determining regions (CDRs) are shown for CDR-L3, -H1, -H2 and -H3, with the humanized Fab 4D5 as the scaffold. Amino acids allowed at each position are represented by their single-letter code. Positions marked X allows for any of the 20 genetically encoded amino acids excluding Cys. Positions highlighted in grey are invariant; whereas positions highlighted in orange represent non-paratope positions where randomization is restricted to any of 2 or 4 amino acid choices. Positions highlighted in blue are randomized with loops containing different lengths of residues (4-6 residues for CDR-L3 and 6-17 residues for CDR-H3). The figure is adapted and modified from already published work (Fellouse et al., 2007); while the nomenclature of the CDR positions is based on the description by Kabat et al.

1.3 Vascular endothelial growth factor (VEGF) signaling and its implication in tumor angiogenesis and lymphangiogenesis

1.3.1 Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs)

Vascular endothelial growth factors (VEGFs) belong to a family of growth factors which in humans consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor PlGF. These growth factors play important roles in angiogenesis, lymphangiogenesis and vasculogenesis, and are implicated both in normal physiology and disease (Roskoski, 2007). Angiogenesis is defined as the formation of new blood vessels from preexisting vasculature, through the process of capillary sprouting; whereas vasculogenesis describes the process of new blood vessel formation from endothelial cells derived from precursor hemangioblasts. Although these two processes are distinct from each other, they are both tightly regulated by mechanisms which involve more than one signaling pathway, encompassing VEGF/VEGF receptor (VEGFR) signaling, angiopoietin/Tie signaling (Eklund & Olsen, 2006) and Eph/Ephrin signaling (Heroult, Schaffner, & Augustin, 2006). Lymphangiogenesis, a closely related process, refers to the
growth of the lymphatic vasculature, and is implicated in tumor progression where tumor cells are disseminated to regional lymph nodes and metastasized to distant sites, as well as lymphedema and other inflammatory disorders.

In adult humans, the vasculature is normally quiescent, with the endothelial cells dividing less than once per decade. Angiogenesis is only transiently activated upon physiological conditions such as wound healing and during the female reproductive cycle. However, in tumor progression, this otherwise quiescent nature becomes corrupted, and angiogenesis is continuously activated, constituting an 'angiogenic switch' that supports continuous vessel sprouting (Hanahan & Folkman, 1996).

VEGF, discovered in the 1980s, is a master regulator of vasculogenesis and angiogenesis, important in early embryonic development and in adulthood. It exerts mitogenic influence and confers survival towards vascular endothelial cells (Roskoski, 2007), as well as enhances endothelial cell and monocyte motility (Clauss et al., 1990). The biology of VEGF-B, on the other hand, is less well understood and has been considered to be required for normal heart development, although interestingly it may be involved in energy metabolism. VEGF-C and VEGF-D are predominantly factors involved in lymphangiogenesis, although they may also contribute towards angiogenesis under certain conditions. Both VEGF-C and VEGF-D are products of proteolytic cleavage, as opposed to VEGF and VEGF-B whose isoforms are derived from alternative splicing (Tammela, Enholm, Alitalo, & Paavonen, 2005). Vegfc-null mice are embryonic lethal due to defects in the early stages of lymphatic development; while a heterozygous disruption of the gene resulted in lymphatic deficiency and lymphedema despite survival till birth (Karkkainen et al., 2004).

VEGF receptors (VEGFRs) belong to a family of receptors that include VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt-4) protein receptor tyrosine kinases; and neuropilin-1 and neuropilin-2 non-protein kinase co-receptors (Roskoski, 2008). The VEGF ligand mediates its function via VEGFR-1 and VEGFR-2 (Hicklin et al., 2001). The importance of VEGFR-1 has already been demonstrated more than 15 years ago where germline Vegfr-1 deletion resulted in embryonic lethality due to aberrant vascular development, although interestingly inactivation of the same receptor by deletion of the tyrosine kinase domain still supported embryonic angiogenesis (Hiratsuka, Minowa, Kuno, Noda, & Shibuya, 1998). In normal physiology, VEGFR-2 stimulation drives angiogenesis of blood vascular endothelial cells (BECs) while VEGFR-3 stimulation drives that for lymphatic endothelial cells (LECs). The expression of VEGFRs by BECs and LECs is differential, with VEGFR-1 and VEGFR-2 expressed by the former, and VEGFR-2 and VEGFR-3 by the latter (Jeltsch, Leppanen, Saharinen, & Alitalo, 2013).
1.3.2 VEGF/VEGFR signaling and the tumor vasculature

VEGFs have been demonstrated to promote angiogenesis and lymphangiogenesis by the activation of VEGF receptor tyrosine kinases. Ligand binding primes the dimerization of VEGF receptor tyrosine kinases, which become activated via transphosphorylation (Lemmon & Schlessinger, 2010). VEGF-C ligand binding induces the heterodimerization of VEGFR-3 with VEGFR-2 in LECs, characterized by a phosphorylation profile that is distinct from receptor homodimerization (Dixelius et al., 2003). VEGF-C-mediated VEGFR-3 activation has also been demonstrated to enable LECs to survive, migrate and proliferate, via downstream signaling that implicates the phosphorylation of serine kinases Akt and Erk (Makinen et al., 2001).

VEGFR-3 is expressed during development in all endothelia of the vasculature, however in adults its expression becomes confined to the lymphatics (Kaipainen et al., 1995). Pathological expression of this receptor has also been observed in the tumor blood vasculature. The importance of VEGFR-3 has already been brought to attention in an early study wherein mice deficient in this receptor are non-viable and suffered from cardiovascular failure by embryonic day 9.5, a result of abnormalities in blood vessel organization (Dumont et al., 1998). Previous studies have demonstrated in murine models of angiogenesis that the inhibition of VEGFR-3 signaling by monoclonal antibodies suppressed endothelial proliferation, sprouting and vessel branching. In addition, the combinatorial use of VEGFR-3 and VEGFR-2 antibodies complemented each other and reported an augmented inhibition of angiogenesis and tumor growth (Tammela et al., 2008). VEGFR-3 is activated by the ligands VEGF-C and VEGF-D, and soluble ligand traps that take the form of the soluble extracellular domain of the receptor have also been successfully employed to disrupt VEGFR-3 signaling and inhibit tumor lymphangiogenesis and metastatic dissemination to regional lymph nodes in a prostate cancer xenograft model (Burton et al., 2008).

1.3.3 The use of antibodies targeting the VEGF/VEGFR axis

The impetus of targeting angiogenesis in order to curb tumor growth and progression has already been recognized more than four decades ago (Folkman, 1971). Fig. 5 below depicts the various routes where antibodies could be used to target the VEGF/VEGFR axis. The monoclonal antibody bevacizumab (Avastin), a potent inhibitor of the ligand VEGF, is now deployed in a plethora of different diseases, including kidney cancer, metastatic breast cancer (Miller et al., 2007) and advanced non-small cell lung cancer (NSCLC) (Sandler et al., 2006), in combination with chemotherapy or immunotherapy (Bagri, Kouroš-Mehr, Leong, & Plowman, 2010). Other antibodies directed towards the receptor arm of the axis have also been reported, and these antibodies target the ligand-binding domains of receptors VEGFR-2 (Witte et al., 1998) and VEGFR-3 (Persaud et al., 2004). Of note, the phase I dose escalation study of the
VEGFR-3 inhibitor IMC-3C5 has recently been completed, for patients with advanced solid tumors refractory to conventional therapy or where no standard therapy could be envisaged (http://clinicaltrials.gov/ct2/show/NCT01288989). Ramucirumab, an inhibitor of VEGFR-2 that also targets the ligand-binding domain, has recently been reported in a completed phase 3 trial, where it conferred survival benefits among patients with advanced gastric or gastro-oesophageal junction adenocarcinoma even when administered as a monotherapy (Fuchs et al., 2014).

**Figure 5. The inhibition of the VEGF/VEGFR axis.**

Antibodies targeting the ligand arm or the receptor arm of the VEGF/VEGFR signaling axis are illustrated, as well as soluble ligand traps that sequester ligands from binding and activating their cognate receptors. The Tie1 and Tie2 receptor tyrosine kinases of another receptor family are also represented. Ab denotes antibodies; Ab* denotes the subset of antibodies inhibiting receptor dimerization. TKI refers to tyrosine kinase inhibitors; Ang2 refers to angiopoietin-2 and Pb refers to a peptibody fusion protein against Ang2. Source: (Alitalo, 2011).
Recent work has expanded the inhibition of the VEGF/VEGFR axis with modalities other than inhibiting ligand binding and using soluble ligand traps, as exemplified by the use of a monoclonal antibody 2E11 that recognizes a conformational epitope present at least in part in the immunoglobulin homology domain 5 (D5) of VEGFR-3, the domain important for receptor dimerization. This murine monoclonal did not recognize the ligand binding domain D1-D3, and thus could inhibit VEGFR-3 signaling independent of ligand concentration (ie. even at high ligand concentrations where receptor-blocking antibodies would have been outcompeted). Indeed, this antibody inhibited VEGFR-3 heterodimerization with VEGFR-2 and signaling from VEGFR-3 homodimerization. More importantly, it exhibited a synergistic effect in inhibiting endothelial sprouting and vascular morphogenesis when used in conjunction with antibodies that inhibit ligand binding (Tvorogov et al., 2010). A graphical representation of the inhibition principle of this antibody is shown in Fig. 6 below.
Antibodies that inhibit ligand binding (shown in blue) are capable of inhibiting receptor homo- or heterodimerization (and their downstream signaling events), but this effect is severely compromised at high ligand concentrations. Antibodies targeted to the dimerization domain (shown in red) could also inhibit receptor homo- or heterodimerization, and remain effective even at high ligand concentrations. The coupled use of antibodies employing both strategies of receptor inhibition serves as a synergistic platform in antagonizing the VEGF/VEGFR signaling cascade. Source: (Tvorogov et al., 2010).

Figure 6. Combinatorial use of ligand-binding inhibitors and receptor dimerization inhibitors.
1.4 Background

The background of this Master’s thesis is based on the work already published (Tvorogov et al., 2010) and is an extension of the same principle of inhibiting VEGFR-3 homo- or heterodimerization, to be used in a synergistic fashion with existing antibodies that inhibit ligand binding in antagonizing the VEGF/VEGFR axis, particularly VEGFR-3-mediated signaling. Because the existing 2E11 monoclonal is of murine origin, and therefore implicates issues with immunogenicity such as HAMA, human antibodies would present as better therapeutics for this endeavor. One method of generating such human antibodies is to survey artificial human antibody phage display libraries for potential binders towards VEGFR-3 dimerization epitope.
2. AIM OF THE STUDY

The aims of this Master's thesis are:

1. To select human Fab antibody fragments targeted to VEGFR-3 dimerization domain by surveying the artificial human antibody phage display library
2. To assess the robustness and performance of the antibody phage display library as a valuable source of specific antibody fragments
3. MATERIALS and METHODS

3.1 Refreshment of antibody phage display library (F-library)

The antibody phage display library (kind gift of Dr. Fellouse) was refreshed from glycerol stocks by transducing logarithmic-phase (OD\textsubscript{600nm} = 0.5) TG1 E.coli in 1L liquid culture of 2xYT media, at a multiplicity of infection (MOI) of 0.1 for 1.5h at +37°C at 180rpm. Ampicillin was added to a final concentration of 100µg/ml for the selection of phage-transduced bacteria and the culture was maintained for an additional 1h. M13KO7 helper phages (kind gift of Next Biomed Technologies Oy) were then introduced at an MOI of 10 for the rescue of phage progeny, wherein the bacterial culture was maintained for 2h at +37°C at 180rpm. Kanamycin was then added to a final concentration of 70µg/ml and the culture was maintained overnight at +37°C at 180rpm. Bacteria were pelleted by centrifugation of the overnight culture at 5,000rpm (4,080g) for 30mins at +4°C on a Sorvall RC 5B Plus centrifuge. The phage-containing supernatant was filtered through a 0.45µm sterile filter unit (Millipore) to remove any bacterial debris. 1 volume of polyethylene glycol 6000 (PEG 6000, Fluka Chemika) containing 2.5M NaCl (VWR) was then added to 4 volumes of the phage-containing supernatant for phage precipitation, and incubated on ice for 1h. Phages were pelleted by centrifugation at 10,000rpm (16,300g) for 30mins at +4°C, then resuspended in PBS, and debris was removed by centrifugation at 5,000rpm (2,300g) for 20mins at +4°C using a table-top microcentrifuge (Eppendorf 5415 R). Finally, phages were ready for infectious titer determination.

3.2 Determination of phage infectious titer

10-fold serial dilutions of the concentrated phage stock were made in PBS to a total volume of 100µl, of which 10µl of the diluted phage preparation was used to transduce 200µl logarithmic-phase TG1 for 30mins at +37°C without shaking. 100µl of transduced bacteria were then plated onto Luria-Bertani (LB) plates containing 100µg/ml ampicillin as a supplement and incubated overnight at +37°C. Infectious titer was determined using the colony count assay where titer = Number of colonies x Dilution factor x 2 x 1000µl/ml x 1/Volume of plated cells (ie. 100µl), therefore expressed as colony-forming units/ml (cfu/ml). Using this equation the infectious titer of the refreshed naïve library was determined to be 1.7 x 10^{13} cfu/ml.
3.3 Affinity selection with VEGFR-3 fragments

Biopanning of the antibody phage display library was performed against VEGFR-3 fragments to identify any VEGFR-3-specific antibody fragments. The VEGFR-3 antigens were kindly provided by Dr. Veli-Matti Leppänen. Affinity selection was accomplished using 2 main strategies. The first strategy of employed a biotinylated version of VEGFR-3 domains 4 and 5 (b-R3D45) as the capture antigen. Specifically, 10µl of streptavidin-coated magnetic beads (Dynabeads M-280, Invitrogen) were washed in PBS according to the manufacturer’s instructions and incubated with 20µg of b-R3D45 in a total reaction volume of 200µl in PBS. The mixture was incubated at room temperature for 30mins with mixing by rotation. The control (mock) experiment involved a parallel set of 10µl beads incubated without the antigen. Beads were then magnetically separated and washed in accordance with manufacturer’s instructions and transducing phage units were introduced, in a total reaction volume of 1ml in blocking buffer PBS containing 5% non-fat milk. The incubation was allowed to proceed overnight at +4°C with mixing by rotation. Beads were washed 5 times in PBS and bound phages were released by trypsin elution, where 100µl of trypsin (Sigma) was added and the reaction incubated for 1h at room temperature. The eluate was used to transduce 10ml of TG1 bacteria in exponential growth phase for 1h at +37°C at 180rpm. 300µl of this culture was removed to prepare 10-fold serial dilutions in 2xYT media. For output titer determination, 100µl of each diluted preparation was plated onto Luria-Bertani (LB) plates containing 100µg/ml ampicillin as a supplement and incubated overnight at +37°C. The output titer was calculated as follows: Output titer = Number of colonies x Dilution factor x 1/Volume of plated cells (ie. 100µl) x 1000µl/ml, therefore expressed as cfu/ml. The b-R3D45-selected phage pool was to be amplified. Ampicillin was added to a final concentration of 100µg/ml and the culture was propagated for a further 1h. Helper phages were introduced as above for the rescue of progeny phage and the culture was propagated overnight with selection using 70µg/ml kanamycin as described above. Phages were harvested and precipitated as described above, resuspended in PBS and used for subsequent rounds of affinity selection.

The second strategy of affinity selection featured VEGFR-3 domains 1 through 7 (R3D17), as the capture antigen. Specifically, the antigen R3D17 was passively-coated onto 6-well plates (Greiner CELLSTAR) at a concentration of 10µg/ml overnight at +4°C, in a total volume of 1ml in PBS. Control wells were incubated in PBS without antigen. Wells were washed 3 times in PBS and blocked for 1.5h at room temperature with PBS containing 5% non-fat milk to prevent unspecific binding. Wells were washed as above and phages were incubated in a total volume of 1ml in PBS containing 5% non-fat milk for 1h at room temperature with rotation on a platform at 90rpm. Wells were washed 5 times in PBS containing 0.1% Tween-20 (PBST) to remove unbound phage. 2ml of logarithmic-phase TG1 bacteria was then added for transduction by bound phage without elution, 30mins at +37°C without shaking. After transduction, a 200µl aliquot was removed for 10-fold serial dilution in 2xYT and 100µl of
each dilution was plated for output titer determination. The output titer was calculated based on the same
equation as above. The remaining 1.8ml of transduced bacteria was combined with 48ml of logarithmic-
phase TG1 bacteria for increased culture volume and subsequent phage recovery. The 50ml culture was
propagated for 30mins at +37°C at 180rpm. Ampicillin was added to a final concentration of 100µg/ml and
the culture was propagated for a further 1h. Helper phages were introduced as above and the culture was
propagated overnight with selection using 70µg/ml kanamycin as described above. Phages were harvested
and precipitated as described above, resuspended in PBS and used for subsequent rounds of affinity
selection.

3.4 Screening for positive clones by monoclonal phage ELISA

To identify VEGFR-3-specific antibody fragments, random colonies were selected from
enriched output titer plates for liquid culture in 2xYT media containing 100µg/ml ampicillin as a
supplement, either as 10ml cultures in 50ml tubes, or in 96-well plates (Nunc), the latter based on the
protocol as described by others (Lee et al., 2007). Phages were produced from individual phagemid clones
as a homogeneous preparation and subsequently screened using a monoclonal phage ELISA. The phage-
containing supernatant was precipitated and prepared as a concentrated stock for the case of 10ml
cultures or used directly without precipitation as a high-titer concentrated phage preparation was not
necessary for phage production from 96-well plates.

The monoclonal phage ELISA protocol was adapted from already published work (Lee et al.,
2007). 6-well plates (Nunc) were coated overnight at +4°C with 5µg/ml b-R3D45 or 2µg/ml R3D17 antigen
diluted in PBS in a volume of 100µl. Wells were washed 3 times in PBS and blocked with 250µl of PBS
containing 5% non-fat milk for 2h at room temperature, with rotation and mixing at 130rpm. Wells were
washed again for 3 times in PBS. In a separate 96-well plate, phage-containing supernatant of each clone
was diluted 1:4 in PBS containing 5% non-fat milk as the blocking buffer to a volume of 100µl and applied to
the wells washed above. Alternatively, precipitated concentrated phage stocks were used at 1:10 dilution in
blocking buffer. The incubation of the phages to allow binding to coated b-R3D45 or R3D17 was carried out
for 1h at room temperature with rotation at 90rpm. Wells were washed 5 times in PBS containing 0.1%
Tween-20 (PBST) and an additional 3 times in PBS to remove all unbound phage. Anti-M13 HRP-conjugate
mouse monoclonal antibody (GE Healthcare) was applied at 1:2000 dilution in PBS containing 5% non-fat
milk at a volume of 100µl, and incubated for 1h at room temperature with rotation at 90rpm. Wells were
washed 3 times in PBST and an additional 3 times in PBS. Finally, 100µl ready-to-use ABTS substrate
(Invitrogen) was applied to the wells, incubated for 15-30mins at room temperature and the absorbance at
405nm was measured with a 96-well plate Multiskan EX spectrophotometer (ThermoLabsystems). Positive
clones were defined as those which gave a signal of at least twice that of a negative control phagemid clone selected from a control output titer plate (Moreland et al., 2012). The positive control was represented by clone P4.1, whenever applicable. These clones were further analyzed and characterized by performing plasmid minipreps (Qiagen) of the corresponding phagemid clones and sequencing using the sense primers FLibF1 and AM126 for probing the light chain and heavy chain respectively.

3.5 Cloning of VEGFR3-specific Fab genes into pET12a expression vector

In order to perform subsequent functional studies, these newly identified phage-displayed human antibody fragments specific to VEGFR-3 dimerization domain were introduced into a different bacterial expression system for periplasmic expression. These human antibody fragments were to be expressed as soluble proteins devoid of any phage component/pIII minor coat protein. The pET12a expression vector was selected for this endeavor, to be used in conjunction with the BL21(DE3)pLysS strain of E.coli. This pET12a vector had previously been engineered to contain a NotI site between its SalI site and BamHI site. Phagemid DNAs of these individual unique clones were prepared using miniprep kits (Qiagen) and their corresponding antibody genes PCR-amplified with the primers YSL01 and YSL02. The antisense primer YSL02 also introduced a TAG stop codon just before and overlapping the NotI site. PCR was performed using Phusion DNA polymerase in accordance with the manufacturer’s instructions on a G-Storm GS482 thermal cycler (Gene Technologies). PCR products were analyzed on agarose gel electrophoresis and purified with PCR product purification kit (Qiagen). Purified PCR products were then digested with SalI and NotI (Fermentas), gel-purified (Qiagen gel purification kit) and ligated into similarly-digested modified pET12a vector using T4 DNA ligase (Fermentas). Ligated products were transformed into the XL1-Blue E.coli strain for plasmid DNA amplification and purification by miniprep kit (Qiagen). All newly cloned pET12a constructs were sequenced verified with the sense primer FLibF1.
3.6 Bacterial expression of human Fab antibody fragments

The BL21(DE3)pLysS strain of E. coli was used for periplasmic expression of the cloned human antibody fragments in modified pET12a vector. Specifically, pET12a vectors containing the antibody inserts were transformed into the above-mentioned E. coli strain. Quadruplicate colonies were picked per each unique antibody clone to inoculate overnight 5ml starter cultures of Luria broth containing 100µg/ml ampicillin as supplement. Overnight cultures were diluted to 100ml Luria broth containing 100µg/ml ampicillin and grown to exponential phase at which point IPTG (Fermentas) was added to a final concentration of 0.5mM. Cultures were propagated overnight at +37°C at 180rpm. Overnight cultures were cleared by centrifugation at 5,000rpm (3,020 g) for 30mins at +4°C, and the Fab-containing supernatants filtered through a 0.45µm filter unit (Millipore) to remove any bacterial debris. These supernatants were subsequently screened by Western blotting to validate the presence of expressed Fabs.

3.7 Western blot validation of expressed anti-VEGFR3 human Fabs

Western blotting was performed to confirm the expression of VEGFR3-specific human Fabs via the FLAG epitope present C-terminal to the light chain antibody sequence. Filtered antibody-containing supernatants from above were boiled at +95°C for 5mins in 1x Laemmli buffer containing β-mecaptoethanol, then loaded onto and resolved by 12% polyacrylamide gel electrophoresis (PAGE). Resolved fragments were transferred onto 0.2µm nitrocellulose membranes (Biorad) by semi-dry transfer apparatus (Biorad Trans-Blot SD), and then blocked with PBS containing 5% non-fat milk for 1.5h at room temperature with mixing by a table-top shaker. After 3 washes with PBS containing 0.05% Tween-20 (PBST), the membranes were incubated with mouse anti-FLAG M2 antibody (Sigma) at 1:5000 dilution in PBS containing 3% non-fat milk, overnight at +4°C with mixing by a table-top shaker. Membranes were washed as above, and goat anti-mouse-IRDye680 secondary antibody (LI-COR Biosciences) was added at 1:2500 dilution in PBS containing 3% non-fat milk, and incubated for 1.5h at room temperature with mixing by a table-top shaker. Membranes were washed as above, and analyzed on the Odyssey infrared analyzer (LI-COR Biosciences).
4. RESULTS

4.1 Affinity selection

4.1.1 Results of first series (P-series) of affinity selection experiments using b-R3D45

A total of 5 rounds of affinity selection (P-series) were performed using the biotinylated capture antigen b-R3D45 via the first strategy (Fig. 7a). In the first round of selection, P1, $6.8 \times 10^{12}$ transducing phage units from the refreshed naïve library were introduced for binding to b-R3D45, which through its biotin moiety had been immobilized on streptavidin-coated magnetic beads. This quantity of phages fully represented the entire repertoire and diversity of the entire Fab antibody library whose size had previously been empirically determined to be at about $3 \times 10^{10}$ (refer to Fig. 4). The input and output phage transducing units were determined by titration of transduced bacteria using the colony count assay as described in Materials and Methods.

There was no overt enrichment of specific b-R3D45-binding clones, as the output titers of both b-R3D45-selected phages and those from the control (mock panning) were of the same order of magnitude (a modest enrichment of 1.3). This represented a canonical result of the first round of biopanning, in which no specific binders would have been enriched at this early stage.

In the second round of affinity selection, P2, a total of $2.9 \times 10^{12}$ phages amplified from the first round were introduced for binding to the capture antigen b-R3D45. As with the case in the first round of selection, there was not an outnumbering fold of b-R3D45-selected phages over the control, although the enrichment factor had slightly increased from 1.3 to 3.0, suggesting that the phage pool might have been tapered towards more specific clones. Interestingly, the output titer was markedly reduced as compared to that obtained in the first round, differing by a few orders of magnitude. Nonetheless, as enrichment was not envisaged at this stage of affinity selection, b-R3D45-selected phages were amplified for the third round of selection.

In the third round of affinity selection, P3, $1.14 \times 10^{12}$ phages amplified from the second round were introduced for binding to b-R3D45. For the first time, a dramatic enrichment of phages was observed with the capture antigen b-R3D45 as compared to control. There was a more than 200-fold enrichment which strongly suggested that b-R3D45-specific clones have been enriched at this stage.

In the fourth round of affinity selection, P4, $2.3 \times 10^{12}$ phages amplified from the third round were introduced for binding to b-R3D45. The results of the fourth round recapitulated that observed in the third. A more than 200-fold enrichment of b-R3D45-selected phages as compared to control was again observed.
In the fifth round of affinity selection, P5, $2.88 \times 10^{13}$ phages amplified from the fourth round of selection were introduced for binding to b-R3D45. There was a more than 60-fold enrichment of b-R3D45-selected phages as compared to control. To exclude the possibility that phages might have been enriched towards the biotin moiety present in the biotinylated capture antigen b-R3D45, a control biotinylated antigen, b-GST Hck SH3 (biotinylated GST-tagged SH3 domain of Hck), was also introduced as a mock capture antigen for affinity selection done in parallel. Intuitively, this control capture antigen was not expected to enrich for any phages at this stage of biopanning when the phage diversity has already been primed towards R3D45-specific clones. Indeed, the b-R3D45-selected phages yielded an enrichment factor of 64.8 over this mock antigen selection (data not shown), demonstrating that the enriched phages were not targeted towards the biotin moiety. At this stage, it was decided that random clones be selected for further analysis of specificity by monoclonal phage ELISA and characterization by sequencing.

4.1.2 Results of second series (Q-series) of affinity selection experiments incorporating the alternative biopanning strategy

In a bid to uncover more promising specific clones to VEGFR-3 dimerization domain, the affinity selection strategy was modified such that b-R3D45 was no longer the only antigen choice in every round of selection. To this end, the extracellular domain of VEGFR-3, domains 1 through 7 (R3D17), was also selected as a capture antigen, to be used in an alternate fashion with b-R3D45. Because R3D17 was not biotinylated, it was passively coated onto the plastic surfaces of a 6-well plate and used to select for phages that bound to immobilized R3D17. The panning strategy was such that the first and third rounds (Q1 and Q3) were performed as before, through the use of streptavidin magnetic beads; while the second and fourth rounds (Q2 and Q4) introduced the new R3D17 method (Fig. 7b). A total of 4 rounds of selection were performed.

As the unique single clone P4.1 had already been uncovered in the previous series of selection experiments by this stage (refer to Results 3.2 below), it served as a useful tool by acting as a positive control in this new panning strategy. In the first selection round Q1, $4 \times 10^{12}$ phages of the refreshed naïve library and $1.2 \times 10^{10}$ phages of the homogeneous positive control clone P4.1 were introduced for selection with and without the capture antigen. Indeed, the positive control clone exhibited a more than 200-fold enrichment over the control (mock) selection (data not shown) demonstrating an exceptional binding towards b-R3D45 and its robustness for use as a positive control. With regards to the naïve library, results in Q1 reflected a modest 4.9-fold excess of b-R3D45-selected phages over the control (mock) selection, although any enrichment of specific clones at this first round of selection was not expected.
For the second round of selection Q2 wherein the R3D17 antigen was first used, 1 x 10^{11} phages amplified from the first round Q1 and 6 x 10^9 phages of the homogeneous positive control clone P4.1 were introduced for selection with and without the capture antigen. Similar to the results of Q1, there was no enrichment of R3D17-specific phages at this stage, concurring with results derived from the previous panning strategy where there was no enrichment after the first 2 rounds of selection. Again, in the second round of panning Q2, there was an incredible enrichment of this positive control clone retained by the R3D17 antigen over the control surface on which the antigen was not coated. This was completely within expectation as it was known that this clone was specific to b-R3D45 and had been prepared as a homogeneous preparation. A negative control clone represented by a homogeneous phage prepared from a phagemid clone selected from a control plate was also included in the selection. 2 x 10^8 phages of this negative control clone were introduced for affinity selection, but there was no enrichment of phages by R3D17 over the control selection (enrichment factor of 1.1).

In the third round of selection Q3, 1.3 x 10^{12} phages amplified from the second round Q2 were introduced for selection by b-R3D45. Essentially, the third round of selection which reverted to the use of b-R3D45 as the capture antigen was in principle the same as that performed previously (as in Q1 and P-series experiments). It was rather surprising that there was no enrichment of b-R3D45-selected phages over the control after 3 rounds of selection, which had already been evident in the previous P-series selections (a more than 200-fold enrichment by this stage). Nevertheless, a final selection round using immobilized R3D17 was performed.

In the fourth and final round of selection Q4, 2.2 x 10^{11} phages amplified from the third round Q3 were introduced for selection by R3D17. With this alternate antigen-switching mode of affinity selection, enrichment was finally observed after 4 rounds of selection, in particular up to 250-fold. Random clones were selected after 4 rounds of panning for further characterization by monoclonal phage ELISA and sequencing.
**Figure 7. Affinity selections for binders targeted to VEGFR-3 dimerization domain.**

(a) Results after 5 rounds of selection with b-R3D45 (P1 to P5) and (b) after 4 rounds of selection with the alternating use of b-R3D45 (Q1 and Q3) and R3D17 (highlighted in purple) (Q2 and Q4). Control (mock) refers to selections without the antigen. Input (and output) refers to the quantity of input (and output) transducing phage units. Enrichment is calculated as the ratio of outputs between antigen selections and control (mock) selections.

### 4.2 Screening for positive clones by monoclonal phage ELISA and analysis by sequencing

#### 4.2.1 Characterization of clones after P-series of affinity selections

Individual unique phagemid clones were randomly selected from the output titer plates of P4 and P5, the fourth and fifth rounds of affinity selection that used the first strategy, and subsequently generated as homogeneous phage preparations. 12 colonies each from P4 and P5 were selected and propagated in liquid culture, and it was hypothesized that clones from P4 may exhibit a certain degree of variability and diversity while those of P5 would probably be saturated with 1 or 2 high-performing clones. These clones were sequenced with the forward primer FLibF1 which probed the randomized CDR-L3 and the forward primer AM126 which probed the randomized CDR-H1, CDR-H2 and CDR-H3. Unexpectedly, the sequencing results revealed only one unique clone (P4.1), and by P4 this clone had already saturated the pool (Fig. 8a). Expectedly, when performed on monoclonal phage ELISA, this clone exhibited convincing
positive results as reflected by an almost instantaneous color change upon substrate addition. This indicated that in the affinity selection experiments 4 rounds were sufficient to isolate one single binder and perhaps there might be more diversity within the pool at the earlier P3 stage. Indeed, when the experiments were repeated and 12 clones (P3.1 to P3.12) were randomly selected and sequenced after the third round of panning, a certain degree of variability and diversity was observed (Fig. 8a), as demonstrated by the presence of 7 different clones. When subsequently screened by monoclonal phage ELISA, 5 of these 7 newly characterized clones yielded positive results. Of note, one clone (P3.3), though represented in 5 copies (out of 12 random selections) and seemingly had been enriched as a specific binder, yielded only weakly positive results in monoclonal phage ELISA, if at all. The other clone clone which did not give positive results was clone P3.11, recovered in 1 copy. Because these clones were randomly selected to be sequenced before being screened by ELISA to be positive, it was possible for nonspecific clones to have been included, such as in the case of P3.11.

In order to screen for positive clones at a much larger scale, a higher-throughput method was established such that 96 random clones could be screened in a single run. Therefore, 92 random clones (controls excluded) from this P3 selection round were also selected for propagation in liquid culture and homogeneous phage supernatant production. These phage supernatants were screened in monoclonal phage ELISA without duplicates which would otherwise decrease the number of screened colonies by half. 8 out of the 92 clones screened yielded promising results on monoclonal phage ELISA, all of which were sequenced and characterized (Fig. 8b). In addition, a random clone (3A4) that gave negative results on the monoclonal phage ELISA was also sequenced to query which background nonspecific clone remained in the phage pool after 3 rounds of selection. 2 positive control samples (independently selected clone P4.1 phagemid colonies) and 2 negative control samples (independently selected phagemid colonies from control output titer plates) were included. Multiple positive and negative control samples were used to minimize random errors arising from phage titer variation. Interestingly, this clone 3A4 was identical to clone P3.3 which had previously been characterized. This result strongly indicated and added weight to the speculation that clone P3.3 was indeed a nonspecific background member of the otherwise enriched phage pool. Intriguingly, 2 new clones were characterized (clones 3H2 and 3H4), which would almost certainly be missed had this new screening method not been established. Clone 3G11 surfaced again, identical to previously described clone P3.6, and was recovered in 4 copies. This clone was unmistakably a specific binder as it had been independently produced and validated by ELISA.
Figure 8. Characterization and CDR sequences of VEGFR-3-selected Fabs.

Positions indicated are based on the nomenclature described by Kabat et al. As previously described (Fellouse et al., 2007), the following residues are highlighted – Tyr, yellow; Ser, red; Gly, green. Invariant positions in the library design are highlighted in grey, while gaps in alignment are represented by dashes. (a) P4.1, the dominant single clone (highlighted in orange) after 4 and 5 rounds of selection with b-R3D45. In addition, 12 clones were randomly selected for sequencing after 3 rounds of selection with the same antigen (before screening by monoclonal phage ELISA). (b) Characterization of all 8 positive clones and 1 negative clone (3A4) after screening by monoclonal phage ELISA (total 9 samples). Clones were derived after 3 rounds of selection with b-R3D45 using the first strategy. Clones highlighted in purple are identical to each other; as is the case for clones highlighted in blue. (c) Characterization of 19 random positive clones after screening by monoclonal phage ELISA (total 91 positive samples). Clones were derived after 4 rounds of selection using the second strategy that alternated between the use of antigens b-R3D45 and R3D17. Clones highlighted in orange are identical to each other.

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4.2.2 Characterization of clones after Q-series of affinity selections

From the Q-series of affinity selection experiments that employed the second strategy in which the 2 capture antigens b-R3D45 and R3D17 were alternately used, 91 random clones (controls excluded) were selected from Q4, the fourth round of selection, in the same fashion as previously performed. These were also propagated in liquid culture in a 96-well plate format and produced as individual homogeneous phage supernatant. Remarkably, all 91 clones screened showed positive results (Fig. 9). It was not known at this stage if the pool had been already dominated by one or few clones, or if there remained a certain degree of diversity. Given that all clones were positive, 19 random clones were sequenced and characterized (Fig. 8c). Multiple positive and negative control samples were included, based on the same principle as above.

The majority of these newly sequenced clones (clone 4F1, 7 out of 19) were the exact clone as the positive control P4.1 that had previously been identified. However, there were also 3 new clones that were not characterized before (clones 4G1, 4D7 and 4A2). In particular, clone 4G1 was represented in 6 copies; clone 4D7 in 5 copies; while clone 4A2 was represented only in 1 copy. It would appear that clones 4G1 and 4D7, being recovered at higher frequency, may represent ‘fitter’ clones as compared to clone 4A2. It is noteworthy, however, that clone 4A2 exhibited a striking resemblance to the positive control clone P4.1, differing only in their CDR-L3 (identical CDR-H1, CDR-H2 and CDR-H3). It was also observed that the Q4 heterogeneous phage pool was somewhat different from that where the previous clones were characterized (P3 pool: P3.1 to P3.12; 3G11 to 3A4).

In summary, a total of 13 individual unique clones were enriched by b-R3D45 and R3D17 selection and sequence identified. With the exclusion of clones P3.3 and P3.11, these 11 clones, namely P4.1, P3.1, P3.2, P3.5, P3.7, 3G11, 3H2, 3H4, 4G1, 4D7 and 4A2, proved to be interesting candidates to be produced as human antibody fragments free of phage-derived material and further analyzed in functional studies. Since clone P3.3 was enriched for reasons not fully understood, it was also selected for subsequent cloning experiments as a non-VEGFR-3-specific negative control. Clone 4F1, a duplicate of the same clone as P4.1, was also selected for cloning.
Figure 9. Monoclonal phage ELISA screen for positive clones.

91 randomly-selected clones were screened after 4 rounds of selection which incorporated the second strategy (Q4), using ELISA with immobilized R3D17 as antigen. 19 individual clones selected for sequencing are shown in orange. The 2 positive controls and 3 negative controls are shown in red and blue respectively.
4.3 Cloning of selected antibody genes into pET12a expression vector

Fig. 10 below shows representative results from clone 4D7 of PCR experiments where the Fab antibody genes were amplified from the phagemid vector using the primers YSL01 and YSL02. To elucidate the optimal annealing temperature, PCR reactions were carried out with a temperature gradient from +60°C to +72°C (lanes 2 to 7). In all lanes, the 1.7kb PCR product was amplified demonstrating that even the lowest annealing temperature tested (+60°C) was a viable annealing temperature. Since no nonspecific products were formed at this temperature, it was standardized for subsequent PCR experiments for the other clones. Lane 8 represents the negative control sample without phagemid DNA template.

Figure 10. Agarose gel electrophoresis analysis of PCR-amplified Fab antibody gene.

The Fab antibody gene encoded by each unique phagemid clone is to be amplified by primers YSL01 and YSL02 and subsequently introduced into the modified pET12a vector for Fab expression. Results for clone 4D7 are shown, with a temperature gradient for annealing temperature being tested from +60°C to +72°C. Lane 1: GeneRuler™ 1kb DNA ladder (Thermo Scientific); Lanes 2 to 7: 1.7kb PCR product containing Fab antibody gene with annealing temperature gradient from +60°C (lane 2) to +72°C (lane 7); Lane 8: Negative control without phagemid DNA template.
4.4 Validation of expressed human Fabs by Western blot

Fig. 11 below shows representative western blot results from clones 3H2 and 4G1, detected by probing the FLAG epitope C’ of the light chain antibody sequence. Because of inter-individual differences between the colonies with regards to their propensity to express human Fabs, quadruplicates were picked per each unique Fab clone, hereafter referred to as a, b, c and d (eg. 3H2a, 3H2b, 3H2c).

The Fab human antibody fragments were expressed and their antibody light chains detected as proteins of approximate 25kDa (heavy chains were dissociated due to reducing conditions with β-mecaptoethanol that disrupted the disulphide linkages). As demonstrated below, all quadruplicate colonies of both clones 3H2 and 4G1 were able to express the desired antibody fragments, albeit to a different extent, reiterating the argument for inter-individual differences in protein expression. This strongly suggested, at least, that the 3H2 clone bacterial supernatant contained a higher concentration of human Fabs as compared to that of the 4G1 clone. The previously expressed Fab of clone P4.1 was used as a positive control in lane 2.

Figure 11. Anti-FLAG epitope Western blot for validation of expressed human Fabs.

Bacterial expression of human Fabs were confirmed by probing the FLAG epitope tag C’ of the antibody light chain sequence. The antibody light chains were detected as proteins of approximately 25kDa. Representative results are shown for clones 3H2 and 4G1 (in quadruplicates) with a previously validated clone P4.1 as positive control. Lane 1: ColorPlus™ Prestained protein ladder, broad-range (10-230 kDa)
Lane 2: Clone P4.1, positive control; Lanes 3 to 6: Clone 3H2 (a to d); Lanes 7 to 10: Clone 4G1 (a to d).
5. DISCUSSION

5.1 Selection of VEGFR-3 dimerization domain-specific Fabs and their characteristics

The selection of a plethora of VEGFR-3 dimerization domain-specific binders from this human antibody fragment library F-Library has attested to its performance and value as a rich resource for isolating desired immunoproteins against a diverse range of antigens (Fellouse et al., 2007).

Remarkably, the two strategies of affinity selection enriched for antibody clones which are distinctly different from one another, comparing clones of Fig. 8a and b to those of Fig. 8c (excluding clone P4.1). The first series of selection experiments were based solely on b-R3D45 immobilized on streptavidin magnetic beads and selection was performed only in mobile phase; while the second series alternated between b-R3D45 selection in mobile phase and R3D17 selection in solid phase. Though R3D17 would in theory encompass the entire sequence found on b-R3D45, it is arguable that the two antigens are not identical, especially within the context of the selection conditions in which certain epitopes may be masked or disproportionately represented. Therefore, in the selections that employed the second strategy the phage pool might have been subjected to a different conformation-specific selection pressure as compared to the phage pool present in selections that adopted only the first strategy. Therefore, the isolation of clones that differ significantly from each other may accrue, at least in part, from antigen-restricted surface determinants that are non-identical between b-R3D45 and R3D17, eg. clone 3H4 vs. 4D7.

Upon closer scrutiny of the characterized clones, numerous interesting points are noted. First, the library design was such that the CDR-L3 was randomized with loops containing 4-6 residues at positions 91-94 (refer to library design in Fig. 4). However, clones 4D7 and 4A2 proved otherwise by having 7 residues at these positions. Tipping the scale to the other side, several clones revealed short sequences at these positions by having only 3 residues, for example clones P3.1, P3.5 and 3H2, among others. In a similar fashion, the CDR-H3 was constructed such that positions 95-100a were randomized with loops between 6 and 17 residues. Yet, results show that several different clones harbored short sequences at these positions, ranging from 3-5 residues, although long CDR-H3 sequences do not preclude interaction and binding to our VEGFR-3 antigens, as exemplified by clone P3.1 that exhibited 17 residues at these positions, the longest possible loop length. This indicates that the library may have been much more diverse than initially thought.

Second, for both CDR-L3 and CDR-H3 where the greatest randomization was imbued, there is a resounding high frequency of Tyr (Y), Ser (S) and Gly (G) residues. This may not be entirely surprising, at least for the CDR-H3, as the library design had been biased towards these 3 residues. Also, this result is consistent with the notion and observation that naturally occurring antibodies indeed have their CDRs
enriched with the residues Y and S (Fellouse et al., 2005). Serine residues (and also alanine) have been reported to play an ancillary role by conferring conformational flexibility in tyrosine-dominated contact interfaces with antigen (Fellouse, Wiesmann, & Sidhu, 2004; Fellouse, Barthelemy, Kelley, & Sidhu, 2006). Intriguingly, some residues surfaced at higher frequencies than would be predicted, such as Trp (W), Val (V), His (H), Phe (F), Ala (A) and Pro (P), yet other residues such as Leu (L), Ile (I) and Met (M), as well as negatively charged Glu (E) and Asp (D) never once surfaced (unless dictated by library design at predefined positions), as if these latter-mentioned residues were omitted from the permitted amino acid choice. The high incidence of aromatic residues such as Y, W and F may translate to the speculation that these residues are responsible for dictating hydrophobic interactions within the binding cleft (the Fab:VEGFR-3 interface).

Third, although some of the characterized clones seem to be totally unrelated to each other, there are few which exhibited immense similarity. For example, clone P4.1 is almost identical to clone 3H2 with only 1 amino acid difference each in CDR-L3, -H1 and -H3. In addition, the CDR-H2 is common between clones P4.1 and 4G1, both of which being high-frequency clones of Q4, and is also present on clones 3H2 and 4A2, suggesting that this string of residues may play a role in specificity. Amazingly, clones P4.1 and 4A2 bear identical CDR-H1, -H2 and -H3, but their CDR-L3 are distinct from each other.

Clones P3.3 and P3.5 also bear an uncanny resemblance to each other, with only 4 residues being different (1 in CDR-L3; 3 in CDR-H1). However, P3.5 exhibited a positive signal on phage ELISA whereas P3.3 did not (weak at best), despite the latter whose several copies of the same clone had been tested. It may be surmised that clone P3.3 was able to perform and be retained within the phage pool upon subsequent rounds of selection by means other than b-R3D45-directed specificity.

Interestingly, clone 4G1 (represented in 6 copies) reflected slightly weaker ELISA signals in general when compared to clones 4F1 and 4D7, although this does not necessarily translate to weaker affinity. As the monoclonal phage ELISA screen employed phage-containing supernatant whose titer had not been pre-determined and normalized between the samples, variations in phage titer may account for differences in ELISA signals, at least in part, although it was reasoned that since these phage supernatants were produced as individual homogeneous preparations, specific binders would render convincingly positive signals even when tested at higher dilutions (ie. even if titer is low).
5.2 Modulation of the affinity selection strategy

The first round of affinity selection is the most critical stage during the selection process, as any specific binders lost would never be recovered in the subsequently amplified phage pools. Therefore, despite the phage pool being highly diverse and saturated with nonspecific clones even after the first selection round, specific clones that are retained within the phage pool, though seemingly outnumbered in the beginning, would easily be enriched upon succeeding selection rounds. The use of a higher antigen concentration in the first selection round as compared to succeeding rounds, which ensures the adequacy of binding sites, therefore represents a good strategy to minimize the loss of any specific binders in the early phase. This antigen concentration could be progressively tapered (lowered) upon each successive selection round to mimic a selection pressure, by exposing the enriched phage pool to a progressively limited binding surface, thus driving the enrichment of phages that exhibit a more favorable binding kinetic profile.

The use of b-R3D45 in solution phase during the first round of selection in both P-series and Q-series affinity selection experiments constitutes a better strategy than the use of R3D17 passively immobilized onto the plastic bottom of a 6-well plate, as the latter method may result in conformational changes in the peptide and/or denaturation, thus severely compromising the abundance of binding sites or even modify the antigenic epitopes to undesirable surfaces. Such corrupted surfaces may enrich for phages that compete with phages that are truly targeted to the desired antigenic surfaces, and intrinsic differences in growth properties between the different phages may further complicate the selection process. Notwithstanding, this does not negate the fact that specific binders could be generated towards passively coated antigens. Indeed, it remains to be verified if three to four rounds of selection using only the R3D17 antigen or performing selections that toggle between the two antigens with the first round using R3D17 would yield the same VEGFR-3-specific clones. Other parameters during the selection process could be modulated to enrich for binders towards a desired profile, for example limiting the interaction time between the amplified phage pool and the antigen after the first selection round, to select for binders that are readily captured by the antigen, ie. clones with high on-rate constant $k_{\text{forward}}$. 

5.3 Bacterial expression of human VEGFR-3-specific Fabs

The differences between the quadruplicate colonies within each unique Fab antibody clone in the degree of Fab expression have already been demonstrated in the results of Fig. 11. Apart from the inherent inter-individual differences, one technical reason that could account for the expression variability may be the fact that IPTG induction was initiated at the exponential phase of bacterial growth, but because induction was carried out for multiple samples simultaneously, it was virtually impossible to standardize the growth conditions of all individual cultures such that the optical density was identical, which therefore could translate to moderate or even significant differences in physiological conditions, growth and expression yield. Other means of expression employ the elegant use of amber-suppressor E.coli supE strains during the selection phase (eg. TG1, XL1-Blue MRF' and DH5a F'), where the TAG codon at the end of the antibody coding sequence encodes a glutamic acid instead of a stop codon hence displaying fusion proteins; and non-amber-suppressor strains during Fab expression (eg. TOP10 F', HB2151 and JS5), where the TAG is recognized as a stop codon, hence enabling the expression of soluble Fabs (Qi et al., 2012).

5.4 Future prospects

5.4.1 Further development and characterization of the selected VEGFR-3 Fabs

To query the efficacy of these selected human Fabs in inhibiting VEGFR-3-mediated signaling and how well they compare to the existing mouse monoclonal 2E11 in synergistic inhibition of VEGFR-3 signaling when used in conjunction with receptor blocking antibodies like 3C5, these newly described Fabs are to be concentrated from bacterial supernatants for instance by ammonium sulphate precipitation and/or purification by FLAG tag-specific affinity gels (or protein A affinity columns) and subjected to subsequent functional analyses, such as the inhibition of VEGF-C ligand-induced BaF3 cell survival (Tvorogov et al., 2010). Though Fabs serve as functional antibody domains which could elicit biological effects such as inhibiting receptor dimerization, it is not fully understood if their smaller size in comparison to a whole antibody may limit their efficacy in this context. Importantly, these Fab genes could also be cloned into expression vectors that permit the eukaryotic expression of whole human IgG antibodies that contain the effector Fcγ domain eg. in cell lines such as NS0 myeloma of murine origin (Persaud et al., 2004) (akin to ‘arming’ the Fab), which are not supported by prokaryotic expression systems like pET12a.

Another approach to assess the performance of these novel Fabs in comparison to the existing murine 2E11 in terms of binding affinity towards the VEGFR-3 dimerization interface is to perform a competitive ELISA, where different concentrations of each of these novel Fabs are applied to immobilized antigen VEGFR-3 dimerization domain in the presence of a constant concentration of 2E11. The extent to
which the novel Fabs would outcompete the binding of 2E11 to its target would elucidate information regarding their relative affinities towards the antigen.

Further studies to interrogate the binding kinetics of these human VEGFR-3-specific Fabs could be undertaken, by means such as the biolayer interferometry-based instrument where various concentrations of the soluble Fabs are introduced to biotinylated antigen immobilized onto a streptavidin biosensor surface, thus yielding a calibration curve from which the association rate constant $k_a$, dissociation rate constant $k_d$ and dissociation constant $K_D (k_d/k_a)$ could be derived (Zhang et al., 2012).

### 5.4.2 Phage display in relation to VEGF/VEGFR drug discovery in the future

The hunt for novel antiangiogenic therapeutics has been relentless, particularly with respect to new targeting modalities such as that reported for the inhibition of VEGFR-3 dimerization interface. Indeed, shortly after this description another study reported a fully human VEGFR-2 antibody 33C3 derived from the XenoMouse technology. In a similar fashion, this antibody specifically targets domains 4-7 of VEGFR-2, the extracellular portion of the receptor implicated in receptor dimerization but not ligand binding, and has been shown to mediate antiangiogenic effects independent of ligand concentration. Both in-vitro and in-vivo models also demonstrated its efficacy in suppressing angiogenesis (Kendrew et al., 2011).

The use of phage display to mine antibodies against other targets of the VEGF/VEGFR axis has been described, an example of which targeted the ligand arm of the axis, where a fully human monoclonal antibody fragment against human VEGF-C inhibited the ligand from binding to its receptors VEGFR-2 and VEGFR-3 (Rinderknecht, Villa, Ballmer-Hofer, Neri, & Detmar, 2010). Another report generated a fully human scFv fragment directed against VEGFR-2 which inhibited VEGF ligand-induced human umbilical vein endothelial cell (HUVEC) proliferation (Zhang et al., 2012).

With the advent of single-domain antibody fragments (also known as nanobodies, $\text{V}_{\text{HH}}$) that originated from the discovery of cameld heavy chain-only antibodies (Hamers-Casterman et al., 1993), it is of great interest to know if such minimalistic antibody domains could be isolated from phage-displayed immune libraries surveyed against antigens such as VEGFR-3, and more importantly if such immunoproteins could inhibit receptor activation and signaling by targeting the ligand-binding domain and/or the dimerization interface. Indeed, a recent study has already described a nanobody targeted towards the VEGF ligand that dose-dependently inhibited VEGF-stimulated HUVEC proliferation (Farajpour, Rahbarizadeh, Kazemi, & Ahmadvand, 2014). The ease of expression of such nanobodies in prokaryotic and eukaryotic hosts, as well as their capability of simultaneous inhibition of various ligands or their receptors,
among others, position them as unparalleled drug candidates of the future (Roovers, van Dongen, & van Bergen en Henegouwen, 2007; Van Bockstaele, Holz, & Revets, 2009).

Simultaneous targeting of VEGFR-2 and VEGFR-3 could work against tumor angiogenesis and lymphangiogenesis, as evidenced in a breast cancer metastasis model (Roberts et al., 2006). However, as these VEGF ligands and receptors govern intricate processes based on myriad ligand-receptor combinations, as well as their expression on diverse cell types, the greater challenge of the future would be finding the best method to home these novel specific therapeutics to the desired tumor locality.
6. CONCLUSION

The human Fab phage display library F-library has presented as an invaluable tool in isolating novel binders against the dimerization domain of VEGFR-3, and serves as a potential source of therapeutic antibodies targeting receptor dimerization and inhibiting receptor activation in tumor angiogenesis and lymphangiogenesis. As these novel antibody fragments are derived from an artificial human antibody library, problems relating to immunogenicity through the use of murine antiangiogenic monoclonals could be circumvented. Phage display technology remains a gold standard of display technology, and its continued use, together with more modern display platforms of the likes of ribosome display and mRNA display, will be entrusted with the task of uncovering novel and more potent therapeutics of the future, addressing the plethora of human pathologies, particularly cancer.
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REFERENCES


APPENDIX

Sequences of primers used in this Master’s Thesis:

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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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