Development of novel antiangiogenic biologics

by

Iacovos P. Michael

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Department of Molecular Genetics
University of Toronto

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Abstract

Current anti-VEGF biologics, such as bevacizumab and VEGF trap, have been successfully used as therapeutic agents for cancer and age-related macular degeneration (AMD). Since these strategies target VEGF systemically, their toxicity profile, including proteinuria and thromboembolic events, and need for frequent eye injections in AMD treatment, prevail. Therefore, the aim of this PhD thesis was to generate novel anti-VEGF biologics that inhibit VEGF activity specifically at the desired target site.

Two classes of biologics were engineered that simultaneously bind VEGF and either: 1) the extracellular matrix (ECM) or 2) target-site specific antigens. The first subgroup, “sticky-traps”, is composed of VEGF trap linked to a sequence of hydrophobic amino acids, with affinity for heparin sulfate proteoglycans of the ECM. The second subgroup, “lassos”, is composed of a C-terminus positioned form of VEGF trap linked to single-chain variable domain antibodies specific for either HER2 (HER2/V lasso) or fibronectin extra domain B (EDB; EDB/V lasso), expressed on breast cancer cell surfaces or in the vascular bed of solid tumours, respectively.
Using a novel transgenic method, piggyBac transposons, biologics were expressed in transgenic cancer cell lines in a doxycycline inducible manner. They were shown to inhibit VEGF activity and also retain the native function of their constituent domains. Specifically, the sticky-traps adhered to the ECM and the HER2/V lasso inhibited the proliferation of HER2 positive cancer cell lines.

Sticky-traps as well as lassos were able to inhibit or delay tumour growth of A-673, Pc-3, SKOV-3 and HT-29 xenografts. In contrast to soluble VEGF trap, sticky-traps were retained at the tumour site and were undetectable in the circulation. Moreover, sticky-traps, in contrast to VEGF trap, did not delay wound healing and regression of trachea blood vessels. Furthermore, transgenic studies indicated that HER2/V lasso is more effective compared to anti-HER2 Ab and VEGF trap used alone or in combination.

These novel classes of antiangiogenic molecules could be advantageous in a clinical setting. Using the principles established in my PhD thesis work, similar dual function biologics can be designed for inhibition of other molecules with disease relevance.
Acknowledgments

It is hard to imagine that my PhD journey, a journey that I had to re-start at one point in my graduate career, is coming to an end. It was definitely not an easy path to travel, but at the end of the day, it definitely helped me to achieve the highest level of academic recognition. I learned not only how to troubleshoot and solve biological problems, but most importantly how to build strong collaborations, accept and give advice/opinions.

I would not be able to travel this path without the huge contribution of an exceptional scientist but foremost a kind human being; my PhD supervisor Dr. Andras Nagy. Andras, thank you for believing in me, supporting me and for not only being my PhD supervisor, but a true mentor. Once I leave your laboratory I will have many good memories varying from the moments of brainstorming during our early 7:00 AM meetings, to drinking Zivania at lab parties.

Along with my supervisor, my committee members Drs. Jim Dennis, Frank Sicheri and Robert Kerbel were like “cardinal points” for me, guiding me in the right direction. I have enjoyed our intense academic discussions and debates. Jim, Frank and Bob thank you for your guidance, continual advice and support. I am also extremely thankful of the continuous support and great mentorship of Dr. Georgia Sotiropoulou, my first research supervisor.

I would also like to express my acknowledgements to all present and past members of the Nagy laboratory for their continual support, friendship and for creating an enjoyable laboratory environment. I would especially like to thank the postdoctoral fellows that were like mentors to me, Drs. Takeshi Baba and Hoon-Ki Sung. I owe a lot to them for being patient and not only teaching me many basic techniques, but always being there and willing to troubleshoot experiments with me.

I am also thankful to my relatives in Toronto, my aunt Soulla and uncle Giwrgo, and my cousins Jacob and Tommy for their support and warm hospitality. I would also like to
thank my parents-in-law, Raquel and José, for their many and wise advices as well as constant encouragement.

There are no words to express my gratitude toward my parents, Chrystalla and Prodromos, my sisters Varvara, Christina and Ifigeneia, and my brother-in-law Antrea. "Αγαπητοί μου γονείς και αδερφές σας ευχαριστώ πάρα πολύ. Αυτό το ταξίδι δεν θα είχε καμιά αξία αν δεν είχα εσάς να ομορφένεται την ζωή μου και να είσαστε μια αστείρευτη πηγή αγάπης από την οποία μπορώ να αντλώ δυνάμεις στις δύσκολες στιγμές της ζωής μου. Σας ευχαριστώ για όλα. Σας έχω πάντα στην σκέψη μου και στα βάθη της χειρότερης μου." 

During this journey I travelled and came a long way but I was always lucky to have my co-pilot, my colleague, friend and beloved wife Carla by my side. ¡Muchas gracias mi esposa querida por estar siempre a mi lado, entenderme y apoyarme! Tomamos la decisión correcta. Este viaje no fue fácil, pero las dificultades que encontramos nos hicieron más fuertes y nos ayudarán a caminar juntos el resto de nuestros días.

"Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference"

“The road not taken”
by Robert Frost (1874–1963)
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<td>amino acid</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADEPT</td>
<td>antibody-directed enzyme-prodrug therapy</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>Ang</td>
<td>angiopoietin</td>
</tr>
<tr>
<td>AS</td>
<td>antisense</td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
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<tr>
<td>BMDC</td>
<td>bone marrow derived cell</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
</tr>
<tr>
<td>CEP</td>
<td>circulating endothelial progenitor cell</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>colony-stimulating factor 1 receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
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<td>DR</td>
<td>diabetic retinopathy</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDA</td>
<td>extra-domain A</td>
</tr>
<tr>
<td>EDB</td>
<td>extra-domain B</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cell</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>Fc</td>
<td>constant domain of antibody</td>
</tr>
<tr>
<td>Fv</td>
<td>variable domain of antibody</td>
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<tr>
<td>FcRn</td>
<td>neonatal Fc receptor</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FIAU</td>
<td>2'-fluoro-5-iodoarabinosyluracil</td>
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<tr>
<td>Flk-1</td>
<td>fetal liver kinase 1</td>
</tr>
<tr>
<td>Flt-1</td>
<td>fms-related tyrosine kinase 1</td>
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<td>GBM</td>
<td>glioblastoma</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>GIST</td>
<td>gastrointestinal stromal tumour</td>
</tr>
<tr>
<td>GOI</td>
<td>gene of interest</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin-and-Eosin</td>
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<tr>
<td>HBD</td>
<td>heparin binding domain</td>
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<td>HPC</td>
<td>haematopoietic progenitor cell</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>heparin sulphate proteoglycan</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFP</td>
<td>interstitial fluid pressure</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMSI</td>
<td>integrase-mediated site-specific insertion</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility antigen</td>
</tr>
<tr>
<td>MVD</td>
<td>microvascular density</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Clinical Excellence</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NRP1</td>
<td>neuropilin 1</td>
</tr>
<tr>
<td>NSLC</td>
<td>non-small cell lung cancer</td>
</tr>
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<td>Description</td>
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</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidyl inositol</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidyl inositol phosphate</td>
</tr>
<tr>
<td>PIGF</td>
<td>placenta growth factor</td>
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<tr>
<td>PSMA</td>
<td>prostate specific membrane antigen</td>
</tr>
<tr>
<td>RfA</td>
<td>reading frame A</td>
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<tr>
<td>RPLS</td>
<td>reversible posterior leukoencephalopathy syndrome</td>
</tr>
<tr>
<td>RMCE</td>
<td>recombinase-mediated cassette exchange</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>scFv</td>
<td>single chain variable domain</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>rtTA</td>
<td>reverse-tetracycline transactivator</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TBS-T</td>
<td>tris-buffered saline-Tween</td>
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<tr>
<td>tgGOI</td>
<td>transgene of interest</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
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<td>TSP-1</td>
<td>thrombospondin-1</td>
</tr>
<tr>
<td>tTF</td>
<td>truncated tissue factor</td>
</tr>
<tr>
<td>TRE</td>
<td>tetracycline responsive promoter</td>
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<tr>
<td>VEGF</td>
<td>vascular-endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>vascular-endothelial growth factor receptor</td>
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<tr>
<td>VDA</td>
<td>vascular disrupting agent</td>
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<td>VSP</td>
<td>VEGF signaling pathway</td>
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Chapter 1

Introduction

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Chapter 1: Introduction

1 Angiogenesis

Blood vessels are essential for the survival of mammalian cells by providing oxygen and nutrients and removing toxic metabolic products. In vertebrates two major mechanisms of blood vessel formation exist: 1) endothelial cell (EC) differentiation from endothelial progenitor cells (vasculogenesis)\(^1, 2\), and 2) sprouting or intussusception from pre-existing vessels (angiogenesis)\(^3, 4\). Apart from its essential role during embryogenesis \(^5-7\), angiogenesis is also involved in physiological processes such as wound healing and regeneration of the endometrial lining during the menstrual cycle. In adult tissues angiogenesis is tightly regulated by angiogenic stimulators and inhibitors \(^8, 9\). An imbalance between these molecules, termed the “angiogenic switch”\(^10\), results in pathologic angiogenesis, which contributes to the pathophysiology of many diseases \(^11, 12\). Cancer, psoriasis, arthritis, age-related macular degeneration (AMD \(^13\)) and obesity are among the many disorders that are characterized or caused by excessive angiogenesis. On the other hand, insufficient angiogenesis can lead to heart and brain ischemia, gastric and oral ulcerations, hypertension, pre-eclampsia, and nephropathy.

1.1 Normal Angiogenesis

The formation of the vascular system is one of the first events during embryo development. The nascent vascular network is formed by \textit{de novo} vessel formation from angioblasts into a primitive vascular plexus (vasculogenesis) \(^1\). The formation of this plexus is then followed by a complex remodeling processes, involving angiogenesis (sprouting, bridging and intussusceptive growth from existing vessels), stabilization by recruitment of mural cells, followed by branching, remodeling and pruning and finally by arterio-venous specialization \(^14\). Every event during the development of a functional circulatory system is tightly orchestrated by various factors \(^15\). In a similar way, angiogenesis during adulthood, \textit{i.e.} during wound healing, is also regulated by the same factors, recapitulating the process that takes place during embryo organogenesis.
VEGF-A, the master regulator, is responsible for migration of angioblasts and endothelial cells which express VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). This process creates leaky, immature and unstable vessels. Vessels become stable upon recruitment of mural cells, pericytes and smooth muscle cells, which cover microvessels and large vessels, respectively. PDGFB is secreted by endothelial cells, presumably in response to VEGF, and facilitates the recruitment of mural cells. Upon recruitment, mural cells establish interactions with endothelial cells through the Ang-1/Tie2 and Ephrin-B2/Eph-B4 signaling pathways. Ang-2 causes vessel destabilization, which leads to vessel regression in the absence of VEGF and sprouting in the presence of VEGF. Secretion of TGF-β1 promotes vessel maturation by ECM production and by inducing the differentiation of mesenchymal cells to mural cells.
1.1.1 Regulation

A number of growth factors have been identified as important regulators of vasculogenesis and angiogenesis. The vascular endothelium-specific growth factors include members of the vascular endothelial growth factor, angiopoietin and ephrin families (Figure 1.1).

- Vascular endothelial growth factors

The vascular endothelial growth factor (VEGF) family includes five members, *i.e.* placenta growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A (from now on referred to as VEGF) was the first to be cloned 16-18, while the remaining members were identified based on their homology to VEGF. The crucial role of VEGF during embryonic development has been shown by genetic approaches using mouse models. The deletion of one allele results in embryonic lethality (haploinsufficiency) 5, 19.

VEGF has the ability to promote growth of vascular endothelial cells (ECs) derived from arteries, veins and lymphatics 20 and is a survival factor, both *in vitro* and *in vivo* 21, 22. VEGF is also known as vascular permeability factor, based on its ability to induce vascular leakage 16, 23.

The human *VEGF* gene has eight exons separated by seven introns (Figure 1.2a). Alternative exon splicing leads to the generation of different isoforms, with VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206}, being the most common ones, containing 121, 165, 189 and 206 amino acids, respectively 24. VEGF_{165} is the predominant isoform. Less frequent splice variants such as VEGF_{145} and VEGF_{183} have also been reported 25(Figure 1.2b). Mouse VEGF isoforms have one amino acid, *i.e.* glycine, missing at the N-terminus thus the corresponding isoforms are VEGF_{120}, VEGF_{144}, VEGF_{164}, and VEGF_{188}.

Due to alternative splicing of exon 8, a second set of splice variants is generated termed VEGF_{xxx}b, xxx denoting the amino acid length of the protein (Figure 1.2b) 26, 27. These isoforms have an alternative C-terminal sequence, STLRKD instead of CDKPRR,
Figure 1.2: VEGF gene, isoform and protein structures
which results in conformational alterations due to the absence of Cys and the corresponding
disulphide bond. VEGF\textsubscript{165}b was unable to induce VEGFR2 phosphorylation to the same
extent as VEGF\textsubscript{165}, and was unable to bind to VEGFR2 coreceptor, neuropilin 1 (see
below)\textsuperscript{28}. Furthermore, recombinant VEGF\textsubscript{165}b was able to inhibit angiogenesis in various \textit{in vitro} and \textit{in vivo} models as well as delay tumour growth in mice\textsuperscript{28}.

Native VEGF is a heparin-binding homodimeric glycoprotein of 45kDa\textsuperscript{17} (Figure
1-2c). The heparin-binding domain is a highly basic area encoded by exons 6 and 7. VEGF\textsubscript{189}
and VEGF\textsubscript{206} contain both exons and thus bind to heparin with high affinity\textsuperscript{29}. VEGF\textsubscript{121}
lacks the residues encoded by exons 6 and 7, and is an acidic polypeptide that does not bind
heparin and is freely diffusible\textsuperscript{29}. VEGF\textsubscript{165} and mouse VEGF\textsubscript{144} lack the residues encoded by
exons 7 and 6, respectively, and have intermediate properties, since once they are secreted, a
significant fraction remains bound to the ECM\textsuperscript{30}. Cleavage at the C-terminus of the ECM-bound
isoforms by plasmin and metalloproteases generates a diffusible/bioactive molecule\textsuperscript{29, 31}.
VEGF binds two related receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (Figure 1.3). The binding site for VEGF (and PlGF) has been primarily mapped to the second immunoglobulin-like domain. Although Flt-1 was the first RTK to be identified, the precise function of this molecule is still under debate. Flt-1 undergoes weak tyrosine autophosphorylation in response to VEGF and also binds PlGF and VEGF-B. Flt-1 has been proposed to have a negative role, either by acting as a decoy receptor or by suppressing signaling through Flk-1. An alternatively spliced/soluble form of Flt-1 (sFlt-1) has an inhibitory effect on VEGF signaling. Flk-1 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF. It undergoes dimerization and ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic and prosurvival signal. Neuropilin 1 (NPR1) enhances
binding of VEGF\textsubscript{165} to Flk-1. It has been proposed that NPR1 presents VEGF\textsubscript{165} to Flk-1 in a manner that enhances the effectiveness of Flk-1 mediated signal transduction\textsuperscript{38}.

- **Angiopoietins**

  Angiopoietins (Ang1, 2, 3 and 4) also have a pivotal role in vascular formation. Their receptors, Tie1 and 2, a family of tyrosine kinases are selectively expressed within the vascular endothelium\textsuperscript{39}. All of the known angiopoietins bind primarily to Tie2.

  Ang1 has a permissive role by optimizing the manner in which endothelial cells integrate with supporting cells, thus allowing them to receive other critical signals from the environment. Ang1 has the ability to maximize the interactions between endothelial cells and their surrounding support cell and matrix, leading to vessels that are resistant to leakage. Thus, Ang1 is thought to counter the effect of VEGF-A on permeability\textsuperscript{40}.  

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**Figure 1.3: VEGF family and VEGFR2 signaling pathway**

The members of the VEGF family and their cognate receptors are shown. Heparin sulphate proteoglycans (HSPs) are responsible for membrane and matrix retention of VEGF-A isoforms as well as PlGF and VEGF-B. VEGFR1 also occurs as a soluble form sVEGFR1, which serves as a negative regulator of VEGFR2 signaling, by acting as a trap for VEGF-A. VEGFR2 is implicated in most, if not all, aspects of VEGF signal transduction on endothelial cells. A number of pathways are responsible for the survival, migration, proliferation and permeability of endothelial cells. Each pathway is indicated by different coloured of arrows, blue for PI3K, purple for Src, green for Cdc/p38 and gray for PLC\textsubscript{γ}. Dashed arrows indicate enzyme/substrate reactions involving PI 4,5-P\textsubscript{2} (PIP\textsubscript{2}), PI 3,4,5-P\textsubscript{3} (PIP\textsubscript{3}) and inositol 1,4,5-P\textsubscript{3} (IP\textsubscript{3}). Yellow stars indicate the tyrosine phosphorylation sites, responsible for initiation of signaling cascades. Neuropilin-1 acts as a coreceptor for VEGFR2. VEGFR3 is found primarily on lymphatic endothelial cells and is critical for their proper function and establishment of the lymphatic vessels. DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; Erk extracellular regulated kinase, HSP27, heat shock protein 27; PI3K, phosphoinositide 3’ kinase; PKC, protein kinase C; PLC\textsubscript{γ}, phospholipase C\textsubscript{γ}.
Ang2 could act as a Tie2 antagonist and might provide a key de-stabilizing signal involved in initiating angiogenic remodeling. Autocrine induction of Ang2 in the endothelium may block the constitutive stabilizing influence of paracrine Ang1, allowing endothelial cells to revert to a more plastic and destabilizing state reminiscent of developing vessels.\textsuperscript{41-44}

• Ephrins

The Eph (receptor) – Ephrin (ligand) system is involved in the organization of the vascular system, \textit{i.e.} demarcation of arterial and venous boundaries, as well as establishment of vascular polarity. Ephrin-B2 and its receptor EphB4 display remarkably reciprocal distribution patterns during vascular development, with ephrin-B2 marking the endothelium of primordial arterial vessels while ephB4 marks the endothelium of primordial venous system. EphrinB2-EphB4 signaling is critical for the establishment of arterial and venous identities, and participates in the formation of arteriovenous anastomoses by arresting EC migration at the arterial-venous interface.\textsuperscript{45-47}

Ephrin-B2 expression extends progressively from arterial endothelium to the surrounding arterial smooth muscle and to pericytes during adulthood. In adult settings of angiogenesis, \textit{e.g.} in tumours, the endothelium of new vessels strongly re-expresses ephrin-B2.

• Platelet-derived growth factor

Platelet derived growth factor (PDGF) B is secreted by ECs, presumably in response to VEGF-A, and facilitates recruitment of mural cells. PDGFR-\(\beta\), which is expressed by mural cells, is responsible for their proliferation and migration during vascular development.\textsuperscript{48}

• Transforming growth factor (TGF-\(\beta\))

TGF-\(\beta\)1, secreted by ECs and mural cells, promotes vessel maturation by stimulating ECM production and inducing differentiation of mesenchymal cells to mural cells.\textsuperscript{49, 50}
1.1.2 Role during adulthood

For many years, the consensus was that quiescent blood vessels in normal adult tissues are not VEGF dependent. However, recent studies have revealed that VEGF signaling pathway (VSP) inhibition causes vascular regression and functional deterioration in a number of adult organs. These findings indicate a role for the VEGF signaling pathway in endothelial cell survival and functional homeostasis in fully developed adult organs even when active angiogenesis is not occurring.

- The cardiovascular system

VEGF deletion in the developing myocardium leads to fewer coronary blood vessels, thinned ventricular walls, depressed basal contractile function and an abnormal response to beta-adrenergic stimulation, indicating a critical role of VEGF signaling in cardiac morphogenesis and function.

Endothelial cell-specific deletion of VEGF with VE-cadherin-Cre causes endothelial cell apoptosis, cardiac hemorrhage and results in an increased risk of early postnatal lethality. Pharmacological inhibition of the VSP causes microvascular regression in adult quiescent vessels, pointing to the requirement of the VSP for vascular maintenance.

- Kidney

The kidney glomerulus of the kidney is the functional structure for blood filtration comprised of capillary endothelial cells, basement membrane and specialized visceral epithelial cells (podocytes). VEGF is expressed by glomerular podocytes and VEGF receptors are expressed on adjacent capillary endothelial cells. Podocyte specific deletion of VEGF, using Podocin- or Nephrin-Cre and a VEGF hypomorphetic allele (Vegf\textsuperscript{lo}), generated an allelic series of VEGF dosage in podocyte: Vegf\textsuperscript{-/-}, Vegf\textsuperscript{10/-}, Vegf\textsuperscript{+/-} that resulted in prenatal death, mesangiolysis and endotheliolsis respectively. These studies demonstrated dosage sensitivity as well as a critical role of podocyte-derived VEGF in glomerular development and functional homeostasis.
Nervous system

VEGF is highly expressed by various cell types of the nervous system, including mitral cells, tufted cells, periglomerular neurons and astrocytes in the olfactory bulb area as well as Purkinje cells in cerebellum. Mice homozygous for nervous tissue specific deletion of the hypoxia-response element in the VEGF promoter show adult onset of neurodegenerative changes similar to those in human amyotrophic lateral sclerosis (ALS). The blood flow was reduced suggesting that the lower perfusion of the nervous tissue might be the underlying mechanism. Mice with CNS specific Vegf deletion by Nestin-Cre displayed reduced vascular density in cortex and retina, which results in structural thickening abnormalities in those areas. The removal of VEGFR-2 from the neuronal lineage with Nestin-Cre does not result in any significant phenotype suggesting that VEGF has a paracrine role in regulating the density of the vascular plexus in the developing CNS. A recent study revealed that VEGF is required for the differentiation and survival of newly born neurons in the olfactory bulb.

The hematopoietic system

In the adult, VEGF signaling in bone marrow mononuclear cell differentiation, migration and survival has also been demonstrated. Further studies have shown that the VSP suppresses functional maturation of T-cell and dendritic cell populations. This phenomenon might be an underlying mechanism behind tumor induced immune system suppression in cancer patients.

The endocrine system

Animals treated with specific VSP antagonist, sVEGFR-1, showed a significant reduction of microvascular density in the thyroid, while treatment with a multitarget kinase inhibitor (AG-013736) resulted in an increased Thyroid-Stimulating Hormone production indicating impaired thyroid function.
Conditional over-expression of VEGF in pancreatic islets increases vascularization in the posterior foregut, which leads to ectopic insulin expressing cells as well as islet hyperplasia \(^{69}\). Islet-specific deletion of VEGF in the mouse with \(Pdx-1-Cre\) resulted in reduced capillary support of beta cells and impaired glucose-stimulated insulin secretion \(^{70}\). In the adult, pharmacological VSP inhibition results in a significant decrease in islet vascular density without apparent impairment of systemic glucose homeostasis \(^{53}\).

Adipogenesis has been thought to be an angiogenesis dependent process \(^{71-73}\). Pharmacological inhibition of angiogenic growth factors has been suggested as weight reduction therapies through suppression of adipogenesis \(^{74, 75}\). However, recent studies have demonstrated that impaired fat tissue perfusion caused by adipose hypoxia could lead to metabolic deterioration such as insulin resistance mediated diabetes \(^{76-80}\).

• The skeletal and integumentary system

Both osteoblasts and osteoclasts are known to express VEGF while the latter also express VEGFR-1 and -2. Previous studies have shown that VEGF directly enhances differentiation of osteoclasts and their bone absorption activity, as well as angiogenesis, indicating both a paracrine and autocrine role of VEGF \(^{81, 82}\). Recently, it was shown that VEGF overexpression in the endochondral skeleton resulted to increased bone formation by enhanced beta-catenin activity through the VEGFR-2 and PI-3 kinase pathway \(^{83}\).

In the skin, mature keratinocytes are constitutive sources of VEGF. Epidermal-specific deletion of VEGF in the basal layer (using the \(K5-Cre\) transgene) failed to show any abnormality in skin and hair growth, suggesting a dispensable role of VEGF in this organ. However, this mouse model shows delayed skin wound healing and impaired skin tumor formation, indicating an essential role of skin derived VEGF in pathological conditions \(^{84}\).
1.2 Pathological Angiogenesis

Abnormal angiogenesis can have a serious impact on the normal physiology of various organs and is implicated in the pathogenesis of many diseases. A number of diseases, such as cancer, psoriasis, arthritis and blindness, are characterized or caused by increased angiogenesis. Obesity, asthma, atherosclerosis and infectious diseases have also been associated with excessive angiogenesis. On the other hand, insufficient or abnormal angiogenesis is associated with heart and brain ischemia, neurodegeneration, pre-eclampsia, respiratory distress and osteoporosis.11, 12, 85

1.2.1 Cancer

The observation that blood supply is closely associated with tumour growth was reported more than a century ago.86 Judah Folkman in a seminal paper suggested that cancer cells, like other mammalian cells, have to be located within 100 to 200 μm from vessels, the diffusion limit of oxygen and nutrients, in order to be able to survive; thus tumour growth is angiogenesis-dependent.87 It soon became obvious that blood supply was not only necessary but also a rate-limiting step for tumour growth and metastasis.85, 86.

• Angiogenic switch and formation of tumour vessels

During tumour progression, the balance between pro- and anti-angiogenic molecules is disrupted and tipped in favour of angiogenesis, the “angiogenic switch”. Pro- and anti-angiogenic molecules are derived from cancer cells, endothelial cells, stroma cells, blood and the extracellular matrix (Table 1.1). Tumour vessels develop via angiogenesis from pre-existing vessels, by sprouting or intussusception, and via vasculogenesis from circulating endothelial progenitor cells derived from bone marrow.85 Moreover, tumour cells can also be involved in vessel lumen formation by mimicking endothelial cells (“vasculogenic mimicry”) and form part of the vessel architecture.88, 89.

• Structure and function of tumour vessels

Tumour vessels are structurally and functionally very distinct from those in normal tissues. The tumour vasculature network is highly disorganized, with tortuous and dilated
vessels of uneven diameter, and excessive branching and shunts. This leads to disrupted blood flow within the tumour and the creation of hypoxic and acidic regions. Within these hypoxic regions, human tumour cells that have lost their apoptotic response to hypoxia are clonally expanded and remain dormant for years. The latter describes “tumour dormancy” and is due to a balance between cell proliferation and apoptosis.

Tumour vessel walls are also abnormal, with having extended endothelial fenestrations, widened interendothelial junctions, and a discontinuous or absent basement membrane, as well as absence of smooth muscle cells. The endothelial cells themselves are abnormal in shape, growing on top of each other and projecting into the lumen. These defects lead to tumour vessels leakiness, which is also heterogeneous over space and time. This, in combination with the fact that functional lymphatics are absent within the tumour, contributes to high interstitial fluid pressure (“IFP”), which interferes with the delivery of therapeutic agents.

Lastly, one of the most important characteristics of tumour vessels is the expression of unique surface proteins (“vascular zip codes”) that are absent or barely detectable in mature, normal vessels. Given their tumour-specificity, these antigens represent novel targets that may be exploited in several treatment strategies, such as specific targeting and delivery of therapeutic agents.

1.2.2 Ocular neovascularization

Abnormal vasculature is associated with the pathophysiology of age-related macular degeneration and diabetic retinopathy, responsible for 50% and 17% of blindness in the United States and Europe, respectively.

1.2.2.1 Age-related Macular degeneration

Abnormal neovascularization is one of the hallmarks of the “wet” form of age-related macular degeneration (AMD), a potentially blinding disease. AMD will affect approximately one in three people to some degree by 75 years of age. AMD is categorized as early, during which symptoms are inconspicuous, and late, during which severe loss of vision is usual.
early phase is characterized by drusen (*i.e.* focal deposition of acellular, polymorphous debris between the retinal pigment epithelium and Bruch’s membrane) or by hyperpigmentation or by small hypopigmentation, without visible choroidal vessels. The late phase is further divided into “wet” and “dry” forms. Dry AMD is characterized by drusen and large areas of retinal atrophy (called geographic atrophy) extended to the center of the macula. The “wet” or neovascular form of AMD is characterized by choroidal neovascularization accompanied by increased vascular permeability and fragility\textsuperscript{13,101}.

Patients with neovascular AMD can have sudden, within days to weeks, visual loss as a result of subretinal hemorrhage or fluid accumulation. Although wet AMD represents only 10 to 15% of the overall prevalence, it is responsible for 80% of case of severe vision loss or legal blindness caused by both forms\textsuperscript{13,101}.

### 1.2.2.2 Diabetic retinopathy

Diabetic retinopathy is a common microvascular complication of diabetes. Thus, as the worldwide prevalence of diabetes continues to increase, diabetic retinopathy remains one of the leading causes of blindness in many developed countries. Chronic exposure to hyperglycemia and other risk factors (*e.g.* hypertension) are responsible for the initiation of a series of changes that lead to microvascular damage and retinal dysfunction\textsuperscript{102}.

The earliest stage, nonproliferative, is characterized by microaneurysms, dot hemorrhages, exudates, and retinal edema. The retinal capillaries leak proteins, lipids, and red blood cells into the retina, which can interfere with visual acuity. As the disease progresses, proliferative retinopathy develops, which is characterized by growth of new capillaries and fibrous tissue grow within the retina and into the vitreous chamber. Angiogenesis results as a consequence of small vessel occlusion, which causes hypoxia. Vitreous hemorrhage or retinal detachment can lead to blindness\textsuperscript{103,104}.
Table 1.1

Endogenous stimulators and inhibitors of angiogenesis

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial growth factors</td>
<td>Arresten</td>
</tr>
<tr>
<td>Fibroblast growth factors acidic and basic</td>
<td>Canstatin</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>Endostatin</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Fibulin</td>
</tr>
<tr>
<td>Transforming growth factor alpha and beta</td>
<td>Fibronectin fragments</td>
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<tr>
<td>Angiogenin</td>
<td>Pigment epithelial derived factor</td>
</tr>
<tr>
<td>Scatter factor/hepatocyte growth factor</td>
<td>Thrombospondin -1 and -2</td>
</tr>
<tr>
<td>Placenta growth factor</td>
<td>Tumstatin</td>
</tr>
<tr>
<td>Interleukin -6, -8</td>
<td>Interferons alpha and beta</td>
</tr>
<tr>
<td>Tumour necrosis factor alpha</td>
<td>Interleukins</td>
</tr>
<tr>
<td>Angiopoietin 1</td>
<td>Platelet factor-4</td>
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<tr>
<td>Endocrine gland VEGF</td>
<td>Angiostatin</td>
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<tr>
<td>Granulocyte-macrophage colony-stimulating factor</td>
<td>Anti-thrombin III</td>
</tr>
<tr>
<td>Ephrin-B2</td>
<td>Plasminogen Kringle 5</td>
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<tr>
<td>Delta-like-4</td>
<td>Prothrombin Kringle 2</td>
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<td>Tissue type plasminogen activator</td>
<td>sFlt-1</td>
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<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>Vasostatin</td>
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<tr>
<td>Matrix metalloproteinase -1, -2, -3, -9</td>
<td>Tissue inhibitor of metalloproteinases -1, -2, -3</td>
</tr>
<tr>
<td></td>
<td>Alphastatin</td>
</tr>
<tr>
<td></td>
<td>Prolaktin (16 KDa fragment)</td>
</tr>
</tbody>
</table>
2 Antiangiogenic therapy

A plethora of antiangiogenic agents and strategies are being currently evaluated in preclinical and clinical studies (Table 1.2). According to Folkman, angiogenesis agents can be divided into two broad categories based on their mechanism, direct and indirect. Direct inhibitors are usually endogenous (see below) and target endothelial cells directly by inhibiting their proliferation and migration. Indirect inhibitors are usually man-made (e.g. recombinant proteins) and target a growth factor crucial for angiogenesis, which is usually produced by a cell type other than endothelial cells, or its receptor.

The current antiangiogenic strategies are broadly classified into three categories. The first includes molecules that directly affect endothelial (progenitor) cells, inhibit angiogenesis and induce vessel regression. The main target in this area is VEGF and its receptors. The second category includes molecules, like PDGF inhibitors, that target the mural and stroma cells and destabilize vessels and lower the IFP, which improves drug delivery. The third area includes molecules, like VEGFR-1 inhibitors, that target haematopoietic cells, reduce the infiltration of pro-angiogenic bone-marrow-derived precursors and mature leukocytes and stimulate the release of endogenous angiogenesis inhibitors in dendritic cells.

Although there are a broad number of potential mediators of tumour angiogenesis, VEGF was considered as an attractive target due to its properties. It is an endothelial specific mitogen and angiogenesis inducer, and its receptors are primarily localized on endothelial cells. In 1993 Jin Kim et al. demonstrated for the first time that in vivo inhibition of VEGF using a neutralizing antibody had an inhibitory effect on tumour growth and hypothesized that blocking VEGF action could have a therapeutic potential for many malignancies with aberrant angiogenesis. Since then a number of approaches for inhibiting the VEGF signaling pathway have been developed (Figure 1.4) and tested in various clinical settings.
Table 1.2

Current inhibitors of angiogenesis and their primary targets

<table>
<thead>
<tr>
<th>Agent</th>
<th>VEGF signaling pathway</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biologics (protein-based)</strong></td>
<td></td>
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<tr>
<td>Bevacizumab (Avastin)*</td>
<td>VEGF-A</td>
<td></td>
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<tr>
<td>Ranibizumab (Lucentis)*</td>
<td>VEGF-A</td>
<td></td>
</tr>
<tr>
<td>VEGF trap (Aflibercept)*</td>
<td>VEGF-A, VEGF-B, PIGF</td>
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<tr>
<td>HuMV833</td>
<td>VEGF-A121, VEGF-A165</td>
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<tr>
<td>TB-403</td>
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<tr>
<td>IMC-1121b (Ramucirumab)</td>
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<td>VEGFR-1, -R2</td>
<td>Tie-2</td>
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<td><strong>Small Molecule Tyrosine Kinase Inhibitors (chemical-based)</strong></td>
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<td></td>
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<td>Sorafenib tosylate (Nexavar)*</td>
<td>VEGFR2</td>
<td>PDGFRβ, RAF</td>
</tr>
<tr>
<td>Sunitinib malate (Sutent)*</td>
<td>VEGFR2</td>
<td>PDGFRβ, c-kit</td>
</tr>
<tr>
<td>Pazopanib hydrochloride (Votrient)*</td>
<td>VEGFR1, -R2, -R3</td>
<td>PDGFR, c-kit</td>
</tr>
<tr>
<td>AZD6474 (vandetanib)</td>
<td>VEGFR2, -R3</td>
<td>RET, PDGFRβ and EGFR</td>
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<tr>
<td>GSK1363089 (foretinib)</td>
<td>VEGFR2</td>
<td>c-MET</td>
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<tr>
<td>SU5416 (semaxanib)</td>
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<td>c-kit</td>
</tr>
<tr>
<td>PTK787/ZK 222584 (vatalanib)</td>
<td>VEGFR1, -R2, -R3</td>
<td>PDGFR-α, -β, c-kit, c-fms</td>
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<tr>
<td>AG-013736 (axitinib)</td>
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<td>PDGFRβ, c-kit</td>
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<td>AZD2171 (cediranib maleate)</td>
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<td>PDGFR-α, -β, c-kit</td>
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<td>AV-951 (tivozanib)</td>
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<td>AMG 706 (motesanib diphosphate)</td>
<td>VEGFR1, -R2, -R3</td>
<td>PDGFR, c-kit, RET</td>
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<td>CEP-11981</td>
<td>VEGFR1, -R2, -R3</td>
<td>Tie-2</td>
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<tr>
<td>BMS-582664 (brivanib alaninate)</td>
<td>VEGFR1, -R2, -R3</td>
<td>FGFR1, -R2, -R3</td>
</tr>
<tr>
<td>BMS-690154</td>
<td>VEGFR2</td>
<td>HER1, HER2</td>
</tr>
</tbody>
</table>
2.1 Antiangiogenic strategies

2.1.1 Agents targeting the VEGF signaling pathway

Current anti-VEGF agents can be divided into two groups based on their molecular entity (Figure 1.4). The first group comprises biologics, i.e. antibodies and recombinant proteins aiming to hinder the interaction between VEGF ligand and receptor (Table 1.3 and 1.4). The second group is tyrosine kinase inhibitors (TKIs), i.e. chemicals that inhibit the activity of the kinase domain of the receptors and thus preventing downstream signaling (Table 1.3 and 1.4).

The biological therapies showed efficacy in the clinical setting only when they were administered in combination with chemotherapy. Multitargeted TKIs, on the other hand, were approved as monotherapy for the treatment of renal cell carcinoma. The most plausible explanation is the fact that TKIs are more efficient due to their ability to target more than one biological pathway simultaneously. Thus, biologics able to target multiple signaling pathways simultaneously might also be more effective.
Figure 1.4: Anti-VEGF targeting strategies.

Current anti-VEGF strategies use entities such as antibodies, recombinant proteins, aptamers, antisense oligonucleotides (AS) and small molecule chemicals. They act (a) by blocking the interaction between VEGF and its receptors (b) by inhibiting the activity of the tyrosine kinase domain (c) by interfering with VEGF translation at the mRNA level. DARPins are small, single domain proteins, derived from natural ankyrin repeat proteins, which can be selected to bind any given target protein with high affinity and specificity. Adnectins are fibronectin type-III domain (FN3)-based molecules with altered binding sites for specific protein with high specificity and affinity. CovX-bodies, chemically programmed antibodies, are a fusion of small synthetic molecules, peptides, with mAb. The peptides determine the specificity of the fusion molecule, while retaining the favorable pharmacokinetic profile and effector functions of the antibody.
2.1.1.1 Biological therapies

Biological therapies show high specificity for their target (i.e. ligand or receptor) and thus they do not exhibit any off-target effects. With respect to cancer patients, these agents are administered intravenously, every two weeks. In the case of patients with eye diseases, they are injected directly into the eye.

- Bevacizumab

Bevacizumab (Avastin, Genentech) is a humanized antibody of the mouse anti-VEGF MAb A.4.6.1\textsuperscript{116}. It binds and neutralizes the activity of all human VEGF-A isoforms but not the activity of the other members of the family, i.e. VEGF-B, VEGF-C and PlGF. Bevacizumab exhibits the expected pharmacokinetic properties of monoclonal Abs with serum half-life of 17-21 days in humans\textsuperscript{117}.

A study by Hurwitz et al. led to the approval of bevacizumab as a therapeutic agent in metastatic colorectal cancer (CRC) when used in conjunction with chemotherapy\textsuperscript{118, 119}. Since then, bevacizumab in combination with chemotherapy has been approved for use in recurrent or metastatic non-small cell lung cancer (NSCLC)\textsuperscript{120}, metastatic breast cancer\textsuperscript{121} and renal cancer\textsuperscript{122}, and as a single agent in recurrent glioblastoma (GBM)\textsuperscript{123, 124}.

A shorter version of bevacizumab (Fab fragment), ranibizumab (Lucentis, Genentech), has been approved for the treatment of neovascular AMD\textsuperscript{125}.

- VEGF-trap

VEGF-trap (Aflibercept, Regeneron) is a recombinant decoy protein able to neutralize VEGF activity\textsuperscript{126}. In order to have optimal pharmacokinetic and an acceptable toxicity profile it has been modified in a number of ways. The parental decoy protein was a fusion of the first three IgG-like domains of VEGFR-1 with the Fc constant region of a human IgG1 Ab. However, due to the positively charged amino acids of the first and third IgG-like domains, the parental trap exhibited poor pharmacokinetics. Thus, in order to improve upon this design, the first IgG-like domain was omitted and the third Ig-G like
domain of VEGFR-1 was substituted with the third IgG-like domain of VEGFR-2. The improved VEGF-trap has adequate pharmacokinetic properties. In addition to all VEGF-A isoforms, it is able to also VEGF-B and PlGF as well.

VEGF-trap is currently under evaluation in various clinical trials, including phase III in NSCLC, prostate, colorectal and pancreatic cancer \(^{114}\), as well as neovascular AMD \(^{127}\).

• **HuMV833**

HuMV833 is a humanized monoclonal Ab that neutralizes the activity of VEGF\(^{121}\) and VEGF\(^{165}\) \(^{128}\). This agent has been evaluated in early-stage Phase I clinical studies and further trials are awaiting in order to prove its true clinical value \(^{129}\).

• **IMC-1121b**

IMC-1121b (Ramucirumab, ImClone) is a fully human monoclonal IgG1 antibody against the extracellular domain of VEGFR-2 \(^{130},^{131}\). Binding to the receptor prohibits the interaction with its ligand, VEGF-A. A Phase I clinical trial demonstrated acceptable pharmacokinetic properties and showed antitumor and antiangiogenic activity \(^{132}\). IMC-1121b is currently enrolled in several Phase II clinical trials for a number of solid tumours. A Phase III clinical trial comparing docetaxel with or without IMC-1121b as first-line therapy for advanced breast cancer is also ongoing \(^{133}\).

• **IMC-18F1**

IMC-18F1 is a fully human monoclonal IgG1 antibody that specifically binds to VEGFR-1 and prohibits the interaction with its ligands (VEGF-A, VEGF-B and PlGF) \(^{134}\). A Phase I clinical trial demonstrated a favorable safety profile \(^{135}\) yet further studies are needed in order to evaluate its clinical value.

2.1.1.2 Small tyrosine kinase receptors inhibitors

Tyrosine kinase receptors (TKR) inhibitors are small chemical molecules that can inhibit the signaling pathway of more than one receptor simultaneously. This allows them to
have a broader antiangiogenic activity, but at the same time it increases off-target side effects. They are administered orally on a daily basis.

- **Sorafenib**

  Sorafenib (Nexavar, Onyx, Bayer) is a dual-action inhibitor that targets the RAF/MEK/extracellular signal-regulated kinase (ERK) pathway in tumor cells and tyrosine kinases VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, c-kit, Fms-like tyrosine kinase 3 (Flt-3) in the tumor vasculature. It is approved as monotherapy for renal cell carcinoma (RCC) and hepatocellular cancer (HCC). Currently, sorafenib is evaluated as monotherapy or in combination with chemotherapy in various Phase III clinical trials for melanoma, NSCLC, HCC, RCC, thyroid and breast cancer.

- **Sunitinib**

  Sunitinib (Sutent, Pfizer) is an inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, c-kit, Flt-3, RET and colony-stimulating factor 1 receptor (CSF-1R). It is now approved as monotherapy for advanced renal cell carcinoma and in imatinib-refractory gastrointestinal stromal tumours (GISTs). Clinical trials are underway to determine the efficacy of sunitinib in other tumor types including metastatic breast, colorectal, hepatocellular and lung cancers.

- **Pazopanib**

  Pazopanib (Votrient, GlaxoSmithKline) is a selective inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, and c-kit. It also has modest activity against fibroblast growth factor (FGF) receptors 1, 2 and 3 (FGFR-1, FGFR-2 and FGFR-3, respectively) and colony-stimulating factor 1 receptor (CSF-1R). Pazopanib has been recently approved as monotherapy for renal cell carcinoma, while current clinical trials are determining its efficacy for breast, thyroid and cervical cancers, as well as soft-tissue sarcomas.
Table 1.3

Antibody-based *versus* chemical-based therapeutic agents

<table>
<thead>
<tr>
<th></th>
<th>Antibody-based</th>
<th>Small molecule chemical-based</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target</strong></td>
<td>Number: One</td>
<td>Number: Multiple</td>
</tr>
<tr>
<td></td>
<td>Specificity: Very high</td>
<td>Specificity: Moderate</td>
</tr>
<tr>
<td></td>
<td>Type: Extracellular</td>
<td>Type: Intracellular</td>
</tr>
<tr>
<td><strong>Mechanisms of</strong></td>
<td>Main: Inhibition of signaling pathway</td>
<td>Main: Inhibition of signaling pathway</td>
</tr>
<tr>
<td><strong>action</strong></td>
<td>Other: (a) Effector functions (ADCC and CDC), (b) Conjugates</td>
<td>Other: None</td>
</tr>
<tr>
<td><strong>Penetration</strong></td>
<td>Tumour: Moderate</td>
<td>Tumour: Good</td>
</tr>
<tr>
<td></td>
<td>Cell membrane: No</td>
<td>Cell membrane: Yes</td>
</tr>
<tr>
<td><strong>Pharmacokinetics</strong></td>
<td>Half-life: Long; days to weeks</td>
<td>Half-life: Short; hours</td>
</tr>
<tr>
<td></td>
<td>Tunable</td>
<td>Non-tunable</td>
</tr>
<tr>
<td><strong>Administration</strong></td>
<td>Route: Intravenous</td>
<td>Route: Orally</td>
</tr>
<tr>
<td></td>
<td>Frequency: Every 3 weeks</td>
<td>Frequency: Daily</td>
</tr>
<tr>
<td><strong>Toxicity</strong></td>
<td>Low to moderate</td>
<td>Moderate to high due to off-target effects</td>
</tr>
<tr>
<td><strong>Immunogenicity</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Resistance</strong></td>
<td>Yes. Due to upregulation/activation of other signaling pathways that can compensate</td>
<td>Yes. Due to point mutations that can alter the kinase binding domain</td>
</tr>
<tr>
<td><strong>Manufacturing and</strong></td>
<td>Development: Complex and time-consuming</td>
<td>Development: Easier and faster</td>
</tr>
<tr>
<td><strong>quality control</strong></td>
<td>Cost: High</td>
<td>Cost: Moderate</td>
</tr>
</tbody>
</table>

2.1.1.3 Aptamers

- **Pegaptanib**

Pegaptanib sodium injection (Macugen) is a pegylated oligonucleotide aptamer that binds to and inactivates VEGF$_{165}$ by neutralizing it ability to bind heparan sulphate proteoglycans and thus to extracellular matrix $^{147}$. It is approved for treatment of AMD $^{147, 148}$. 
2.1.2 Endogenous inhibitors

Apart from the aforementioned synthetic/recombinant molecules, natural/endogenous inhibitors of angiogenesis are also widely tested for their clinical utility. The majority are fragments of the extracellular matrix molecules like endostatin, TPN-40, canstatin and tumstatin. Non-matrix derived endogenous inhibitors include interferons (e.g. IFN-α), interleukins (e.g. IL12) and other molecules such as angiostatin. Endogenous inhibitors usually have a broad spectrum of action by downregulating many proangiogenic pathways and upregulating antiangiogenic ones. For example, endostatin is known to downregulate proangiogenic factors, such as VEGF, bFGF, and HIF-1α, and upregulate antiangiogenic factors such as thrombospondin-1, maspin and TIMP-2. Its pharmacological entity, Endostar, is approved for lung cancer in China.

2.1.3 Vascular disrupting agents

Vascular disrupting agents (VDAs) are low-molecular-weight chemicals that aim to cause the rapid and selective shutdown of the established tumour vasculature and thus lead to tumour necrosis. Thus VDAs can be used in combination with the aforementioned inhibitors whose aim is to inhibit the formation of new vessels. Treatment with VDAs results in vascular shutdown within minutes, which can typically last for 24 h. This vascular disruption leads to extensive tumour necrosis, however it leaves a viable rim of neoplastic cells at the periphery that are responsible for regrowth. It has been hypothesized that these cells survive because their nutrition support is derived from vasculature in the adjacent normal tissue. Rapid recruitment of CEPs from bone marrow and establishment of new vessels has also been shown to be an important process for the establishment of this viable ring.

The VDAs currently enrolled in clinical trials belong to three categories. The first one, combretastatins, includes molecules that are able to bind tubulin and prevent microtubule assembly, such as the phosphate prodrug of CA4. The second group includes flavanoids, such as vadimezan, that can induce the intratumour release of cytokines, such as TNFα. The third one includes N-cadherin inhibitors, such as ADH-1. The VDAs are considered for tumour angiogenesis.
is important for establishment of adhesion between endothelial cells and pericytes, thus disruption of this interaction induces vascular damage and extensive tumour necrosis.

2.1.4 Metronomic chemotherapy

Metronomic chemotherapy refers to chronic administration of a chemotherapeutic agent at a low, non-toxic, dose on a frequent schedule with no prolonged drug-free breaks. Studies have shown that metronomic chemotherapy can directly cause growth arrest and/or apoptosis of activated endothelial cells and sustain the mobilization of EPCs from bone marrow. Indirectly, it could upregulate endogenous inhibitors such as thrombospondin. Several clinical trials are underway evaluating its antitumour efficacy.

2.2 Mechanisms of action of anti-VEGF/angiogenic therapy

Since the initial hypothesis by Judah Folkman that vessel growth inhibition should be able to keep cancer in a dormant state it has become apparent, from the plethora of clinical trials, that antiangiogenic therapies exhibit antitumor effects by various mechanisms. The combination of several mechanisms rather than one single mechanism is responsible for the efficacy of the antiangiogenic agents in the clinic.

- Anti-angiogenic effects

VEGF inhibition results in vessel regression through three different mechanisms. First due to the role of VEGF as a pro-survival factor for endothelial cells, its inhibition leads to endothelial cell apoptosis and thus regression of existing vessels. Second, ablation of VEGF in the tumour microenvironment negatively affects the sprouting and migration of endothelial cells, thus the generation of new vessels (angiogenesis) is prohibited and hence, anti-VEGF therapy acts as a cytostatic agent for blood vessels. Finally, it has been shown that during tumour growth, bone marrow derived haematopoietic progenitor cells (HPCs) and circulating endothelial progenitor cells (EPCs) are recruited into the tumour site and have an important role for the establishment of new vessels through the mechanism of vasculogenesis. VEGF is an important chemotactic factor for both types of bone marrow-
derived cells, thus VEGF inhibition can hinder their mobilization and incorporation at the
tumour site.\textsuperscript{154, 165}

- **Effects on vessel function**

  VEGF is responsible for controlling vessel dilation by controlling the expression of
nitric oxide and prostaglandins. Thus, VEGF inhibition, in theory, can result in
vasoconstriction and decreased blood perfusion at the tumour site. The latter could lead to
ischemia and tumour necrosis. Vasoconstriction is also observed systemically and is one of
the major side effects of anti-angiogenic therapy.

  Antiangiogenic therapy has been proposed to have a remodeling effect on the tumour
vessels, termed “normalization”.\textsuperscript{166, 167} Tumour vasculature is characterized by highly
disorganized, leaky and immature vessels, with uneven diameter, and excessive branching
and shunts. During VEGF inhibition, normalization of the vasculature results in less leaky,
less dilated, and less tortuous vessels with a more normal basement membrane and greater
coverage by pericytes. These morphological changes are accompanied by functional changes
such as decreased interstitial fluid pressure and increased tumor oxygenation. Most
importantly, normalization is expected to allow for improved penetration of drugs in tumors
\textsuperscript{167, 168}. Even though the aforementioned morphological changes have been observed with
many mouse models, it will be difficult to apply them in the clinic due to the fact that the
normalization window seems to be very narrow.\textsuperscript{169, 170} In addition, at the moment, there are
no available biomarkers allowing us to monitor this process.

- **Effects on tumour cells**

  VEGFRs have been detected in a variety of tumour types.\textsuperscript{171} Various studies have
also shown that VEGF signaling pathway is implicated in cell survival, migration and
apoptosis of tumour cells, such as breast cancer and pancreatic cancer cells.\textsuperscript{172-175} Thus, it
has been hypothesized that anti-VEGF agents might have direct effects on the tumour in
addition to their antiangiogenic effects.
• **Immune modulation**

VEGF has a dual role in the function of dendritic cells. Along with other tumour-derived factors it is responsible for the recruitment of immature dendritic cells to the tumour site. However, VEGF also inhibits their maturation after they engulf tumour antigens, so they are unable to present them \(^{67}\). The latter results in immune privilege of the tumour cells \(^{68}\). Inhibition of VEGF at the tumour site could improve the immune responses \(^{176}\). Preliminary, phase I, studies with VEGF trap were not conclusive due to the small number of subjects \(^{177}\).

• **Chemosensitizing activity**

Antiangiogenic therapy might act synergistically and increase the potency of chemotherapy \(^{178}\). This is possible due to three different scenarios. First, it is expected that due to the vessel normalization process, the delivery of chemotherapeutic agents at the tumour site will increase even though there will actually be less vessels \(^{167},^{168}\). However, as mentioned above, due to the fact that the normalization window seems to be very narrow, the optimization of such co-administration is difficult \(^{169},^{170}\).

Second, a recent study showed that anti-VEGF therapy does not increase the delivery of chemotherapy to the tumour, but instead it increases the antiangiogenic effect of chemotherapy itself \(^{179}\). Indeed, this could be the second mechanism through which antiangiogenic and chemotherapeutic agents act synergistically. Anti-VEGF therapy may sensitize the endothelial cells to cytotoxic agents and thus increases the vascular damage \(^{180},^{181}\).

The third scenario during which antiangiogenic therapy can be used as a chemosensitizing method is when it is applied after each round of chemotherapy \(^{182}\). The rationale behind this schedule is based on the fact that tumour cells need a high amount of oxygen and nutrients after each round of cytotoxic therapy in order to recover. Thus, by applying an antiangiogenic agent after each round of cytotoxic therapy, tumour recovery may be inhibited.
2.3 Models of resistance of anti-VEGF/angiogenic therapy

Targeting tumour vessels was an attractive approach due to the belief that resistance to antiangiogenic therapy would be minimal. Vessel endothelial cells, in contrast to tumour cells, were thought to have a stable genome and their survival to be VEGF dependent. Even though antiangiogenic strategies are able to prolong progression-free survival of cancer patients, the impact in overall survival is minimal, usually translated to weeks to months. It is now clear that tumours are able to escape the pressure created by antiangiogenic agents by various mechanisms. These can be either acquired during the course of antiangiogenic therapy and be mediated either by the tumour or the host, or by preexisting, intrinsic mechanisms characteristic of the tumour type.

• Acquired mechanisms of resistance

During antiangiogenic therapy, the tumour and host microenvironment might resist treatment by various mechanisms.

1. Upregulation of alternative angiogenic factors.

It has been observed in both mouse models as well as during clinical studies that VEGF inhibition and tumour dormancy was followed by regrowth. During this other angiogenic factors, such as fibroblast growth factors (Fgf-1 and Fgf-2), ephrin A1, angiopoietin, PlGF, PDGF and TNF-α, were upregulated.

2. Recruitment of vascular progenitor bone marrow derived cells (BMDCs).

Antiangiogenic therapy results in an increase in hypoxia at the tumour site and thus upregulation of HIF-1α and its downstream effectors, such as SDF-1α, IL-6 and granulocyte colony-stimulating factors (G-CSF). These cytokines act as chemoattractants for a number of bone marrow derived cells, such as Gr1+CD11b+ myeloid-suppressor cells, Tie-2 expressing monocytes, tumour-associated macrophages as well as circulating endothelial progenitor cells. BMDCs may contribute to angiogenesis by secretion of various
angiogenic factors, such as Bv8 by myeloid and macrophages, while circulating endothelial progenitor cells (CEPs) might contribute to vasculogenesis.

3. **Pericyte coverage of tumour blood vessels.**

Pericytes have a productive role for endothelia cells by secreting paracrine factors that stimulate survival, vessel stabilization and maturation \(^{199, 200}\). Thus, in treated tumours endothelial cells that are tightly covered by pericytes resist antiangiogenic therapy. Preclinical studies suggest that dual targeting of endothelial cells and pericytes, for example by VEGF and PDGF traps, might be a more efficacious antiangiogenic strategy \(^{201}\).

4. **Abnormal endothelial cells.**

Tumour associated endothelial cells were assumed to be genetically stable and thus it was expected that they will develop less drug resistance than tumour cells \(^{202}\). However, recent studies have shown that tumour endothelial cells can be aneuploid and acquire genotypic alterations \(^{203-205}\). Thus, they have the capability to develop mutations that convey drug resistance.

- **Intrinsic mechanisms of resistance**

1. **Expression of a plethora of angiogenic factors.**

It is now recognized that tumours are capable of expressing more than one pro-angiogenic factor. In the case of breast cancer, it was shown that late-stage tumours expressed up to six factors \(^{206}\). Thus, inhibition of VEGF alone would not be sufficient in such cases \(^{207}\).

2. **Vascular mimicry and co-option.**

In highly vascularized organs, such as lungs, brain and liver, tumour cells co-opt quiescent normal blood vessels, and thus there is no need for development of new blood vessels \(^{42, 208-210}\). During vascular mimicry, tumour cells line the blood vessels next to
endothelial cells. Existing blood vessels are less sensitive to antiangiogenic therapy and thus tumours can survive in these organs.

3. Selection of more invasive tumour cells.

Antiangiogenic therapy increases local hypoxia. Mutant tumour cell clones carrying inactivating mutations in the \textit{TP53} tumour suppressor gene are more tolerant and can survive under these conditions. Hypoxia also forces the tumour cells to invade the surrounding area and eventually metastasize.

2.4 Side effects of anti-VEGF/angiogenic therapy

As a result of rather unexpected side effects observed during completed and on-going clinical trials, the crucial role of VEGF for the proper function of a range of organs has become apparent. Numerous detailed reviews describing the side effects of anti-angiogenic therapies have been published. Below is a summary of bevacizumab, the only anti-VEGF-specific entity approved, side effects that point to the active role of VEGF in various physiological processes.

\textbf{Hypertension} is the most frequent side effect of bevacizumab occurring at an overall incidence of 8-67\% and seems to be dose-dependent. It has been attributed to two main mechanisms: 1) VEGF decreases the production of two major vasodilators, NO and PGI$_2$, resulting in vascular resistance, and 2) VEGF inhibition causes a decrease in the number of small arteries and arterioles, rarefaction. Both of these possibilities have been suggested based on indirect observations and therefore further studies are needed to investigate the exact mechanism. Hypertension is also the cause of \textit{reversible posterior leukoencephalopathy syndrome} (RPLS) reported in patients treated with bevacizumab, characterized by the onset of headaches, cortical blindness and seizures.

Treatment with bevacizumab also increases the risk of \textit{bleeding}. Mild spontaneous mucocutaneous bleeding presenting as mild epistaxis has been reported in up to 44\% of patients. More serious tumor-related and sometimes fatal bleeding complications may
### Table 1.4

Approved anti-angiogenic agents

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cancer</th>
<th>Combination</th>
<th>Common adverse effects (&gt;20%)</th>
<th>Serious side-effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bevacizumab (Avastin)</strong></td>
<td>Metastatic Colorectal Cancer</td>
<td>5-FU–based chemotherapy</td>
<td>Epistaxis, headache, hypertension, rhinitis, proteinuria, taste alteration, dry skin, rectal hemorrhage, lacrimation disorder, back pain and exfoliative dermatitis.</td>
<td>Non-Gastrointestinal fistula formation, arterial thromboembolic events, reversible posterior leukoencephalopathy syndrome, surgery-wound healing complications, severe or fatal hemorrhage</td>
</tr>
<tr>
<td></td>
<td>Non-Small Cell Lung Cancer</td>
<td>Carboplatin and paclitaxel chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metastatic Breast Cancer</td>
<td>Paclitaxel chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glioblastoma</td>
<td>Monotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metastatic Kidney Cancer</td>
<td>Interferon alpha</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sunitinib (Sutent)</strong></td>
<td>Advanced renal cell carcinoma</td>
<td>Monotherapy</td>
<td>Fatigue, asthenia, diarrhea, nausea, mucositis/stomatitis, vomiting, dyspepsia, abdominal pain, constipation, hypertension, rash, hand-foot syndrome, skin discoloration, altered taste, anorexia, and bleeding.</td>
<td>Left ventricular ejection fraction declines, prolonged QT intervals, hemorrhagic events, thyroid dysfunction</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal stromal tumor after disease progression on or intolerance to imatinib mesylate</td>
<td>Monotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sorafenib (Nevaxar)</strong></td>
<td>Advanced renal cell carcinoma</td>
<td>Monotherapy</td>
<td>Fatigue, weight loss, rash/desquamation, hand-foot skin reaction, alopecia, diarrhea, anorexia, nausea and abdominal pain</td>
<td>Cardiac ischemia and/or infarction, bleeding, hypertension, gastrointestinal perforation, surgery-wound healing complications</td>
</tr>
<tr>
<td></td>
<td>Unrespectable hepatocellular carcinoma</td>
<td>Monotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pazopanib (Votrient)</strong></td>
<td>Advanced renal cell carcinoma</td>
<td>Monotherapy</td>
<td>Diarrhea, hypertension, hair color changes, nausea, anorexia, and vomiting.</td>
<td>Prolonged QT intervals, hemorrhagic events, arterial thrombotic events, gastrointestinal perforation or fistula, hypothyroidism, proteinuria, surgery-wound healing complications</td>
</tr>
</tbody>
</table>
occur in cases of lung, colorectal, gastric, pancreatic and ovarian cancers. At the moment, there is no definite explanation for the bleeding events, however it could possibly be associated with the fact that VSP inhibition can suppress the renewal capacity of endothelial cells. This might also be the reason for the healing complications observed in patients that undergo surgery while on bevacizumab. Impaired wound healing might also be associated with platelet function. Platelets have an active role during wound healing since after activation, they secrete survival factors including VEGF. A recent study showed that platelets are able to take up bevacizumab from the blood circulation and release it upon activation. However, it has not yet been clarified if this occurs happening during wound healing.

Disturbed platelet function as well as lower NO and PGI2 levels due to inhibition of VEGF might also be the underlying mechanism for the two-fold increase of arterial thromboembolic events in patients treated with bevacizumab. Recently, Meyer et al. suggested that complexes of bevacizumab with VEGF activate the platelet FcgammaRIIa receptor, which leads to their aggregation and thus the formation of thrombi. More in depth studies are needed in order to further understand the molecular connection between inhibition of the VSP and thrombosis.

In agreement with studies in mouse models, the frequency of ventricular dysfunction and congestive heart failure is also increased during treatment with bevacizumab.

Gastrointestinal perforations and fistula formations are rare but potentially fatal side effects of anti-VEGF therapies. Bowel perforations are closely associated with bleeding complications and are more prominent in patients with intra-abdominal tumors. Fistula formations have been observed during treatment of both lung and colon cancer with bevacizumab. Animal studies have shown that blood vessels in the adult gastrointestinal tract are sensitive to VEGF levels. Hence, reduced gut epithelial regeneration due to the vascular regression might be the underlying mechanism in these pathological processes.

The adverse effects of bevacizumab in kidney function are well documented and
have been demonstrated using transgenic mice models with the podocyte specific alteration of VEGF $^{61, 62}$ as well as pharmacological $^{53}$ animal studies. These investigations have demonstrated the critical role of the VSP in maintaining functional filtration in the kidney. Hence, it is not surprising that proteinuria is another predominant side effect of bevacizumab.
3 Targeted drug delivery

“All Ding sind Gift und nichts ohn Gift; alein die Dosis macht das ein Ding kein Gift ist” (“All things are poison and nothing is without poison; only the dose makes a thing not a poison”). This statement by the 16th century pharmacologist Paracelsus emphasized, for the first time, the importance of controlling the exposure to a drug, i.e. dose, in order to avoid side effects. Three centuries later, at the end of the 19th century, Paul Ehrlich (1908 Nobel Laureate) proposed that “wir müssen chemisch zielen lernen” (“we have to learn how to aim chemically”) and introduced the “magic bullet concept”. These two concepts revolutionized the field of pharmacology and laid the foundations for targeted therapies.

Targeted-delivery of therapeutic agents, drugs, aims to achieve a beneficial therapeutic index by increasing efficacy and at the same time reducing toxicity in the body. This can be accomplished by spatiotemporal delivery of a drug to its target such that over time there is an increase in the local drug concentration in order to achieve the effective, while at the same time limiting the exposure to the rest of the body.

Following systemic administration, drug delivery involves three steps: (a) transport within the vascular system, (b) transport across microvascular walls, and (c) transport through the interstitial space within the tissue target. These processes are determined by the physicochemical properties of a drug or particle (e.g. molecular or particle size, charge) and the biologic properties of a tissue (e.g. vasculature, extracellular matrix components, interstitial fluid pressure, tissue structure and composition). Accordingly, specific delivery of a molecule at the target tissue can be achieved by two approaches: (a) passive targeting, due to intrinsic properties, and (b) active targeting, due to extrinsic properties.

Passive targeting can be due to the molecular weight or charge of the molecule. In the case of molecular weight, passive targeting is achieved via the enhanced permeability and retention (EPR) effect. Under normal conditions, only small molecules (e.g. chemotherapeutic agents, kinase inhibitors) can cross the endothelial barrier and accumulate
in tissues. This is facilitated through convection (i.e. bulk motion of fluid) and diffusion (i.e. due to concentration gradients). However, under pathological conditions, such as cancer and inflammation, the endothelial barrier is disrupted and blood vessels have leaky morphology and the endothelial cells are poorly aligned with wide fenestrations. This result in “enhanced permeability” in which large molecules (e.g. antibodies, micelles, liposomes), in addition to small molecules, are also able to accumulate in tissues via diffusion. In the case of solid cancers, macromolecules are localized and trapped within the tumour microenvironment due to “enhanced retention” owing to increased interstitial pressure, dysfunctional hydrodynamics and lack of lymphatic vessels, which reduces fluid drainage and therefore reduces transport by convection. In the case of passive targeting due to molecular charge, it has been shown for example that cationic liposomes and liposome-DNA complexes are preferentially taken up by angiogenic endothelial cells.

Active targeting is based on specific recognition. In order to achieve active targeting two components are necessary: (a) a target-specific molecule, e.g. antigen, and (b) a carrier system, e.g. antibody, that will deliver the bioactive effector molecule.

It is important to emphasize at this point that the terms “specific-antigen” and “targeted-therapy” are not referred to the inhibition of a target molecule in a signaling pathway (e.g. the inhibition of VEGF signaling by bevacizumab or the inhibition of the HER2 receptor by trastuzumab). Instead, a tumour or vascular-specific antigen is used as a distinct binding site for the carrier system, facilitating its localization to the target site, i.e. tumour.

### 3.1 Specific antigens

Drug delivery strategies rely on the identification of antigens that are specific for the target, easily accessible and highly expressed. Methods such as *in vivo* phage display, serial analysis of gene expression, microarray-based gene expression profiling and proteomic-based technologies have been successfully used for the identification of specific antigens expressed by tumour cells and tumour vasculature.
## Table 1.5

Tumour specific and/or overexpressed antigens and antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Site of overexpression</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid Tumours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2/neu</td>
<td>25% of breast cancers, adenocarcinomas of ovary, prostate, lung and gastrointestinal tract</td>
<td>- Trastuzumab*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Ertumaxomab (HER2/CD3)</td>
</tr>
<tr>
<td>EGFR (Epidermal growth factor receptor)</td>
<td>Non-small lung, breast, colorectal, head and neck, prostate cancer</td>
<td>- Cetuximab*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Panitumumab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Matuzumab</td>
</tr>
<tr>
<td>Ep-CAM (Epithelial cell adhesion molecule)</td>
<td>Colorectal, pancreatic, non-small lung cancer</td>
<td>- Edrecolomab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Catumaxomab (EpCAM/CD3)</td>
</tr>
<tr>
<td>CEA (Carcinoembryonic antigen)</td>
<td>Gastrointestinal tract neoplasm</td>
<td>- T84.66</td>
</tr>
<tr>
<td>GD2</td>
<td>Neuroectodermal cancers such as melanoma and neuroblastoma</td>
<td>- Ch14.18</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Melanoma</td>
<td>- MDX-010</td>
</tr>
<tr>
<td>G250</td>
<td>Kidney cancer</td>
<td>- WX-9250</td>
</tr>
<tr>
<td>Lewis (Y)</td>
<td>Breast, colon, stomach, pancreas, ovarian, and lung cancer.</td>
<td>- Hu3s193</td>
</tr>
<tr>
<td><strong>Lymphomas</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20</td>
<td>Non-Hodgkin lymphomas</td>
<td>- Rituximab*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Ibritumomab tuixetan* ((^90)Y conj)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Tositumomab*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(^{131}I conj)</td>
</tr>
<tr>
<td>CD22</td>
<td>Non-Hodgkin lymphomas</td>
<td>- Epratuzumab</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Non-Hodgkin lymphomas</td>
<td>- Hu1D10</td>
</tr>
<tr>
<td>CD33</td>
<td>Acute myeloid leukemia</td>
<td>- Gemtuzumab ozogamicin* (Conj with calicheamicin)</td>
</tr>
<tr>
<td>CD52</td>
<td>B-cell chronic lymphocytic leukemia</td>
<td>- Alemtuzumab*</td>
</tr>
</tbody>
</table>
### Tumour Stroma

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tissues</th>
<th>Antibody/Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP (Fibroblast activation protein)</td>
<td>Colorectal, breast, pancreas, lung cancer</td>
<td>- Sibrotuzumab</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>High-grade astrocytomas, breast, lung, squamous cell carcinoma</td>
<td>- 81C6, F16, G11</td>
</tr>
</tbody>
</table>

### Tumour Vasculature

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tissues</th>
<th>Antibody/Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMA (Prostate specific membrane antigen)</td>
<td>Clear cell renal, neuroendocrine, pancreatic duct, non-small lung, breast carcinoma, colorectal, prostate adenocarcinoma, malignant melanoma, soft tissue sarcoma</td>
<td>- J591</td>
</tr>
<tr>
<td>EDB (Fibronectin extra-domain B)</td>
<td>High-grade gliomas, breast, lung, squamous cell carcinoma</td>
<td>- L19</td>
</tr>
<tr>
<td>ROBO4</td>
<td>- Tumour vasculature</td>
<td></td>
</tr>
<tr>
<td>Annexin A1</td>
<td>- Tumour vasculature</td>
<td></td>
</tr>
<tr>
<td>Integrin αvβ3</td>
<td>- Tumour vasculature</td>
<td>- Vitaxin</td>
</tr>
<tr>
<td>Endoglin</td>
<td>- Tumour vasculature</td>
<td>- TEC-11, K4-2C10</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>- Tumour vasculature</td>
<td></td>
</tr>
<tr>
<td>CD44 isoforms</td>
<td>- Tumour vasculature</td>
<td>- TED-23</td>
</tr>
<tr>
<td>TEM-1, -7, -8</td>
<td>- Tumour vasculature</td>
<td></td>
</tr>
</tbody>
</table>

*: Approved and used in the clinic.
3.1.1 Tumour-cell specific antigens

The transformation of normal cells into tumor cells involves the alteration of the genome signature and thus the expression profile of the cells. The latter can potentially create unique signatures resulting in altered protein expression. Indeed, a number of receptors and other membrane proteins (Table 1.4) have been identified as potential candidates for targeted delivery of drugs by using carriers such as antibodies (see below). Although a wide range of tumour-specific antigens have been linked to the transformation of normal cells into cancer cells, no single common feature exists to allow unambiguous targeting to the tumour. Thus, specific antigens have to be used for each cancer (sub)type.

Another obstacle associated with utilizing tumour antigens for drug delivery is the fact that they are not readily accessible. Thus, drug delivery systems have to overcome various physical barriers in order to reach them. Furthermore, targeting a tumour antigen can also have a direct antitumour effect. For example, an antibody-carrier that recognizes a signal-transducing molecule crucial to cell growth or differentiation (e.g. anti-HER2, anti-EGFR1) could inhibit its function and at the same time deliver the drug.

3.1.2 Tumour-vascular specific antigens

Unlike normal vasculature, tumour vasculature is disorganized, tortuous, leaky, with vascular shunts and abnormal blood flow. Thus, the endothelium in tumours is exposed in hypoxic and acidic areas, and is often deprived of nutrients. In addition, the endothelial cells in tumour vessels are constantly proliferating and contributing to the formation of new vessels, in contrast to normal tissues, in which they are quiescent. All these aspects contribute to the different expression profile of the tumour vasculature.

In contrast to tumour cell antigens, vascular antigens, also referred to as vascular “zip codes”, can be common across a broad spectrum of solid tumours (e.g. prostate specific membrane antigen and fibronectin extra domain B). Thus, in principle a single antigen could be used for targeted therapy of various cancer types. Furthermore, vascular antigens are
readily accessible from the bloodstream to systemically administered agents. An ideal vascular antigen should not only discriminate between physiological and tumour angiogenesis, but also regenerative angiogenesis, which occurs in the menstrual cycle and wound healing.

Using the aforementioned techniques a number of studies let to the identification of tumour vascular antigens, “zip-codes”, and are currently underway preclinical and clinical evaluation (Table 1.4). These include both endothelial specific antigens (e.g. ROBO4) as well as antigens of the stroma-vascular bed (e.g. fibronectin and tenascin-C isoforms).

3.2 Carrier systems

Carrier systems can be divided into three types: (a) particle, (b) soluble and (c) cellular carriers. Particle type carriers include liposomes, lipid particles, microspheres, nanoparticles, polymeric micelles and (modified) viruses. Soluble carriers comprise of monoclonal antibodies, recombinant proteins, aptamers, peptides, polysaccharides, and biodegradable carriers consisting of polymers. Finally, bacteria, immune cells and stem cells have also been studied as possible cellular delivery systems.

From the aforementioned carriers, aptamers, peptides and antibodies have an intrinsic ability to interact with high affinity and specificity with ligands specifically expressed at the target site. Using chemical or genetic approaches, the latter can be conjugated to carriers such as liposomes and nanoparticles in order to achieve targeted delivery of the payload.

3.2.1 Aptamers

Aptamers are short single-stranded oligonucleotides that recognize a target on the basis of its 3D shape. Aptamers are identified from randomly synthesized oligonucleotide libraries, following a process known as SELEX (Systematic Evolution of Ligands by Exponential enrichment). Their specificity and affinity to targets can be compared to that of antibodies, thus they are also referred to as “chemical antibodies”. Several unique properties of aptamers, including high binding specificity, low immunogenicity, structural
stability and ease of synthesis, have made aptamers promising agents for directed therapy against cancer targets.

Targeted delivery has been performed using aptamers selected against vascular “zip-codes” such as membrane proteins (e.g. PSMA) and matrix components (e.g. tenascin C). A variety of different cargoes have now been successfully delivered both in vitro as well as in vivo, via aptamers, such as siRNA, toxins, nanoparticles and chemotherapeutic agents.

3.2.2 Peptides

Peptides are short, random amino acid sequences, identified in in vivo phage display libraries, in vivo biopanning. This technique, was first developed in 1985 and used for the first time in vivo in 1996 by Pasqualini and Ruoslahti. Since then a number of peptides were identified that specifically target normal and tumor blood vessels and tumor lymphatic vessels.

Peptides, also referred to as “homing peptides”, show high specificity for their targets and exhibit low immunogenicity. Their small size allows them to more efficiently penetrate tissues compared to antibodies or proteins. Another advantage is the fact that they are inexpensive and very easy to synthesize. However, due to rapid renal clearance, their short half-life compromises their clinical applicability.

Homing peptides have been successfully used as delivery vehicles to target imaging agents, drug molecules, oligonucleotides, liposomes, and inorganic nanoparticles to tumors and other tissues. The most widely used peptides in targeted delivery applications so far are RGD (Arg-Gly-Asp) and NGR (Asn-Gly-Arg), the first homing peptides discovered. Both appear to be independent of tumor type. The RGD peptides have high specificity for \( \alpha_v \) integrins and NGR for aminopeptidase N (APN). Both have also been studied for specific delivery of proteins, such as TNF-\( \alpha \), chemotherapeutic agents, including doxorubicin, and pro-apoptotic peptides, like the antimicrobial (KLAKLAK)\(_2\).
3.2.3 Antibodies

The seminal development of hybridoma technology by Georges Köhler and César Milstein for production of monoclonal antibodies (mAbs) in 1975 changed the route of medicine and biological therapies 306, 307 (Figure 1.5). The initial in vivo human studies showed that mouse mAbs had limited clinical utility because of their short half-life, an inability to trigger human effector functions and the production of human anti-mouse antibodies (the HAMA response) 308. Antibody bioengineering allowed for the development of chimeric antibodies, that is, antibodies with human constant regions and mouse variable regions 309, 310 (Figure 1.6). Further studies gave rise to “humanized antibodies” through engraftment of the mouse mAb complementarity-determining region (CDR) loops, responsible for antigen recognition, into the human variable domain framework 311. Finally, technologies such as phage display libraries of human antibody fragments and mice that are transgenic for the human Ig locus give rise to completely human mAbs 312-316.

Monoclonal antibodies 317 or engineered antibody fragments 318 are now widely used therapeutically for various diseases including cancer 319-321. Their high specificity, long serum half-life as well as their ability to interact with components of the immune system (see below) makes them a useful biological tool. They can be used in their native/unconjugated form or as conjugates (see below) 322.

3.2.3.1 Important properties

One of the main reasons for the great success of mAbs as biologics is the ability to change/fine many of their properties in order to increase their clinical potential. Apart from the immunogenicity and effector function (see above and next section), below are some of their most important properties.

• Antigen-binding specificity and affinity

Antibodies are typically highly specific for their target. However, techniques such as phage-display technology may be used to further enhance their specificity 323. The antigen-
binding affinity ($K_d$) can be increased through affinity maturation using various display libraries, such as phage, yeast and ribosome \(324\). Yet, it is important to note that increased affinity does not always correlate with increased \textit{in vivo} biological potency. In contrast, studies have shown that antibodies with lower affinity may have better tumour penetration compared to those with higher affinity. This phenomenon, first described by Weinstein, is known as “binding-site barrier” \(325\). According to it, the antibody binds strongly to the first targets encountered and fails to diffuse further into the tumour \(326,327\).

**Figure 1.5: Basic structure of an IgG antibody.**

All immunoglobulin monomers are composed of two identical light (L) and two identical heavy (H) chains. Light chains are composed by one constant domain (C\textsubscript{L}) and one variable domain (V\textsubscript{L}), whereas heavy chains are composed of three constant domains (C\textsubscript{H1}, C\textsubscript{H2}, and C\textsubscript{H3}) and one variable domain (V\textsubscript{H}). The heavy chains are covalently linked in the hinge region (h) and the light chains are covalently linked to the heavy chains. The variable domains of both heavy and light chains comprise the antigen-binding site. Each variable domain contains three loops designated complementary-determining regions (CDRs) 1, 2, and 3, which define the antibody specificity. The Fc portion is glycosylated and contains sites that interact with effector molecules, such as complement factors and macrophages receptors (e.g. Fc\textgamma{R}), as well as sites for interaction with the neonatal receptor FcRn. Modifications of the CDRs, glycosylation and Fc binding sites can alter antibody properties, such as binding affinity and specificity, effector functions (i.e. ADCC and CDC) and pharmacokinetics. ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.
Pharmacokinetics

The extended half-life of IgG antibodies is due to the interaction of their Fc region with the salvage neonatal Fc receptor (FcRn). Proteins in the circulation, including antibodies, are taken up in the fluid phase through micropinocytosis. IgG can bind FcRn in endosomes and be recycled to the cell surface and thus released back to the circulation. FcRn is expressed in certain cells, such as vascular endothelial cells, kidney podocytes, intestinal and lung epithelial cells, and antigen-presenting cells (monocytes, macrophages and dendritic cells) \(^{328,329}\).

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Figure 1.6: Evolution of therapeutic antibodies.

Mouse monoclonal antibodies showed poor pharmacokinetics and activity due to the development of human anti-mouse antibodies (HAMA response). Thus, initially, chimeric recombinant antibodies were generated by genetically fusing the variable domains from mouse monoclonal antibodies with the human constant regions. Engraftment of the murine CDRs into a human antibody allowed for the development of humanized antibodies. Finally, recent advances such phage display and transgenic mice led to the production of fully human monoclonal antibodies.

---

- Pharmacokinetics

The extended half-life of IgG antibodies is due to the interaction of their Fc region with the salvage neonatal Fc receptor (FcRn). Proteins in the circulation, including antibodies, are taken up in the fluid phase through micropinocytosis. IgG can bind FcRn in endosomes and be recycled to the cell surface and thus released back to the circulation. FcRn is expressed in certain cells, such as vascular endothelial cells, kidney podocytes, intestinal and lung epithelial cells, and antigen-presenting cells (monocytes, macrophages and dendritic cells) \(^{328,329}\).
The long serum half-life of antibodies is a crucial aspect for their applicability in the clinic for treatment of any disease. This allows for improved efficacy but also for reduced dose and frequency of administration. Various studies have shown that different mutations can increase the affinity of the Fc region to FcRn and thus further increase the half-life\textsuperscript{330, 331}. On the other hand, in cases such as imaging and/or radioimmunotherapy, short half-life is required in order to ensure rapid clearance and avoid toxicity. In this case, antibody fragments with reduced half-life due to truncated Fc region can be used (Figure 1.7a)\textsuperscript{318, 330}.

- **Molecular architecture**

The extensive knowledge about antibody structure and molecular domain architecture allowed for the creation of a broad range of antibody formats with different molecular weights (antibody fragments) (Figure 1.7a), binding sites for the cognate antigen (valency) and antigen binding specificities\textsuperscript{318, 320, 332}. Bispecific antibodies are of particular interest (Figure 1.7b). They can be utilized for inhibition of two different mediators involved in pathogenesis of a disease, or to recognize two cell surface proteins on two different cell types and bring the two cell populations into close proximity. Such antibodies might be used to redirect cytotoxic immune cells to destroy pathogenic target cells, including tumors or virally infected cells, or to bring two cell populations together for activation\textsuperscript{333}. Various methods have been published for engineering of bispecific antibodies, such as use of chemical linkers, fusion via peptide linkers using genetic approaches and recently phage libraries\textsuperscript{334-338}.

Genetic fusions of antibodies with other proteins, such as cytotoxic agents (see below), has also increased the repertoire of antibody-based proteins and endowed it with novel functions\textsuperscript{321} (Figure 1.7c).
Figure 1.7: Representation of antibody formats.
3.2.3.2  Mechanism of action

The antitumour biological activity of antibodies can be the result of various mechanisms, acting alone or in combination \[^{317, 339}\]. Through their effector functions, antibodies can interact with components of the immune system in order to direct its cytotoxic activity to tumour cells. By recognizing antigens expressed on the tumour cells, antibodies can modulate signaling pathways important for tumour survival and progression. Finally, antibodies can be utilized as vehicles to target toxic agents at the tumour site \[^{321, 322}\] (Figure 1.8).

• **Effector functions**

The *in vivo* antitumour activity of some antibodies approved in the clinic (*i.e.* anti-CD20 mAb rituximab \[^{340}\]) is solely due to their effector functions. These functions occur upon binding to the target antigen and are mediated through the Fc region of IgG1 isotype. Switch to a different isotype, such as IgG2 or IgG4 could abolish their effector functions, while point mutations and glycan modifications could improve them \[^{341-344}\].

*Antibody-dependent cellular cytotoxicity* (ADCC) is based on the interaction of the Fc domain of the antibody with cellular Fc receptors (FcR) of immune accessory cells, such as
natural killer cells, neutrophils mononuclear phagocytes and dendritic cells \(^{345-347}\). The binding of FcR to the Fc domain results in crosslinking and activation of the immune cells. This leads to direct killing of the antigen presenting cells by NK cells, release of cytokines and chemokines that can inhibit proliferation and angiogenesis, as well as increase of immunogenicity through increased cell surface expression of major histocompatibility antigens (MHC) antigens \(^{348}\).

Complement-dependent cytotoxicity (CDC) is another cell killing mechanism directed by antibodies \(^{349}\). Upon binding of the antibody to the antigen conformational changes allow for exposure of binding sites for C1q complement factor in the Fc domain of the antibody. This results in complement activation as well as formation of a “membrane attack complex” (MAC) on the surface of the target cell. MAC formation results in pore formation and subsequent cell destruction, while complement activation results in recruitment of immune effector cells, such as neutrophils.

- **Modulation of signal transduction**

  Recognition of the target antigen by antibodies can affect signaling pathways involved in tumour progression. Binding to cell membrane receptors can either mask the binding site of the ligand (\(i.e.\) anti-ERBB2, trastuzumab \(^{350}\)) or prevent receptor dimerization (\(i.e.\) anti-ERBB2, pertuzumab \(^{351}\)). Binding to the ligand (\(i.e.\) anti-VEGF bevacizumab \(^{352}\)) can also prevent its binding to the receptor. Either of these mechanisms prevents activation of intracellular signaling pathways important for the proliferation and survival of the target cell.

- **Targeting of cytotoxic agents**

  The ability of mAbs to specifically recognize antigens expressed at the tumour site has been utilized in many ways in order to deliver cytotoxic agents \(^{321, 322, 353}\). Fusion of various molecules to the N- or C-terminus side of antibodies or antibody fragments has been successful. Both functions of the fusion molecule were preserved in all reported cases.
Figure 1.8: Anti-tumour mechanisms of therapeutic antibodies.

(a) Monoclonal antibodies can block ligand-receptor interaction and thus inhibit important signaling pathways in cancer progression, such as those involved in proliferation. (b) Targeting of cytotoxic agents, such as cytokines, toxins and radionucleotides, can modulate the immune response against the tumour and induce apoptosis. (c) Bispecific antibodies can target circulating cells, such as macrophages, to the tumour site and enhance the immune response. (d) Targeting monoclonal antibodies to the tumour can result in the destruction of tumour cells by antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).
These agents include:

a) **Radioisotopes**: Targeted delivery of beta emitters (e.g. $^{131}$I, $^{90}$Y) and alpha-emitters (e.g. $^{213}$Bi, $^{211}$At) is mainly used for immunotherapy. A key advantage of therapy using radiolabeled Abs compared with unconjugated Abs is their ability to kill neighboring cells in addition to the directly targeted cells, “bystander effect”.

b) **Toxins**: Immunotoxins are recombinant proteins of a mAb fused to a bacterial (e.g. *Pseudomonas aeruginosa* exotoxin A, diphtheria toxin) and plant (e.g. gelonin, ricin) toxins. Even though they are very effective, their application for solid tumours is limited by poor penetration as well as by the development of neutralizing antibodies by the host. They are particularly effective for hematological cancers such as lymphomas and leukemias since the patients are immunosuppressed.

c) **Drugs**: Various chemotherapeutic agents, such as doxorubicin, geldanamycin and methotrexate, have been conjugated with mAbs. For this method to be effective the antibody has to be internalized after binding to cell surface receptor of the target cell, or the linker between the Ab and the cytotoxic drug has to be cleavable so that the drug can be released.

d) **Enzymes**: An alternative method for targeted therapy is the delivery of enzymes at the tumour site that can subsequently activate prodrugs, referred as antibody-directed enzyme-prodrug therapy (ADEPT). For example, $\beta$-lactamase can activate drugs such as paclitaxel and doxorubicin and cytosine deaminase can convert 5-fluorocytosine to the active drug 5-fluorouracil.

e) **Cytokines**: Genetic fusion of cytokines, such as interleukin-2 (IL-2) and tumour necrosis factor alpha (TNF-$\alpha$), to tumour-specific antibodies is expected to alleviate the systemic side effects, increase the local immune response as well as target micrometastases. Phase I clinical trials testing anti-GD2-IL2 and anti-EpCAM-IL2 fusion proteins for the treatment of metastatic melanoma and prostate cancer, respectively, demonstrated biological activity and were well tolerated.
f) **siRNA**: Blocking the expression of proteins within the tumour at the mRNA level using siRNA can also be used as an antitumour strategy. Recent studies have demonstrated that siRNA covalently bound to mAbs recognizing tumour receptors and specifically delivered at the tumour site, display both antitumour activity and specific silencing of the target gene 366.

g) **Other molecules**: Genetic fusions of antibodies with other proteins such as ligands that can induce apoptosis (e.g. sFasL367 and sTRAIL 368, 369) or trigger coagulation specifically at the tumour site (e.g. truncated tissue factor; tTF 97, 370) have also been successfully explored using mouse models.

### 3.3 Antigen-antibody paradigms

#### 3.3.1 HER2 and 4D5 Ab

The human epidermal growth factor receptor 2 (HER2; also known as ERBB2 or c-neu) along with EGFR (HER1), ERBB3 (HER3) and ERBB4 (HER4) constitute the epidermal growth factor family of type 1, single, transmembrane receptors 371, 372. The extracellular region consists of four domains and the intracellular of two kinases. Upon binding of their ligands (epidermal growth factor-like molecules, TGF-α and neuregulins) the receptors undergo a conformational change that allows them to form homo- and heterodimers 373, 374. Following ligand-mediated dimerization of EGFR, the two EGFR kinase domains form an asymmetrical dimer in which one kinase allosterically activates the other. Downstream signaling leads to activation of two key signaling pathways, the MAPK pathway, which stimulates proliferation, and the PI3K–Akt pathway, which promotes tumour cell survival 372. EGFR and HER4 have known ligands and active kinase domains, while HER3 lacks intrinsic kinase activity. HER2 always exists in the active conformation, and thus is readily available for heterodimerization. Indeed, HER2 is the preferred partner for heterodimerization and the HER2/HER3 heterodimer the most potent with respect to strength of interaction, ligand-induced tyrosine phosphorylation and downstream signaling and
functions as an oncogenic unit. HER2 activation can also be mediated through proteolytic cleavage of the extracellular region, leaving behind an active kinase \(^{372, 374}\).

HER2 is amplified and overexpressed in 20-30% of breast cancers, as well as in subsets of patients with ovarian, gastric, lung and prostate cancers \(^{375, 376}\). In the case of breast cancer, its overexpression correlates with large tumour size, metastasis to lymph nodes, high grade, high percentage of S phase cells, aneuploidy, higher metastatic potential and decreased survival \(^{376, 377}\). Blocking HER2 signaling by preventing its dimerization arrests cancer cells in the G1 phase of the cell cycle and inhibits tumour growth \(^{378, 379}\). Trastuzumab (Herceptin), the humanized form of the 4D5 murine anti-HER2 monoclonal antibody \(^{380-382}\), blocks HER2 signaling by (a) preventing its activation through proteolytic cleavage, (b) inhibiting dimerization with other HER receptors, (c) downregulating the HER2 receptor by increasing membrane shedding, and (d) through its effector function, that is, its ability to initiate antibody-dependent cellular cytotoxicity \(^{383-385}\). Trastuzumab has antitumour activity and has been approved for treatment of breast cancer. It improves survival in the first-line setting when combined with chemotherapy in patients with advanced disease \(^{384, 386, 387}\).

A number of derivatives of antibodies targeting HER2 have been described. These include conjugates with cytokines (e.g. IL12 and G-SCF \(^{388}\)) as well as bispecific antibodies targeting HER2 and either a NK cell antigen (e.g. CD16 \(^{389}\)) or a mature T cells antigen (e.g. CD3\(^{390}\)) aiming to bring immune cells into close proximity with tumour cells. The latter trispecific antibody, ertumaxomab, showed promising preliminary results during Phase I clinical trials \(^{391}\). HER2 antibodies have also been conjugated with drugs, such as geldanamycin \(^{392}\) and DM1, a maytansine derivative \(^{393}\). The latter one, T-DM1, has shown encouraging preclinical and early clinical anti-tumour activity with limited toxicity \(^{394}\).

Recent studies have shed light into another important role of HER2 signaling as modulator of the equilibrium between pro- and antiangiogenic factors. The proangiogenic factors VEGF, IL-8, TGF-\(\alpha\), Ang-1, Ang-2 and PAI-1 (plasminogen-activator inhibitor-1) were all reduced, whereas expression of the anti-angiogenic factor TSP-1 (thrombospondin-1) was increased in trastuzumab-treated tumours relative to control-treated
Table 1.6
Examples of clinical trials evaluating the therapeutic value of combining bevacizumab with trastuzumab

<table>
<thead>
<tr>
<th>Identifier-Name</th>
<th>Phase</th>
<th>Arms</th>
<th>Primary Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00364611</td>
<td>Phase II</td>
<td><strong>1: HER2 Negative:</strong> Docetaxel and bevacizumab IV q3w</td>
<td>Progression-free survival of docetaxel and bevacizumab ± trastuzumab for the first-line treatment of patients with metastatic breast cancer</td>
</tr>
<tr>
<td>Pilot Study of Docetaxel &amp; Bevacizumab +/- Trastuzumab in First-Line Treatment of Patients With Metastatic Breast Cancer</td>
<td></td>
<td><strong>2: HER2 Positive:</strong> Docetaxel, bevacizumab, and trastuzumab IV q3w</td>
<td></td>
</tr>
<tr>
<td>NCT00625898</td>
<td>Phase III</td>
<td><strong>1A: Active Comparator</strong> Docetaxel, Carboplatin, and Trastuzumab followed by Trastuzumab</td>
<td>Invasive Disease-free Survival</td>
</tr>
<tr>
<td>BETH Study: Treatment of HER2 Positive Breast Cancer With Chemotherapy Plus Trastuzumab vs Chemotherapy Plus Trastuzumab Plus Bevacizumab</td>
<td></td>
<td><strong>1B: Experimental</strong> Docetaxel, Carboplatin, Trastuzumab, Bevacizumab followed by Trastuzumab and Bevacizumab</td>
<td></td>
</tr>
<tr>
<td>NCT00520975</td>
<td>Phase III</td>
<td><strong>2A: Active Comparator</strong> Docetaxel and Trastuzumab followed by 5-fluorouracil, Epirubicin, and Cyclophosphamide followed by Trastuzumab</td>
<td>Efficacy of the addition of bevacizumab to first-line chemotherapy and trastuzumab, by assessing the progression-free survival after initiation of combination therapy, in patients with HER2 overexpressing metastatic breast cancer.</td>
</tr>
<tr>
<td>First-Line Chemotherapy and Trastuzumab With or Without Bevacizumab in Treating Patients With Metastatic Breast Cancer That Overexpresses HER2/NEU</td>
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<td><strong>2B: Experimental</strong> Docetaxel, Trastuzumab, and Bevacizumab followed by 5-Fluorouracil, Epirubicin, and Cyclophosphamide followed by Trastuzumab and Bevacizumab</td>
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tumours in vivo. Clinical studies showed positive association between HER2 and VEGF expression, implicating VEGF in the aggressive phenotype exhibited by HER2 overexpression. These data support the use of combination therapies directed against both HER2 and VEGF for treatment of breast cancers that overexpress HER2. Various clinical trials are evaluating the combination trastuzumab and bevacizumab as therapy of HER2-positive advanced breast cancer (Table 1.6). Molecules that can inhibit both HER2 and VEGF, such as a recent derivative of trastuzumab that can also bind and neutralize VEGF, might find clinical utility in the treatment of breast cancer.

Finally, it is worth mentioning that a recent study using a radiolabeled anti-HER2 antibody described a predominantly perivascular accumulation of the injected antibody in the tumours, indicating a vascular pattern of HER2. Thus, HER2 could be used as a vascular “zip-code” for specific targeting of tumour vasculature.

3.3.2 EDB and L19 Ab

Fibronectin is a large glycoprotein found in body fluids, extracellular matrices and in most basement membranes. It has a role in many different processes such as cell adhesion, migration and differentiation. Alternative mRNA splicing has given rise to fibronectin isoforms such as the extra-domain A (EDA) and extra-domain B (EDB) of fibronectin. EDB is a 91 amino acid type III homology domain, first identified as a secreted protein from human transformed cells. Further studies showed that EDB is essentially undetectable in healthy adult tissues but it is abundantly found in many aggressive solid tumours, either in the tumour stroma or in the abluminal side of tumour vessels.

The EDB sequence is identical in the mouse, rat, rabbit, dog, monkey and human. Targeted deletion of the exon coding for the EDB domain in mice resulted in animals with normal vasculature, both physiological and tumour-associated. Double EDB⁻/⁻:Trp53⁻/⁻ knockouts did not show any difference in survival. These results suggest that the EDB domain is not important for angiogenesis and is dispensable. It high specificity (i.e. tumour vasculature), anatomical location (i.e. abluminal) and redundant, if any, function, makes this vascular zip-code one of the best candidates targets for specific drug delivery at the tumour
Due to the high conservation, the isolation of anti-EDB antibodies through hybridoma technology is not possible. This was achieved using phage display technologies \(^{407, 408}\). The anti-EDB human antibody L19 \(^{409}\) has high affinity and has been shown to efficiently localize to tumour vasculature of mouse models \(^{410-412}\) as well as in patients with different types of cancer \(^{413, 414}\). Given its high specificity, L19 was used for the derivation of a large number of therapeutic derivatives, such as conjugates with fluorophores and photosensitizers \(^{415, 416}\), liposomes \(^{417}\), procoagulant agents \((i.e. \ tTF \ ^{418})\), cytokines \((i.e. \ L2 \ and \ L12 \ ^{363, 419, 420})\), enzymes \((i.e. \ human \ propyl \ endopeptidase \ ^{421})\) and other proteins \((i.e. \ \text{TNF} \alpha \ ^{422})\). Three of the aforementioned derivatives (L19-L2, L19-\text{TNF} \alpha \ \text{and} \ L19-^{131}I) are currently under clinical development \(^{423}\).
4 Rationale and hypothesis

Angiogenesis is implicated in the pathophysiology of various diseases, including cancer and AMD. Current anti-angiogenic therapies, *e.g.* anti-VEGF, are clinically promising. However, the side effects of these current strategies prevent their utilization at effective biological dosage and schedules, particularly for chronic treatment. Therefore, we hypothesize that *target-specific anti-angiogenic therapy* will allow for optimal clinical outcomes by: (a) allowing for maximal effective dose of the anti-angiogenic therapy to be specifically delivered at the target site, (b) allowing for chronic treatment, (c) with minimal side effects.

As a *proof of principle*, in the proposed study we will target VEGF locally in tumour. In order to achieve target-specific anti-angiogenic therapy, I further hypothesize that:

(i) Fusion of a *heparin-binding domain* (HBD) with an existing anti-VEGF molecule, *i.e.* VEGF-trap, will: (a) allow for tumour-specific anti-angiogenic targeting, (b) be more efficient compared to the soluble form, *i.e.* without HBD, and (c) have minimal side effects, especially during chronic treatment. From now on such a fusion molecule will be referred to as *VEGF sticky-trap* (Fig. 1.9).

(ii) Fusion of a *tumour-specific antibody* with an existing anti-VEGF molecule, *i.e.* VEGF-trap, will: (a) allow the specific delivery of the anti-angiogenic molecule at the target site, and (b) retain the anti-angiogenic function of the anti-angiogenic molecule as well as the effector and biological specific functions of the antibody. From now on such a fusion molecule will be referred to as *VEGF-lasso* (Fig. 1.9).
Figure 1.9: Proposed strategy for development of novel antiangiogenic biologics.

Two new classes of second-generation VEGF traps are proposed, VEGF sticky-traps and VEGF lassos. VEGF sticky-traps consist of a VEGF trap-domain at the amino terminal and a heparin-binding domain (HBD) at the carboxyl-terminal of an IgG1 Fc region. VEGF lassos consist of a single-chain variable (scFv) antibody region at the amino terminal and a VEGF trap-domain at the carboxyl-terminal of an IgG1 Fc region.
Chapter 2

Background-free and doxycycline-inducible transgene integration at specific loci in cancer cell lines
Chapter 2: ΦC31-mediated site-specific insertion

1. Preface

During my thesis I intend to develop a series of recombinant proteins and test their function, ultimately using xenograft mouse models. Traditionally, protein production and purification is used for such studies. However, these methods are both time-consuming and very expensive.

In this chapter I explored a novel genetic approach in cancer cells that allows for inducible expression of transgenes from a defined loci. This method has been previously used successfully in mouse embryonic stem cells. Successful application of this method in cancer cells will allow for high-throughput generation of cancer lines expressing in an inducible manner the various biologics described in chapters 3 and 4.
2. Abstract

Cancer cell lines offer invaluable powerful tools for understanding cancer development and progression. Transgenic approaches further increase their values by helping the identification of genes and genetic pathways involved in this disease. Transgenes or certain DNA sequences often require stable insertion of into the genome of a particular cancer cell line. In this study, we characterized a ΦC31 integrase-mediated site-specific insertion system (ΦC31-IMSI) that allows for introduction of a gene of interest (GOI) into a predetermined genomic locus. Using various cancer lines we showed that the insertion is reproducible and reliable. Our selection system ensured that all of the surviving stable lines were harboring the correct integration site. We also showed that the expression level of various reporter genes from the same locus were comparable among the sister, isogenic, clones. Thus, we also coupled our insertion strategy with the doxycycline inducible system, comprises of the second-generation reverse-tetracycline transactivator (rtTA) and second-generation tetracycline responsive promoters (TRE). Furthermore, we introduce the luciferase gene into the insertion cassette allowing for possible live imaging of the cancer cells transplantation assays. Our intermediate insertion cassette (where the only missing part was the GOI) are Gateway cloning-compatible. We also showed that ΦC31-IMSI can be achieved in a 96-well plate format and thus allowing for high-throughput generation of stable isogenic gene expression libraries in cancer cells. Finally, with xenografts we showed that the genetically altered cancer cell lines retained the properties of the parental line.
3. Introduction

Mutations and polymorphisms in various genes and/or their regulatory elements are implicated in tumor initiation, progression and drug resistance. Elucidation of the consequences of these genetic changes relies largely on the available genetic and cancer models. Stable transgene expression is one of the most powerful and informative of the genetic tools. The generation of stable lines by random integration of a transgene of interest (tgGOI) is appropriate when examining the effect of a single transgene. However, when a comparative analysis of a series of mutations within the tgGOI is required, the generation of stable lines using random integration is inefficient. Foremost, expression levels between clones vary significantly due to chromosomal position effects and frequent copy number variation. Thus, the screening and identification of various stable lines to identify with the desired characteristics is extremely laborious. In addition, random integration may lead to genome alterations, such as inactivation of endogenous genes, which may alter cellular phenotype. Inserting the tgGOI to a predetermined genomic locus can eliminate all of these confounding factors.

Site-specific integration can be achieved by homologous recombination-based targeting of a specific genomic position by recombinase-mediated cassette exchange (RMCE) or integrase-mediated site-specific insertion (IMSI). Homologous recombination, although it has been widely used for gene targeting in mouse embryonic cell lines, in the case of human cell lines becomes laborious, time consuming and inefficient, due to difficulties involving the design of targeting vectors and the lack of isogenecity required for efficient homologous recombination. Furthermore, in the case of cancer cell lines homologous recombination becomes even more difficult. This is due to the fact that these cells acquired many genetic alterations during tumorigenesis, which are increasing even more during in vitro propagation since they are inherently genetically unstable. Recently, alternative techniques, such as the use of recombinant adeno-associated virus and the introduction of double-strand breaks for stimulation of targeting.
have been developed, however the targeting efficiency remains very low, between 1% and 5\%\textsuperscript{441-443}.

RMCE and IMSI overcome the aforementioned limitations allowing for both higher efficiency and easier vector construction\textsuperscript{434, 435}. Current methods involve the introduction of the GOI using bacterial or yeast DNA recombinases into a pre-generated chromosomal locus. The majority of the described strategies use the tyrosine recombinases Cre or Flp and their recognition sites loxP and FRT, respectively\textsuperscript{444-448}. Cre recombinase is a more efficient enzyme than the Flp recombinase. Regarding site-specific insertions, however, both recombinases have a drawback. They perform bidirectional recombination events since they also recognize their recombination products as substrate; hence the inserted GOI could be subsequently excised\textsuperscript{449}. In order to overcome this limitation, chimeric Cre recombinases or mutated loxP sites have been generated, such that the recombination products cannot be recognized by Cre recombinase\textsuperscript{450-453}. This however, has resulted in decreased efficiency of recombination\textsuperscript{451}. An alternative solution is to utilize integrases that only facilitate the insertion reaction.

Phage ΦC31 integrase catalyzes unidirectional recombination between two heterotypic sites, attP and attB (attachment site for Phage/Bacteria)\textsuperscript{454}. The resulting sites, attL and attR, are no longer substrates for ΦC31, thus the integration reaction is not reversible\textsuperscript{455, 456}. ΦC31 has been previously used for integration of an attB-containing plasmid into attP sites pre-inserted into the mammalian genome\textsuperscript{457-459}. Studies have also shown that pseudo attP sites exist in the genome of human and mouse lines and that it is possible to achieve ΦC31-mediated integration into these sites\textsuperscript{457, 458, 460}.

In this study, we characterized our unique IMSI system that utilizes the ΦC31 integrase (ΦC31-IMSI) for specific integration of the GOI in a preinserted genomic landing site (docking site) in various cancer lines, expressed in both a constitutive and inducible manner. We demonstrate that selection system applied has 100% fidelity and that sister clones express comparable levels of various reporter genes. In addition, we coupled this
system to a doxycycline inducible system allowing for tight and robust control of the expression of the GOI. Furthermore, we also introduced the luciferase gene that could be used for monitoring tumor progression \textit{in vivo} using live imaging. Finally, we constructed a series of Gateway compatible intermediate incoming vectors and showed that this system can be applied for high-throughput applications as well. The combination of ΦC31-IMSI with inducible transgene expression, the potential for live \textit{in vivo} imaging and its compatibility with Gateway vectors, represents a novel, valuable tool for the robust characterization of mutations and polymorphisms in unlimited aspects of cancer biology and treatment.
4. Materials and methods

**Plasmid vector construction**

The construction of the basic docking site (DockZ) vector was described by Monetti *et al.* (in preparation) and the structure shown in Fig. 1a. For construction of a slightly modified docking site (DZL), we generated a fusion gene comprising puromycin and luciferase linked via the T2A peptide, *i.e.* puromycin-T2A-luciferase. The T2A junction along with part of the C-terminus of puromycin and N-terminus of luciferase was synthesized and cloned into pBluescript between *Bst*AP1 and *Cla*I sites. This fragment was then subcloned into DockZ using *Bst*AP1 and *Cla*I. The C-terminus of luciferase was subcloned from a pBluescript (pBS) luciferase-containing vector after digestion with *Bsr*GI and *Eco*NI.

The basic incoming vector, Inc-basic, as well as Inc-CAG-MCS-pA and Inc-CAG-EGFP-pA were described by Monetti *et al.* (in preparation) and shown in Fig. 1b. After digestion of Inc-CAG-MCS-pA with *Hpa*I, the Gateway RfA cassette was inserted to generate Inc-CAG-Gateway-pA. Inc-TRE-EGFP-pA was constructed by excision of the pCAGGs promoter and insertion of second-generation tetracycline-regulated promoters, TRE by *Bam*HI and *Hind*III digestion. Subsequent excision of EGFP by *Bsu*36I and *Nhe*I and cloning of MCS gave rise to Inc-TRE-MCS-pA. The Gateway RfA cassette was inserted after *Hpa*I digestion in order to generate Inc-TRE-Gateway-pA. The IRES-EGFP digested with *Eco*RI (polished) and *Cla*I was inserted into Inc-TRE-MCS-pA after *Bsu*36I (polished) and *Cla*I digestion generating Inc-TRE-MCS-IRES-EGFP-pA. The Gateway RfA cassette was inserted after *Hpa*I digestion, constructing Inc-TRE-Gateway-IRES-EGFP-pA.

The reverse-transactivator of tetracycline (rtTA; Tet-ON Advance) was subcloned from the pTet-On Advanced vector (Clontech) into pBluescript using *Eco*RI and *Bam*HI sites. Subsequently, it was subcloned into pcDNA6 digested with *Hind*III and *Sac*II deriving pcDNA6-rtTA.
Cell culture

The cancer cell lines Pc-3, Du145 and SKOV-3 were purchased from ATCC and maintained in RPMI media containing 10% fetal bovine serum (Invitrogen). Cells were transfected with plasmids using ExGen500 (Fermentas) according to the manufacturer's protocol. Stable clones were selected using 1.0 µg/ml puromycin (Sigma), 7.0 µg/mL blasticidin (Sigma) and 750 µg/mL G418 (Invitrogen). Two µg/mL of doxycycline was used to induce gene expression under the control of the TRE promoter. Cell lines were incubated at 37°C with 5% CO₂.

Generation and screening of stable cell lines

Stables lines for DockZ and DZL were derived by transfection of 10⁶ cells (Pc-3 for DockZ; DU145 and SKOV-3 for DZL) with 10 µg linearized with Eam1105I DNA. Forty-eight hours after transfection, cells were trypsinized and replated at limited dilutions. Resistant colonies were selected with puromycin and picked using cloning cylinders after two weeks of selection.

Single-copy stable lines were screened by Southern blot. Ten micrograms of genomic DNA was digested with EcoRI overnight, resolved by gel electrophoresis and transferred to Hybond N+ (GE Healthcare). Single copy integrants were detected using a neomycin phosphotransferase (neoR) probe.

ΦC31-mediated recombination and selection for IMSI derivatives

Recombination was performed by cotransfection of 10⁶ cells with 7.5 µg of incoming vector and 2.5 µg of pCAGGS-ΦC31 using ExGen 500 (Fermentas). Forty-eight hours after transfection, resistant colonies were selected with G418. For 96-well format ΦC31-mediated integration, 0.66 µg of incoming vector and 0.33 µg of pCAGGS-ΦC31 were used. Correct integrants were verified with standard PCR using primers recognizing the attL site (CCAGGGCGTGCCCTTGAGTTCTCTC) and neoR gene.
(CGATGAATCCAGAAAAGCGGCCATTTTTC) and/or Southern using the thymidine kinase probe (Fig 2a).

**Reporter assays**

The Luciferase assay (Promega) was performed according to the manufacturer’s protocol. In brief, cells grown on 24-well plates were washed twice with phosphate-buffered saline (PBS) and incubated in 200 µl lysis buffer for 30 min on ice. Twenty microliters of cell lysate was mixed with 25 µl of luciferase reagent and luciferase activity was read on the luminometer. Luciferase activity was normalized to total protein measured using the BCA kit (Pierce).

The FACSAria™ cell sorter (BD biosciences) was used for single cell analysis of fluorescent expression. Cells grown on 6-well dishes were trypsinized and suspended in PBS. 7-AAD or propidium iodide, diluted 1:100 in PBS, was used for detection of apoptotic cells in the case of GFP and dsRed stably transfected cells, respectively. Ten thousands cells were analyzed per sample.

Soluble Flt1-Fc and free human VEGF were measured using commercially available sandwich ELISAs (R&D systems, catalog # MVR100 and DVE00, respectively) following the manufacturer’s protocol.

**Xenograft assays**

For xenograft assays, 5x10⁶ cells were suspended into 150 µl RPMI media containing 33% of matrigel and injected subcutaneously in both dorsal flanks of SCID mice. Doxycycline was administered using doxycycline-containing pellets (0.625g/kg, Harlan Laboratories). Tumour size was monitored using calipers and the volume was calculated using the formula V= (LxWxH)π/6.
Chapter 2: ΦC31-mediated site-specific insertion

5. Results

ΦC31-mediated site-specific transgene integration system: validation of its fidelity in cancer cell lines

In this study, we utilized our recently developed ΦC31 integrase-mediated transgene insertion system (Monetti, C. et al, in preparation) that allows for introduction of a GOI in a predetermined genomic locus. In this system, a docking site containing an attP site (i.e. DockZ or DZL shown in Fig. 2.1a) is randomly inserted in the genome of the desired cell line using puromycin selection and deriving resistant clones. The docking site also carries an inverted promoter-less neomycin phosphotransferase (neo^R) gene at the 5' end of the attP site. The GOI is inserted with the incoming vector through ΦC31-mediated integration. The incoming vector contains an attB site (Fig. 2.1b) that is subjected to recombination along with the attP site of the docking site. Selection of correct integrants is achieved through activation of the promoter-less neo^R gene at the docking site, since after integration of the incoming vector a promoter is positioned upstream of the neo^R gene which drives its expression (Fig. 2.1b).

In addition, the original system allows for removal of all selection markers through Flp resolution (Monetti, C. et al, in preparation) and negative selection for removal of the thymidine kinase gene by means of selection for FIAU resistance (Fig. 2.1b). In our cancer cell lines, however, we are not planning to perform this resolution step, since we envision the advantage of a built-in positive and negative selectable marker system for future drug selection for or against the cancer cells.

This vision led us to further develop the docking site by adding luciferase for in vivo imaging. Thus, we constructed a docking site that expresses luciferase i.e. DZL (Fig. 2.1a) by creating a bicistronic transgene of T2A sequence _461_ linked luciferase and puromycin resistant genes. The transcript will give rise to two separate proteins since the ribosome skips the Gly-Pro peptide bond of the T2A peptide _461_. Both proteins were expected to remain functional despite the addition of 10 amino acids at the carboxy-terminus.
Chapter 2: ΦC31-mediated site-specific insertion

Figure 2.1: Docking-incoming system.
of puromycin and a proline at the amino terminus of luciferase. To test the docking site and establish the properties of this system, a series of incoming vectors (Fig. 2.1c) and three human cancer cell lines (Pc-3, DU145 and SKOV-3) were used.

After establishing stable lines for the docking site transgene, single transgene insertion colonies were identified by Southern blotting using a neo\textsuperscript{R} probe (Fig. 2.2a). For the Pc-3 line, a total of 36 clones were screened, three of which contained a single copy of DockZ (data not shown). For the SKOV-3 and DU145 lines, 6 out of 48 and 12 out of 48 clones, respectively, contained a single copy of DZL (data not shown), showing that the puromycin was active. Furthermore, we performed the luciferase assay, showing that the luciferase gene was also active in those clones (Fig. 2.2b and 2.2c).

Single-copy docking site clones were screened for ΦC31-mediated integration of an incoming vector encoding for EGFP. Integration was successful for one out of three clones for the Pc-3 line (\textit{i.e.} Pc-3-A7) and two out of six for the SKOV-3 line (\textit{i.e.} SKOV-3-13 and SKOV-3-33). We were not able to achieve successful integration in any of the DU145 DZL...
lines. Subsequently, these clones were subjected to further $\Phi C31$-mediated integration of various incoming vectors and the fidelity of the integration was characterized. A total of 66 colonies (60 for Pc-3-A7 and 6 for SKOV-3-13) were analyzed for correct integration using a pair of PCR primers specific to the $attL$ site and the $\text{neo}^R$ gene (Fig. 2.3a). The expected 700 bp fragment was successfully amplified in all screened colonies (Fig. 2.3b), therefore, all integrations were correctly happened into the docking site. Furthermore, nine colonies were also analyzed by Southern blot using a $\text{tk}$ probe in order to demonstrate single integration of the incoming vector (Fig. 2.3d). A single band was detected in all nine colonies, indicating that after $\Phi C31$-mediated integration a single recombination event between the $attP$ site of the docking site and the $attB$ site of the incoming vector occurred (Fig. 2.3c). The band from colonies for Inc-EGFP was bigger than 10.5Kb, since the second BamHI was in the genomic area. A 9.2Kb band was identified from colonies for Inc-IRES-EGFP as predicted.
Figure 2.3: Fidelity of the docking-incoming system.

(a) Position of the primers pair used for screening of integration (IntF and IntR) as well as the tk probe used for Southern analysis. (b) PCR amplification of the integration junction using primers recognizing the attL site and the neoR probe. Sixty-six colonies (60 colonies for Pc-3-A7 and six for SKOV-3-13) were screened and all had the correct integration site. (c) Southern blot analysis of subclones derived by integration of two different incoming vectors (IncCAG-EGFP and IncCAG-tCD4-IRES-EGFP) into line Pc-3 A7 using the tk probe. Arrows denote single copy of the incoming vector. 1-Kb marker is shown.
Uniform expression of the gene of interest (GOI)

We used two different reporter assays in order to examine the expression levels between clones derived from the same parental line. The incoming vector, expressing luciferase under the control of pCAGGs, was introduced into the DocZ docking site containing Pc-3-A7 line and nine clones were isolated. Luciferase activity was measured and normalized to total protein. The normalized levels indicate that the expression levels between isogenic clones were similar (Fig. 2.4a). One-way ANOVA analysis indicated that the expression levels were similar between isogenic clones ($P=0.509$).

In order to further examine the expression between isogenic clones, we used an incoming vector encoding EGFP or dsRed under the control of pCAGGs either as a single or bicistronic gene, coupled with an IRES to a truncated form of CD4 (tCD4), missing the intracellular and extracellular domains responsible for interaction with other proteins (I. P. Michael and A. Nagy, unpublished data). Using flow cytometry for single cell analysis, we showed uniform GFP expression in each clone as well as a similar mean fluorescent value between isogenic clones derived from Pc-3-A7 and SKOV-3-13. Furthermore, essentially all cells (99%) were positive for GFP (Fig. 2.4b, 2.4c, Table 2.I and data not shown).

Mean GFP expression was one order of magnitude lower when EGFP was expressed as a bicistronic gene using IRES (Table 2.1). After establishing xenografts, we were able to show that the difference in expression level between pCAGGs EGFP and pCAGGs TM-IRES-EGFP was maintained at the tumor site as well (Fig. 2.4di and 2.4dii). Similar results were obtained when dsRed was used instead of EGFP (not shown).

Generation of the doxycycline-inducible system

To obtain temporal control on transgene expression, we coupled the IMSI system with the doxycycline-inducible system. We created a range of incoming vectors containing second-generation tetracycline responsive elements coupled with the minimal CMV promoter (TRE) (Fig. 2.1b). The second-generation reverse tetracycline transactivator was random integrated in lines Pc-3-A7 and SKOV-3-13 using linearized pcDNA6-rtTA-Adv (Fig. 2.5a)
Figure 2.4: Expression of reporter genes from isogenic clones.

(a) Normalized luciferase levels of nine isogenic clones derived after integration of an incoming luciferase containing plasmid under the control of pCAGGs (IncCAP-luciferase) into line Pc-3-A7. There was no significant difference at the luciferase between isogenic clones (P=0.509). Error bars show the standard-deviation of the mean. (b) Histogram plots of EGFP expression of ten isogenic clones derived after integration of (i) IncCAP-EGFP and (ii) IncCAP-TM-IRES-EGFP into the Pc-3-A7. The GFP levels were an order of magnitude higher (Table I) when EGFP was expressed as a single gene. (c) Fluorescent analysis of EGFP expression of three isogenic subclones derived after integration of Inc-CAP-EGFP into the SKOV-3-13. (d) Fluorescent images of xenografts derived from Pc-3 A7 lines stably integrated with (i) IncCAP-EGFP and (ii) IncCAP-tCD4-IRES-EGFP. The relative ratio of EGFP levels of the two vectors was maintained in vivo as well.
Figure 2.5: Generation of an inducible system coupled to the docking system.

(a) Plasmid encoding the second generation of rtTA under the control of CMV. Stable lines were established after transfection and blasticidin selection. (b) EGFP expression after integration of IncTRE-EGFP into Pc-3-A7 rtTA sublines #23 and #25, before and after induction with dox (2 µg/ml) for 24 h and 48 h. bf; brightfield, gfp; fluorescent. (c) Fluorescent analysis of EGFP expression of three subclones derived after integration of Inc-TRE-EGFP into SKOV-3-13 stably transfected with rtTA before and after dox. (d) Expression of GFP after removal of doxycycline. PC-3-A7-23-EGFP cell culture was exposed to doxycycline for 48hrs. Then it was split into two plates and doxycycline was withdrawn from one of them while kept for the other one. 96hrs after doxycycline withdrawal, GFP levels were significantly decrease and were undetectable 144hrs after withdrawal.
and clones, resistant to blasticidin, were selected. An incoming vector for EGFP under the control of TRE was introduced through ΦC31-IMSI. After addition of doxycycline, strong GFP expression was observed for both Pc-3-A7 and SKOV-3-13 rtTA lines, while no GFP was expressed in the absence of doxycycline (Fig. 2.5b and 2.5c). Withdrawal of doxycycline resulted to disappearance of GFP expression indicating that this system can be turned off (Fig. 2.5d).

A second incoming vector, encoding a secreted protein able to trap Vascular Endothelial Growth Factor (VEGF), \textit{i.e.} Flt1-Fc\textsuperscript{35,464}, under the control of the TRE promoter and coupled to EGFP through IRES was stably introduced into line Pc-3-A7-23. Using flow cytometry, we showed that after 72 h of doxycycline administration, uniform expression of GFP was observed in three different sister clones (Fig. 2.6a). Furthermore, using a sensitive ELISA for Flt1-Fc, we demonstrated that its expression was tightly regulated; no protein was detected in the absence of doxycycline and high levels were measured after 72h of doxycycline (Fig. 2.6bi). Flt1-Fc can also trap VEGF, secreted by Pc-3 cells. By using an ELISA that detects only free VEGF, we were able to show that after 72h of doxycycline and secretion of Flt1-Fc, VEGF was ablated from the supernatant, while no reduction of its levels was observed in the absence of doxycycline (Fig. 2.6bii). Inducible expression of EGFP alone did not affect the levels of VEGF in the media (Fig. 2.6bii).

In order to examine if inducibility is maintained \textit{in vivo}, we established xenografts for Pc-3-A7-23 expressing Flt1-Fc or EGFP under the control of TRE. Since previous studies indicate that Flt1-Fc can slow tumour growth through inhibition of angiogenesis\textsuperscript{35}, we used this as a functional assay to test the stability and inducibility of our system \textit{in vivo}. After establishing xenografts for Pc3-A7-23 transfected with Inc-TRE-EGFP (wild type control) and Pc-3-A7-23 transfected with Inc-TRE-Flt1Fc-IRES-EGFP, no difference in tumour growth rate was observed during the first five weeks, indicating that tight regulation is maintained (Fig. 2.6c). After inducing the expression of the GOI by feeding the xenograft bearing animals with doxycycline containing pellets, a reduction in tumour size was observed for Pc-3-A7-23 stably transfected with Flt1-Fc, indicating that strong inducibility is also maintained (Fig. 2.6c).
Chapter 2: ΦC31-mediated site-specific insertion

Figure 2.6: Generation of an inducible system for an angiogenesis inhibitor.

(a) Histogram plots of EGFP expression of three isogenic clones derived after integration of IncTRE-Flt1Fc-IRES-EGFP into Pc-3-A7-23. (b) Expression levels of (i) Flt1-Fc and (ii) VEGF in the supernatant of Pc-3-A7-23 stably transfected with IncTRE-EGFP or IncTRE-Flt1Fc-IRES-EGFP, before (white columns) and 48h after (grey columns) dox induction. No Flt1Fc was expressed in the absence of doxycycline, while it was expressed in high levels after 48h of doxycycline addition to the media. This resulted in trapping of VEGF, which was undetectable with ELISA. (c) Tumour volume of Pc-3-A7-23 xenografts stably transfected with IncTRE-EGFP (empty grey squares) or IncTRE-Flt1Fc-IRES-EGFP (filled blue squares). Before induction of transgene expression both xenografts grow at the same rate. After initiation of feeding with doxycycline containing pellets (arrow), the xenografts expressing Flt1Fc decreased in size, while the ones expressing EGFP continued growing.
High-throughput system

The generation of a series of incoming vectors for derivation of isogenic clones would require both the cloning of GOIs in the desired vector as well as the establishment of stable cell lines. To facilitate and accelerate this procedure, we coupled our system with the Gateway cloning system. We inserted reading frame A (RfA) of the Gateway system into three different incoming vectors, generating Inc-pCAGGs-RfA-pA (Inc-CAP), Inc-TRE-RfA-pA (Inc-TAP) and Inc-TRE-RfA-IRES-EGFP-pA (Inc-TAG) (Fig. 2.1b). This allows for high-throughput insertion of cDNA libraries into the desired incoming vector.

We then examined if it was feasible to achieve ΦC31-mediated integration in a 96-well cell culture plate format. Using the Pc-3-A7 clone we were able to establish stable clones in a 96-well format using three different incoming vectors expressing EGFP, dsRed and luciferase. On average, four G418 resistant colonies were observed per well (Fig. 2.6 and Table 2.2). One-way ANOVA analysis indicated that the number of resistant colonies was independent from the transgene that it was introduced \( (P=0.298) \). Out of 48 wells, 47 had at least one resistant colony (98%, Table 2.2). Similar results were observed when an incoming vector for Flt-1Fc-IRES-EGFP under the control of TRE was introduced in line Pc-3-A7-23 (data not shown).

![Figure 2.7: High throughput ΦC31-IMSI.](image)

Number of resistant colonies after ΦC31-IMSI of incoming vector for EGFP, dsRed and luciferase. There was no difference in the number of colonies per well for the three different transgenes (One-way ANOVA, \( P=0.298 \)).
**Table 2.1**

Characterization of EGFP expression of isogenic clones

<table>
<thead>
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<th>Clone</th>
<th>GFP+ median</th>
<th>% GFP+</th>
<th>Clone</th>
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Average 5814.5 99.35  Average 432.0 98.98
### Table 2.2

Integration frequency in a 96-well plate format

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<table>
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<td>Positive wells*</td>
<td>93.75%</td>
<td>100%</td>
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On average, each well was containing 4.2 resistant colonies. In total, 98% of the wells were containing at least one resistant colony.
6. Discussion

Precise control of chromosomal insertion and expression of various genes involved in tumor progression will allow for the creation of models useful for studying cancer biology, screening and optimizing therapeutic agents and schemes. In this study, we characterized a ΦC31-IMSI system in cancer cell lines. This system allows for the control and precise insertion of the GOI in a predetermined genomic locus. In addition, this system was combined with inducible expression of the GOI as well as luciferase expression allowing for \textit{in vivo} live imaging.

The phage serine recombinase ΦC31 is utilized in this system in order to facilitate the integration of the GOI into the host genome. First, a docking site carrying an \textit{attP} site upstream of a promoter-less neo\textsuperscript{R} gene is inserted as a single copy into the host genome by means of selection for puromycin resistance. The incoming vector, carrying an \textit{attB} site in front of a promoter, is subsequently inserted via ΦC31-mediated integration into the genome (Fig 2.1a). The incoming vector also carries the GOI under the control of the desired promoter. A similar strategy has been previously described and applied in human embryonic kidney 293 cells using zeocin and hygromycin for selection instead of G418 and puromycin, respectively \cite{466}. In addition, using the Flp recombinase and thymidine kinase as a negative selection marker, the selection markers can be excised leaving behind a clean integration of the GOI only. However, this aspect was not examined in the present study. Maintenance of the selection markers offers the advantage for easy isolation of pure populations of cancer cells from tumour models that, for example, resist or develop resistance to various therapeutic schemes. In order to further advance the IMSI we introduced the expression of luciferase that allows for \textit{in vivo} live imaging and monitoring of tumour growth and metastasis. We implemented this feature by constitutively expressing luciferase as a fusion transgene with the puromycin gene of the docking site (Fig. 2.1 and 2.2). As far as we know this is the first luciferase-containing IMSI.

Using this system, we were able to show that all of the resistant colonies in two different cancer cell lines were a result of the integration of the incoming vector in the
desired genomic locus. In agreement with previous studies describing pseudo-\textit{attP} sites human cell lines, we did not observe any multiple integrations in the same clone \cite{457,460,466}. It is possible that integration at pseudo-\textit{attP} sites may occur yet G418 selection selects against these events.

Insertion of various transgenes in the same genomic locus resulted in similar expression levels between isogenic clones. Similar results have been previously reported using different RMCE strategies \cite{467,468}. Such reproducible expression allows for various applications, such as the characterization of methylation and transgene orientation on transgene expression, as well as the characterization of promoter and enhancer elements \cite{469-472}. In this study, we compared the levels of EGFP expressed either directly under the control of a strong promoter or as an IRES-based bicistronic gene under the control of the same promoter. The GFP levels were different in the two cases, with the levels dropping an order of magnitude when EGFP was expressed in a bicistronic fashion (Fig. 2.4 and Table 2.1). We were also able to show that the difference in expression levels was maintained after establishment of \textit{in vivo} xenografts, implying that this system is robust and can be utilized for \textit{in vivo} studies (Fig 2.4).

It is often required that the expression of the GOI is inducible. Thus, we coupled our system with the doxycycline inducible system. After creating stable lines for the docking site we derived sublines constitutively expressing rtTA-Advance \cite{463}. In combination with incoming vectors containing the TRE promoter \cite{462}, we showed that this system allows for tight and inducible regulation of EGFP (Fig 2.5). Furthermore, in a proof-of-principle experiment, we derived stable lines expressing soluble Flt-1 receptor as a chimeric protein linked to the constant domain of an IgG antibody (Flt1-Fc) in an inducible manner \cite{35,464}. We showed that the expression of Flt1-Fc was tightly regulated \textit{in vitro}. Furthermore, this tight regulation was also maintained in xenograft assays, adding to the evidence that this system can be used for \textit{in vivo} studies (Fig 2.6).

Finally, in an attempt to make this system more flexible we combined it with Gateway technology (Fig. 2.1). All incoming vectors were made Gateway compatible, which
allows for fast and reliable generation of incoming-expression libraries. This, in combination with the fact that this system can be used in a 96-well set-up, could allow for high-throughput generation of co-isogenic stable lines for the expression of gene libraries (Fig. 2.7 and Table 2.2).

In conclusion, the IMSI described here offers reliable and directional integration of a GOI into a specific locus of the genome of cancer cell lines utilizing the ΦC31 recombinase. It also allows for reproducible and inducible expression of the GOI as well as derivation of expression libraries in a high-throughput manner. These characteristics were maintained in vivo during xenograft assays, which along with its live imaging feature due to luciferase expression, makes this system an attractive tool for cancer research.
Chapter 3

Local acting VEGF sticky-traps inhibit tumour growth without systemic side effects
1. Preface

In the previous Chapter, I was able to successfully apply the ΦC31-IMSI system in cancer cell lines. Although this system allowed for high-throughput generation of cancer cell lines expressing the transgene from a defined region in an inducible manner, it had two major drawbacks. First, the inducible expression of a transgene was not consistent among isogenic clones in certain cancer lines. Second, the process for the derivation and characterization of cancer lines with the desirable docking site was lengthy, up to six months, and complicated with multiple steps. Hence, I decided to apply the robust transposon-mediated transgenic system for my further thesis work and develop a new approach for fast and high-throughput generation of cancer cell lines expressing the transgene of interest in an inducible fashion. This system was not only efficient but allowed me to characterize the molecules described in this chapter and Chapter 4.

Given the side effects of antiangiogenic therapy, the main aim of this chapter the development and characterization of VEGF sticky-traps. These molecules are composed of a VEGF trap-domain responsible for inhibiting angiogenesis and a heparin-binding domain responsible for targeting and anchoring the sticky-trap at the tumour site.
2. Abstract

Vascular endothelial growth factor-A (VEGF) signaling plays an important role in a broad range of normal physiological processes. Therefore, it is not surprising that current treatments for cancer and age-related macular degeneration based on systemic VEGF targeting strategies often cause severe side effects. Here we report the generation of novel multifunctional anti-VEGF molecules that induce strong local inhibition without disturbing systemic VEGF levels. By combining the tetracycline transgene inducible system with transposon-based gene delivery, we efficiently introduced temporally regulated expression of these new molecules into cancer cell lines for rapid testing of their tumour suppressing activity. Our shortened half-life VEGF trap molecules equipped with extracellular matrix retention domains borrowed from VEGF itself (“VEGF sticky-traps”), were able to delay or even inhibit tumour growth in xenograft studies to the same extent as the original VEGF trap. Moreover, VEGF sticky-traps, unlike the original VEGF trap, were not detectable in the serum or urine and did not cause adverse systemic effects, such as delayed wound healing and regression of tracheal blood vessels. This novel class of antiangiogenic molecules holds promise for future clinical applications and opens up avenues for designing similar multifunctional biologics to treat a variety of diseases.
3. Introduction

The vascular system is the first functional organ to be developed in the embryo. Its proper formation is precisely orchestrated by a number of positive and negative regulators and disruption frequently leads to embryonic lethality. Vascular endothelial growth factor-A (VEGF-A, hereafter referred to as VEGF) is the most important mitogen for endothelial cells. Null mutation of the VEGF gene results in heterozygous embryonic lethality (haploinsufficiency) at midgestation. VEGF primarily mediates its signaling through VEGFR-2 and its co-receptor neuropilin-1. The second receptor, VEGFR-1, undergoes weak tyrosine autophosphorylation upon binding of VEGF and its role is not fully understood.

Abnormal vessel formation is implicated in the pathophysiology of a number of diseases such as cancer, diabetic retinopathy, age-related macular degeneration (AMD) and obesity. Inhibition of angiogenesis has proven to be successful in delaying overall cancer progression and improving the vision of patients suffering from AMD. Over the past decade, a number of different approaches to inhibit VEGF signaling have been developed. These include recombinant trap proteins (VEGF trap), monoclonal antibodies against VEGF itself (bevacizumab) or its receptor VEGFR-2 (DC101) and small multikinase inhibitors (sorafenib and sunitinib). These strategies are promising and some have been approved for clinical use. However, a number of side effects were observed during clinical trials including, hypertension, proteinuria, thromboembolic events, ventricular dysfunction, congestive heart failure, gastrointestinal perforations and fistula formations, bleeding and wound healing complications, due to the disruption of proper vessel function in various organs such as the kidney and gastrointestinal tract. It is now becoming apparent that long-term use of such antiangiogenic based therapy may not be plausible due to the adverse side effect profile.

The significant side effects underscore the important role of VEGF signaling and vessel network maintenance in the proper function of many organs during adulthood. Characterization of the role of VEGF in adulthood is incomplete because of limitations imposed by the available mouse models. Tissue or cell type-specific deletion of VEGF using sticky-traps.
Cre recombinase has elucidated important autocrine and paracrine roles of VEGF. For example, VEGF was recently shown to be important for endothelial homeostasis and proper kidney function respectively \(^{56,61,62}\). However, this model was limited by its irreversibility. In addition, VEGF derived from blood components, such as platelets \(^{476}\) could mask conditional VEGF knockout phenotypes. Expression of a soluble form of VEGFR-1 (sVEFGR-1 \(^{63}\)) in an inducible spatiotemporal manner during adulthood solves the reversibility problem and partial inhibition of VEGF. Yet, sVEGFR-1 entry into the circulation could affect the function of other organs and thus any observed phenotypes might be due to an unknown secondary action.

In this study, we developed VEGF inhibitors that have the ability to inhibit VEGF locally, only at the site of production or administration. We modified the original VEGF trap, composed of domain-2 of VEGFR-1 and domain-3 of VEGFR-2 fused to the constant region (Fc) of human IgG \(^{126}\) by adding a number of novel components. First, we created an alternative form, VEGF sh-trap, which has a short serum half-life. Subsequently, we generated VEGF sticky-traps by fusing VEGF sh-trap with the heparin binding domains (HBDs) of VEGF itself; HBDs have the ability to bind to heparan-sulphate proteoglycans (HSPs) and thus to the extracellular matrix (ECM). We showed that VEGF sticky-traps maintain their primary function, \(i.e.\) to trap VEGF while sticking to the ECM, which consequently allows inhibition of angiogenesis only at the site of expression or administration. This new generation of VEGF sticky-traps is unique and possesses clinical applicability, especially for the long-term inhibition of angiogenesis in various diseases, where the original VEGF trap or other ways of systemic VEGF suppression may fail.
4. Material and methods

Construction of VEGF traps and piggyBac expression system

VEGF traps were generated using basic molecular biology techniques. The classic VEGF trap (1479 bp; 492 a.a.; M.W. 54.8 kDa) is composed by (1) the signal peptide (26 + 5 a.a.; borrowed from human VEGFR-1, NP_002010, a.a. 1-31), (2) domain-2 of human VEGFR-1 (101 a.a.; borrowed from VEGFR-1, NP_002010, a.a. 131-231), (3) domain-3 of human VEGFR-2 (102 a.a.; borrowed from VEGFR-2, NP_002244, a.a. 226-327), and (4) Fc region of human IgG1 (H domain: 15 a.a.; P01857.1, a.a. 99-113 plus CH2 domain: 110 a.a.; P01857.1, a.a. 114-223 plus CH3 domain: 107 a.a.; P01857.1, a.a. 224-330). Two epitope tags (FLAG: DYKDDDDK and His: HHHHHHHHH) were added at the carboxy-terminus with GS1 linkers (GGGS) in between (Fig 3.1a). For the generation of VEGF sh-trap (1227 bp, 408 a.a., M.W. 44.8 kDa), domain CH2 was substituted by (1) H’ domain (17 a.a.; EPKSCDTPPPCPAR) and (2) GS2 linker (GGGSGGGSGGGS). For the generation of the VEGF sticky-traps (hbdVtrap68: 1332 bp, 443 a.a., M.W. 51.0 kDa; hbdVtrap78: 1392 bp, 463 a.a., M.W. 51.0 kDa; and hbdVtrap678: 1464 bp, 487 a.a., M.W. 54.0 kDa) the mouse VEGF-A exons 6 (24 a.a.; NP_001020421, a.a 319-342), 7 (44 a.a.; NP_001020421, a.a 343-386) and 8 (6 a.a.; NP_001020421, a.a 387-392) were used. The GS3 linker (GGGAS) was used between the CH3 and VEGF-A exons (Fig 3.1a and b).

The transposons, PB-TAG and PB-rtTA, were generated by modifying PB-TET and pcDNA-rtTA.neo (IMP, AN unpublished) (Fig 3.2a).

Cell culture and generation of cancer cell lines

The human prostate cancer Pc-3, human rhabdomyosarcoma A-673 and human colon adenocarcinoma HT-29 cell lines were purchased from ATCC and maintained in RPMI-1640, DMEM and McCoy’s 5A media, respectively, containing 10% fetal bovine serum (Gibco Invitrogen, Burlington, ON, Canada). For the generation of stable cell lines, 1*10^6 cells were plated in a 6-well plate and were transfected 16 hrs after with 0.5 µg of PB-rtTA.neo and 2.5 µg of PB-TAG-GOI (1:5 molar ratio) along with 0.5 µg PBase using ExGen500 (Fermentas)
according to the manufacturer's protocol. Stable clones were derived after two weeks of selection using 750 µg/mL G418 (Invitrogen). At the end, all resistant clones were trypsinized and pooled together. For induction of gene expression, we used 2 µg/ml of doxycycline (Sigma). Cell lines were cultured at 37°C in 5% CO₂ containing atmosphere.

**Protein isolation**

For isolation of cytoplasmic and secreted protein from stably transfected cancer lines, one million cells were cultured in 6-well plates with and without doxycycline for 48 hrs. Supernatant were collected, centrifuged and stored at -70°C. Subsequently, the cytoplasmic proteins along with ECM proteins of the cell monolayer were isolated using RIPA lysis buffer containing protease inhibitors (Roche). Plates containing 250ul of lysis buffer per well were incubated for 30 min on ice. The lysis was collected and stored at -70°C.

Protein from xenografts was isolated from frozen tumour sections. Around a half gram of tissue was quickly homogenized on ice in 3 ml ice-cold RIPA lysis buffer containing protease inhibitors (Roche) using homogenizer. Homogenates were incubated for 1 h at 4°C and centrifuged at 15 000 x g for 30 min at 4°C. The supernatant was collected and frozen at -20°C. Aliquots of supernatant were collected for protein determination by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Richmond, CA).

**Western blot assays**

Cell culture extracts and supernatants, tumour protein extracts and plasma were resolved by 4–20% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in TBS-T buffer (10mM Tris pH 7.5, 150mM NaCl and 0.1% Tween 20). A goat anti-human Fc IgG1-HRP conjugated antibody (1 in 5,000; Jackson Immunoresearch, catalog # 109-035-098) was used for detection of VEGF traps. Loading for cell culture and tumour extracts was assessed with rabbit antibody against human beta-actin (1 in 10,000; Sigma) followed by anti-rabbit IgG1-HRP conjugated antibody (1 in 10,000; Sigma).
Chapter 3: VEGF sticky-traps

**Flow cytometry analysis**

For flow cytometry analysis, cells were cultured on 6-well plates with or without doxycycline for 48 hrs, trypsinized and suspended into PBS containing 1% v/v of 7-AAD (BD Pharmingen) for detection of apoptotic cells. The FACSARia™ cell sorter (BD Biosciences) was used for single cell analysis.

**ELISA assays**

Human VEGF was measured using a commercially available sandwich ELISA (R&D systems, catalog # DVE00) following the manufacturer’s protocol. One hundred microliters of tissue supernatant was used for each sample.

**Extracellular binding assays**

One million cells were cultured in 6-well plates containing microscope cover slips with or without doxycycline for 48 hrs. Subsequently, supernatant were collected for Western blot analysis and cell monolayers were washed three times with PBS. After 10 min fixation with 1% paraformaldehyde in PBS, cell monolayers were incubated with a goat anti-human Fc IgG1-cy3 conjugated antibody (1 in 500 in PBS; Jackson Immunoresearch, catalog # 109-165-098) at 4°C overnight.

**Xenografts**

For the xenograft assays, we used male nude mice (8-12 weeks old) for the Pc-3 prostate and A-673 rhabdomyosarcoma cancer lines and female nude mice (12-16 weeks old) for the HT-29 colon cancer line. Briefly, cells were trypsinized and suspended into serum-free media containing 33% Matrigel at a final concentration of $3.3 \times 10^7$ cells per ml. Mice were anesthetized using isoflurane and injected subcutaneously with $5 \times 10^6$ cells per site in both dorsal flanks (150 µl per site). Doxycycline was administered using doxycycline-containing pellets (0.625g/kg, Harlan Laboratories). Tumour size was monitored using calipers and the volume was calculated using the formula $V = (L \times W \times H) \pi / 6$. 
Chapter 3: VEGF sticky-traps

**Wound healing assay**

To test wound healing, wounding was performed and healing was assessed as previously described \(^{479, 480}\). Wounds were created in the dorsal area, close to the neck, of male nude mice (8-12 weeks of age) bearing bilateral subcutaneous tumours in the flank areas. For these studies, we used the A-673 cell lines stably transfected with VEGF traps. Once the combined size of both tumours was approximately 1.5 cm\(^3\), doxycycline was administered for 8 days in order to induce the expression of VEGF traps. At day 8, mice were anesthetized using isoflurane, and full thickness wounds were created using 6mm sterile Biopsy Punch (Miltex) at the nape area. One drop of liquid bandage was applied at the wound area. Postoperatively, liquid Metacam (meloxicam) was given subcutaneously for 3 days for pain management, and Novo-Trimel (trimethoprim and sulfamethoxazole) was given *per os* for five days in order to avoid any bacterial infections. The wound was photographed every other day for a period of 12 to 14 days. Blood samples were also collected before doxycycline administration, at day 8 and at the end of the study, as described below. At the end of the study (day 12 to 14) the mice were euthanized and the wound area was dissected and further analyzed using hematoxylin and eosin (H&E) staining.

**Plasma collection**

Blood samples were collected in Microtainer plasma separating tubes (Becton Dickinson, Franklin) from retro-orbital sinus during the study and by cardiac puncture of mice under anesthesia with isoflurane at the end of the study. Samples were centrifuged, aliquoted, and stored at -70°C until assayed. All capillary tubing, syringes, and needles used for bleeding were heparin-coated to avoid clotting.

**Hypoxia assessment**

Tumour hypoxia was detected using a Hypoxyprobe Plus kit (Natural Pharmacia International Inc.) following the manufacturer’s instructions. Briefly, 60mg/kg of pimonidazole hydrochloride was injected i.p. into tumour bearing mice 1.5 hrs before tissues were harvested. Pimonidazole adducts were detected as described below.
Immunostaining

Blood vessels were detected by staining for endothelial cells using hamster anti-CD31 (PECAM) monoclonal antibody (1:200; Chemicon, catalog # MAB1398Z). Vascular basements were examined with rabbit polyclonal antibody against type-IV collagen (1:5000; Cosmo Bio CO., Tokyo, Japan). Pimonidazole adducts were detected using hypoxyprobe rabbit antisera (1:50, Natural Pharmacia International Inc.). Proliferation was assessed by phospho-Histone H3 (PH3) antibody (1:200; Upstate, catalog # 06-570). Secondary antibodies were anti-hamster Cy-3 conjugated for CD31 (1:500), anti-rabbit Cy-5 conjugated for collagen, PH3 and pimonidazole (1:500).

Tumour samples were frozen using O.C.T. compound (Sakura Tissue-Tek) immediately after dissection and stored at -70°C. Cryostat sections were cut 18 µm in thickness and stored at -70°C until assayed. For immunostaining, sections were fixed with cold methanol for 10 min at room temperature (RT) and left to dry. Tracheas were fixed with 1% paraformaldehyde in PBS for 1 hr at RT. Specimens were incubated in 5% goat serum in PBS containing 0.3% Triton X-100 (PBS-T) for 1 hr at room temperature, in order to permeabilize them and block any nonspecific antibody binding. Primary antibodies were incubated at 4°C overnight, and secondary antibodies for 2 hrs at RT.

Image acquisition and quantification

Sections were visualized using Zeiss LSM510 Meta two-photon microscope. Images were captured with a Zeiss Axiocam camera connected to the microscope using AxionVision software. Images of six fields were taken for each tumour section (from at least three different tumours), while images of 8 to 12 fields were taken for each trachea specimen. Images were analyzed and quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Histopathology

Tumour samples were fixed in 10% neutral buffered formalin prior to paraffin embedding. H&E staining followed. Digital slides were created from microscope slides using
Chapter 3: VEGF sticky-traps

ScanScope CS and images were acquired using ImageScope viewing software (Aperio Technologies, Inc.).

**Statistical analysis**

Results are reported as mean ± SD. Statistical significance of differences were assessed by one-way ANOVA followed by the Newman-Keul test, using PRISM, Ver. 4.0 (Graph-Pad, San Diego, CA).
5. Results

*Design of VEGF sticky-traps*

To design our novel VEGF sticky-traps, we used the original VEGF trap domain structure composed of IgG-like domains 2 and 3 of VEGF-R1 and VEGF-R2, respectively, fused to the constant region (Fc) of immunoglobulin IgG1 and extended with two epitope tags (Fig. 3.1a). The Fc region is composed of two IgG-like domains, CH2 and CH3, which provides molecules such as antibodies and VEGF trap with a long serum half-life. This is due to its ability to bind to the FcRn receptor located on endothelial, epithelial and circulating blood cells after which it is recycled back into the circulation instead of undergoing degradation.

In order to shorten the half-life, we replaced the CH2 domain, which has been previously shown to be necessary for binding to FcRn, with a hinge domain (H') and a poly-glycine-serine linker (Fig. 3.1a). We named this molecule “short VEGF trap” and designated it VEGF sh-trap.

In order to derive VEGF sticky-traps, we further modified the VEGF sh-trap by inserting the VEGF retention domains (*i.e.* HBDs), responsible for binding to HSPs, between CH3 and the epitope tags. The VEGF HBDs are encoded by exons 6 and 7 and alternatively spliced to form different VEGF isoforms that provide different strengths of retention. VEGF145 and VEGF165 splice variants contain either exon 6 or 7, respectively, yet can both bind to HSPs and circulate in the bloodstream. In contrast, the VEGF189 variant, which contains both exons 6 and 7, has very strong affinity for HSPs and does not circulate. We also included VEGF exon 8 which codes for 6 amino acids, since the first amino acid, a cysteine, is necessary for proper disulphide-bound formation of the HBD. In total, we generated three types of VEGF sticky-traps; hbdVtrap-68, hbdVtrap-78 and hbdVtrap-678 (Fig. 3.1a and 3.1b). In addition, we generated a construct coding only for the short Fc (sh-Fc), used as a negative control (Fig. 3.1a).
Chapter 3: VEGF sticky-traps

Figure 3.1: Schematic representation of VEGF trap design.
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**Figure 3.1: Schematic representation of VEGF trap design.**

(a) VEGF trap is composed by the VEGF trap region (domain 2 and 3 of VEGFR-1 and -2, respectively), the Fc region of IgG1 (hinge, CH2 and CH3 domains) and the FLAG and His tags. A poly-glycine-serine linker (GS1) was included between CH3 and FLAG tag and between FLAG and His tag. VEGF sh-trap contains an alternative hinge domain (H’) along with a poly-glycine-serine linker (GS2), instead of the CH2 domain. The VEGF sticky-traps, hbdVtrap-68, hbdVtrap-78, and hbdVtrap-678 contain the amino acids encoded by exons 6 and 8, 7 and 8 and 6, 7 and 8 of VEGF-A, respectively, at the carboxy-terminal end of the CH3 domain. A poly-glycine-serine linker (GS3) was included in between. See methods for further details. (b) Sequence of exons encoding amino acids with affinity for heparan sulphate proteoglycans (HSPs). Amino acid sequence of exons 6, 7 and 8 of mouse VEGF-A are shown and basic amino acids, Arg (R) and Lys (K), are highlighted. The “strength” of each combination is also shown as a numeric value of Lys (K). According to Muñoz et. al. one Arg corresponds to two and a half Lys. Thus, hbdVtrap-68 has 18K, hbdVtrap-78 has 25K and hbdVtrap-678 has 43K.

**Development of a transposon-mediated transgene delivery system in cancer cell lines for the characterization of novel VEGF traps**

We created a novel transgene expression system using the piggyBac transposon to generate stably transfected cancer cell lines and a tetracycline-based ON/OFF switch for transgene expression. This system is composed of two transposons: 1) PB-TAG containing the second-generation tetracycline-regulated promoter (TRE) driving the expression of the transgene coupled to EGFP through an internal ribosomal entry site (IRES) and 2) PB-rtTA containing the reverse-transactivator of tetracycline (rtTA) and the neomycin resistance gene, both driven by separate constitutive promoters (Fig. 3.2a). We and others have previously shown that transposase-catalyzed multiple transposon integrations can be achieved very efficiently by co-transfection of a transposase expression vector and a transposon vector containing a transgene of interest. Here we used a 5:1 molar ratio of PB-TAG to PB-rtTA and selected for G418-resistant cells to detect PB-rtTA stable integrations. Due to the molar ratio difference, most PB-rtTA transgenic G418 resistant cells also integrated the PB-TAG and expressed the VEGF trap molecules in a doxycycline dependent
Figure 3.2: Schematic representation of piggyBac transposon system and characterization of piggyBac transposon-based expression from stable lines.
manner in all three cancer lines (Pc-3, A-673 and HT-29) studied (Fig. 3.2b-d). To simplify
the method and maintain a heterogeneous cancer cell population, clonal establishment of
transgenic cell lines was not performed at this point. Instead, all G418 resistant cells were
pooled. In the absence of doxycycline, none of the transgenes were expressed in any of the
cancer cell lines, showing that this system has zero background expression. On the other
hand, in the presence of doxycycline, all of the transgenes were expressed at a high level in
comparable amounts (Fig. 3.2b-d). Flow cytometry for EGFP in the induced (+ dox) state
showed that 85% of cells expressed the transgene, indicating that a very high proportion of
the resistant cells became double transgenic and responded to doxycycline (Fig. 3.2f and
Table 3.1). Taken together, these results, along with the ability for in vivo fluorescent imaging
(see below), indicate that the transposon system developed for this study is a robust method
for generating cancer cell lines with inducible transgene expression.
Table 3.1

Percentage of cells expressing GFP after 48hrs doxycycline determined by single-cell flow cytometry

<table>
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<th></th>
<th>Pc-3</th>
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<th>HT-29</th>
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<td>95</td>
<td>80</td>
<td>88.3</td>
</tr>
<tr>
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<td>95</td>
<td>93</td>
<td>79</td>
<td>89</td>
</tr>
<tr>
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<td>87.3</td>
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</tr>
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<td>75</td>
<td>87</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>94.16</td>
<td>92.5</td>
<td>76.6</td>
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VEGF sticky-traps have dual function: sequestering VEGF and binding to the ECM in vitro

All three cancer lines express and secrete VEGF into the conditioned media. Therefore, we used a competitive ELISA that only detects free VEGF in order to test the functionality of VEGF traps. Media was collected after 48 hrs of culture with or without the addition of doxycycline. After addition of doxycycline, the VEGF sh-trap and the VEGF sticky-traps (hbdVtrap-68, -78 and -678), similar to VEGF trap, blocked the appearance of free VEGF in the supernatant, indicating that they retain their ability to bind VEGF (Fig. 3.3a-d). To exclude the possibility that this binding takes place intracellulary, e.g. during the secretory pathway, we also assayed a 1:1 mix of wild type and transgenic Pc-3 cells. VEGF was completely sequestered from the supernatant, indicating that binding occurs extracellularly, after secretion (Fig. 3.3b).
Figure 3.3: Functional characterization of VEGF sticky-traps: VEGF inhibition.

The ability of VEGF traps to bind VEGF was assessed using a binding assay (ELISA) that measures unbound-free VEGF in the conditioned media of cancer transgenic cell lines (Pc-3 (a, b), A-673 (c) and HT-29 (d)) before and after doxycycline. White bars show the levels of VEGF secreted by various cell lines in the absence of doxycycline. Addition of doxycycline induces the secretion of the corresponding transgene. Secretion of VEGF traps sequestered VEGF within the supernatant and therefore VEGF was undetectable by ELISA (grey bars). In order to exclude the possibility that VEGF traps bind to VEGF during the secretion pathway, transgenic Pc-3 cells were mixed in a one to one ratio with wild type Pc-3 cells. As shown in panel b, co-culture of Pc-3 wild type cells with those expressing any of the VEGF traps also resulted in complete sequestration of free VEGF after doxycycline exposure for 48 hrs, indicating that binding occurs extracellularly in the media.
We expected the VEGF sticky-traps to bind to ECM components. We used an anti-human FcIgG1-Cy3 antibody to show that VEGF sticky-traps are able to bind to the ECM secreted by the cells. sh-Fc, VEGF trap and VEGF sh-trap did not bind to the ECM (Fig. 3.4a and b). In contrast, the VEGF sticky-traps were able to bind and be retained by ECM components secreted by Pc-3 (Fig. 3.4a) and A-673 cells (Fig. 3.4a), indicating that the affinity of the “VEGF-borrowed” retention domains is retained in our chimaeric molecules. In addition, using Western blot analysis with an anti-human FcIgG1-HRP antibody, we detected soluble (free) VEGF traps in the culture media of the cells taken prior to immunostaining. High levels of sh-Fc, VEGF trap and VEGF sh-trap were found while VEGF sticky-traps were hardly detectable (Fig. 3.4a and b). Collectively, these data imply that VEGF sticky-traps are able to both trap VEGF and bind to HSPs of ECM components.

**VEGF sticky-traps block tumour growth by inhibiting angiogenesis in vivo**

To explore the *in vivo* characteristics of our novel VEGF sticky-traps, we established xenograft models for all transgenic cancer cell lines by injecting them subcutaneously into nude mice. Once the xenografts reached a volume of 0.5 to 0.75 cm$^3$, we induced the expression of the transgene by feeding the mice with doxycycline containing food pellets (*dox*-chow). We were able to monitor the expression of the transgene by visualizing the expression of EGFP within the xenograft using a 388 nm wavelength (blue) LED light source, the appropriate cut-off filters and a conventional camera. Before *per os* administration of doxycycline, none of the xenografts expressed EGFP (Fig 3.5a and b). In the A-673 xenografts, strong EGFP expression was detected as early as 2 days post doxycycline administration while the onset of strong EGFP expression occurred around day 4 in tumors formed by HT-29 cells. Both A-673 and HT-29 lines expressed intense EGFP at day 4 and 8, respectively, post doxycycline (Fig. 3.5a and b). On the other hand, the EGFP expression from xenografts established by Pc-3 cancer lines were weak and almost undetectable in some cases with the conventional camera (data not shown). The expression of all VEGF traps was also monitored by Western blot analysis of tumour extracts and serum from blood samples collected pre and post administration of *dox*-chow (see below).
Figure 3.4: Functional characterization of VEGF sticky-traps: ECM binding.

The ability of VEGF traps to bind to the ECM was determined by immunostaining and Western blot analysis. Immunostaining of cell monolayers (Pc-3 (a) and A-673 (b)) with an anti-human FcIgG1-Cy3 antibody, revealed that only VEGF sticky-traps were able to bind to the ECM secreted by the cells, as shown by the red signal. The protein level of the VEGF traps in the supernatant of the corresponding biological replica of transgenic cell cultures was determined by Western blot analysis using an anti-human FcIgG1-HRP antibody. High levels were detected in the case of soluble VEGF traps, VEGF trap and VEGF sh-trap, while low levels or no protein was detected in the case of VEGF sticky-traps. Scale bar, 100 µm.
Chapter 3: VEGF sticky-traps

Figure 3.5: Xenograft model.
We analyzed the effect of VEGF trap expression on tumour angiogenesis, hypoxia, proliferation and apoptosis (Fig. 3.6a-f). A-673 and HT-29 xenografts were sectioned and immunostained with antibodies recognizing the endothelial marker CD31 (PECAM). Expression of VEGF traps resulted in a dramatic decrease in microvascular density and increase of hypoxic regions in the tumours compared to control tumours expressing sh-Fc (Fig. 3.6a-f). This decrease in blood supply resulted in an increase in hypoxia of HT-29 xenografts (Fig. 3.6b) and a 2-fold decrease in proliferation of A-673 xenografts expressing VEGF traps compare to the sh-Fc expressing control (Fig. 3.6 f). Histological analysis revealed extensive areas of necrosis in both A-673 and HT-29 tumour types (Fig. 3.7a and b).

The doxycycline induced VEGF traps had considerable effects on tumour growth. Almost all of the VEGF traps blocked the growth of xenografts from all three types of tumours (Fig. 3.8a-c). The prostate cancer Pc-3 derived xenografts significantly shrunk; some completely regressed (Fig. 3.8a) with only small foci remaining under the skin (Fig. 3.7c). The xenografts derived from the A-673 cancer lines expressing VEGF traps stopped growing and the average tumour volume remained constant during the treatment (Fig. 3.8b). The stasis of the tumour was independent of the initial volume of the A-673 xenograft tumours. The inhibition of tumour growth was especially remarkable in the case of the very aggressive and fast growing A-673 xenografts (tumour volume doubling time of approximately 3 days,

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**Figure 3.5: Xenograft model.**

Bright field and EGFP images of A-673 (a) and HT-29 (b) transgenic line xenografts before and after addition of doxycycline. EGFP expression was monitored using an appropriate filter set and a conventional camera after excitation with 388nm wavelength light source. No EGFP was detected before feeding the tumour-bearing mice with dox-chow (Day 0). Exposure to doxycycline resulted in high EGFP expression levels as shown at “day 4” which was maintained until the animals were euthanized at “day of dissection”. A-673-sh-Fc tumour bearing mice were euthanized at day 4 and the rest of the groups at day 20. HT-29 sh-Fc tumour bearing mice were euthanized at day 24 and the rest at day 30. Dissected tumours are also shown. Note the red colour due to blood vessels of the dissected tumours in the case of the sh-Fc group in contrast to the VEGF traps groups; indicating the antiangiogenic activity of the latter.
Figure 3.6: In vivo characterization of VEGF trap activity through xenograft studies
Figure 3.6: In vivo characterization of VEGF trap activity through xenograft studies (previous page).

Confocal images of A-673 (a) and HT-29 (b) xenograft sections were immunostained for vessels using an anti-CD31 antibody and hypoxia using pimonidazole rabbit antisera. Expression of VEGF traps resulted in vessel regression and an increase in hypoxic regions at the tumour site. Expression of VEGF traps resulted in vessel regression. Green represents the EGFP expression from the xenografts. Blue represents DAPI staining. Scale bar, 50 µm (a) and 100 µm (b). (c) Confocal images of A-673 xenograft sections immunostained for phospho-histone H3 using an anti-PH3 antibody. Expression of VEGF traps resulted in a 2-fold decrease of the proliferation index. Green represents EGFP expression from the xenografts. Scale bar, 50 µm. Quantification of microvascular density (MVD) and proliferation index are shown in panels d, e and f. Area density positive for CD31 immunoreactivity and proliferating cells (PH3 positive) were quantified using ImageJ. The number of proliferating cells was normalized to EGFP positive area per section in order to calculate the proliferation index. Expression of VEGF traps resulted in significant decrease of the MVD and proliferation compared to control groups expressing sh-Fc (***p<0.001 (a), ***p<0.001 (b), and ** p<0.01(c)).

Figure 3.7: H&E staining of A-673 (a), HT-29 (b) and Pc-3 (c) xenograft sections (next page).

Viable tumour tissue with nuclei stained purple is clearly distinguishable from diffusely pink stained necrotic regions (a and b, asterisks). For Pc-3 xenografts, small viable tumour nodules remain under the skin 50 days after initiation of transgene expression of VEGF traps (c, asterisks). Arrows indicate blood vessels supporting tumour survival. Scale bars, 500 µm (left column) and 25 µm (right column).
Figure 3.7: H&E staining of A-673 (a), HT-29 (b) and PC-3 (c) xenograft sections
Figure 3.8: Tumour growth kinetics after induction of transgene expression.

Expression of VEGF traps resulted in tumour shrinkage in the case of Pc-3 (a), stasis in the case of A-673 (b) and slower growth or stasis in the case of HT-29 (c) xenografts. Error bars represent standard error of the mean. $n=10-12$ tumours for Pc-3, $n=12$ for A-673, and $n=12$ for HT-29 (**$p<0.001$, *$p<0.05$, #$p>0.05$).
in contrast to Pc-3 and HT-29, which exhibit a doubling time of 10 days). In HT-29 xenografts, we observed tumour-stasis when using the hbdVtrap-678 sticky-traps and the VEGF trap, while a small increase in tumour size was observed with the hbdVtrap-68 and hbdVtrap-78. The short half-life VEGF trap, i.e. VEGF sh-trap, was not as effective and tumours kept growing albeit at a much slower rate when compared to the sh-Fc expressing control (Fig. 3.8c). These results show that our VEGF sticky-traps are at least as potent as the original VEGF trap in their ability to inhibit angiogenesis and tumour progression in vivo.

**A-673 enter VEGF trap expression dependent dormancy**

Next we asked if the stasis of A-673 xenografts in the presence of VEGF traps is equivalent to clinical dormancy whereby the cancer cells remain highly proliferative but growth is blocked by increased cell death. We allowed the tumours to reach a combined size of 350 mm³ per mouse and then fed the animals with dox-chow for one week (ON), followed by one week of regular chow (OFF) and then 18 more days of dox-chow (ON) (Fig. 3.9a). We were able to monitor the ON/OFF state of transgene expression by simply observing EGFP expression in the xenografts (Fig. 3.9b).

The first round of VEGF trap expression resulted in tumour stasis, consistent with our previous results (Fig. 3.9c and d). Stopping VEGF trap expression, however, resulted in dramatic re-growth of the tumours that was comparable among VEGF traps (Fig. 3.9c and d). There was a 1.5 fold increase in tumor size within first 3 days that grew to 2.5 fold within a week (Fig. 3.9e). Despite the fact that the original VEGF trap has a long half-life and serum levels did not decrease during the “OFF” period (Fig. 3.9g), it did not exhibit any delay in tumour re-growth compared to VEGF sh-trap or VEGF sticky-traps that are undetectable in the circulation. Re-activating VEGF trap expression a second time resulted in a 25% decrease in tumour size, which was also comparable among all VEGF traps (Fig. 3.9c and f). This second round of tumour shrinkage was also followed by tumour stasis (Fig. 3.9c and d). The tumour re-growth seen after stopping VEGF trap expression suggests that the xenografts are in a state resembling clinical dormancy and are entirely dependent on VEGF trap expression.
Figure 3.9: *In vivo* ON and OFF switch of transgene expression.
Figure 3.9: In vivo ON and OFF switch of transgene expression.

(a) Tumours were allowed to reach a combined size of ~350 mm$^3$ (day 4) per mouse at which time the mice were fed dox-chow for 7-days; “ON-period”. This period was followed by 7-days during which mice were fed regular chow, “OFF-period”, and then by a second “ON-period” for 18 days. (b) Bright field and EGFP images of A-673 transgenic xenografts during cycles of doxycycline (ON) and no doxycycline (OFF) exposure of the tumour bearing mice. High EGFP expression was observed during the “ON” periods, which was undetectable during the first “OFF” period and slowly decreased and became undetectable during the second “OFF” period. The first round of VEGF trap expression resulted in tumour stasis (c and d). Upon withdrawal of doxycycline, tumours increased 1.5 fold within 3 days and 2.5 fold within 7 days (e, d and e). There was no significant difference between groups ($P=0.14$ for day 14 vs 11 and $P=0.07$ for day 18 vs 11). Re-exposure to doxycycline resulted in a 25% decrease of tumour volume within the first 7 days, followed by a period of stasis (c, d and f). There was no significant difference between the groups ($P=0.8$). (n=4 for sh-Fc, n=8 for VEGF trap, n=8 for VEGF sh-trap, n=4 for hbdVtrap-68, n=4 for hbdVtrap-78, n=8 for hbdVtrap-678) (g) Western blot analysis of serum levels of VEGF trap using an anti-human FcIgG1-HRP antibody. VEGF trap concentration did not decrease during the “OFF” periods. Arrowheads show dimers. Two µl of serum were loaded.

VEGF sticky-traps remain at the target site

We designed the VEGF sticky-traps in order to stay at the area of expression (the tumor site) and limit their entry into the circulation. To analyze the location of the traps, we collected serum before and after doxycycline induction of the transgene and measured circulating VEGF traps by Western blot analysis. Without exception, VEGF traps were detected at the tumour site after doxycycline induction (Fig. 3.10 and 3.11). However, in contrast to the sh-Fc and VEGF trap, the VEGF sh-trap and VEGF sticky-traps were undetectable in the serum and urine (Fig. 3.10b-h and Fig. 3.11b-h). The absence of VEGF sh-trap was likely due to rapid uptake and degradation by cells, while the absence of VEGF sticky-traps was due to their ability to bind and remain at the expression site. The latter is also indicated by the fact that degraded hbdVtrap678 was detected in the urine (Fig. 3.10b, asterisks). This is likely due to the massive degradation of the extracellular matrix components at the tumour site by proteases such as matrix metalloproteinases, shown to be upregulated under hypoxic conditions in tumour microenvironments. Degradation products of VEGF sticky-traps were also detected at the tumour site (Fig. 3.10a, asterisks).
Figure 3.10: Western blot analysis of transgene expression at the tumour site (a), urine (b) and the circulation of A-673 tumour bearing mice (c-h)
Figure 3.10: Western blot analysis of transgene expression at the tumour site (a), urine (b) and the circulation of A-673 tumour bearing mice (c-h) *(previous page).*

All transgenes were expressed and detected at the tumour site (a, arrowheads show dimers). However, only VEGF trap was detected intact in the urine and serum (b and d, arrows indicate monomers). VEGF sh-trap and VEGF sticky-traps were undetectable in the urine and serum (b and e-h). Low amounts of degraded VEGF trap as well as hbdVtrap678 were detected in urine (b, asterisks). sh-Fc was also detected in serum (c). For Western blot analysis two µl of serum were loaded for the VEGF trap group and ten µl for the rest. Ten µl of urine were loaded for all samples. Serum and urine from non-tumour bearing animals fed with dox-chow were used as “w.t.” controls. The tumour extracts and serum samples are from the matching samples, while the urine samples are from different replica. Recombinant hIgG was used as Western blot control. Mouse IgG was also detectable by the anti-human IgG1-HRP antibody (arrow).

Figure 3.11: Western blot analysis of transgene expression at the tumour site (a), urine (b) and the circulation of HT-29 tumour bearing mice (c-h) *(next page).*

All transgenes were expressed and detected at the tumour site (a, arrowheads show dimers). However, only VEGF trap was detected in the urine and serum (b and d, arrows indicate monomers). VEGF sh-trap and VEGF sticky-traps were undetectable in the urine and serum (b and e-h). Low amounts of degraded VEGF trap were detected in urine (b, asterisks). For the serum Western blot analysis, two µl of serum were loaded for the VEGF trap group and ten µl for the rest. Serum from non-tumour bearing animals fed with dox-chow was used as “w.t.” controls. Recombinant hIgG was used as Western blot control. The mouse IgG was also detectable by the anti-human FcIgG1-HRP antibody (arrow).
Figure 3.11: Western blot analysis of transgene expression at the tumour site (a), urine (b) and the circulation of HT-29 tumour bearing mice (c-h)
**VEGF sticky-traps do not delay wound healing nor cause tracheal vessel regression**

Next, we sought to obtain functional evidence for the lack of negative effects of the retained VEGF sticky-traps outside of the tumour environment. Since impaired wound healing is one of the major side effects of antiangiogenic therapy, we assayed this aspect specifically in A-673 xenograft bearing animals, where there was tumour stasis upon VEGF trap expression. Once the xenografts reached the combined size of 1.5cm³ per mouse, we induced the expression of the various transgenes by feeding the mice with dox-chow and created full-thickness excisional wounds using a punch biopsy eight days after the initiation of transgene expression. The size of the wound as well as the day in which the scab fell off the wound was monitored. Four days after wounding, we found that the wound size of animals with tumours expressing the soluble VEGF trap was larger than that of those expressing VEGF sticky-trap. Animals with tumors expressing VEGF sticky-traps and control non-tumor-bearing animals fed with dox-chow showed the same wound size on day 4. Similarly, we observed that a longer time period was required for the scab to fall off from the wounds in animals bearing tumours expressing the soluble VEGF trap compared to control animals and those with tumours expressing VEGF sticky-traps. No difference was observed between the latter two.

Finally, we examined tracheal micro-vessels, a sensitive measure of systemic VEGF suppression in mice, by whole mount immunostaining for antibodies recognizing endothelial cells (anti-CD31) and their basement membrane (anti-collagen IV). Functioning vessels are double positive for CD-31 and collagen IV. During vessel regression due to antiangiogenic activity, areas positive for collagen IV and negative for CD31 (called empty sleeves) are formed. Non-tumor bearing mice fed with dox-chow had very few empty sleeves and the majority of the vessels were double positive for CD-31 and collagen IV. However, in the case of animals with tumours expressing the soluble VEGF trap, we observed a five-fold increase of empty sleeves. This showed the regression of preexisting vessels due to VEGF inhibition by circulating VEGF trap and...
Figure 3.12: Wound healing assay and detection of regressed vessels in the trachea.
endothelial cell apoptosis. In contrast, in animals with tumours expressing VEGF sticky-traps (i.e. hbdVtrap678), the number of empty sleeves was the same as in non-tumour bearing animals (Fig. 3.12f, g, \( P > 0.05 \)).

These results clearly demonstrate that our novel VEGF sticky-traps are able to bind VEGF and inhibit tumor growth at least as well as the original VEGF trap. Most importantly however, due to their retention in the ECM and absence in the circulation, our traps do not create systemic side effects.

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**Figure 3.12: Wound healing assay and detection of regressed vessels in the trachea.**

(a) Tumours were allowed to reach a combined size of \(~1500 \text{ mm}^3\) (day 0) per mouse at which time the mice were fed *dox*-chow. At day 8, wounds were generated in the neck area of mice bearing tumours expressing either VEGF trap or VEGF sticky-traps. Non-tumour bearing mice fed with *dox*-chow were used as wild-type controls. Wound size and spontaneous removal of the scab were monitored daily (b-e). At day 20, some of the animals were euthanized and the trachea was dissected (a, asterisk). Serum was collected at day 0, 8 and 20 (a, arrows). VEGF trap expressing tumour bearing mice showed significant delays in wound healing at day 4 compared to non-tumour bearing animals fed with *dox*-chow or those bearing tumours expressing VEGF sticky-traps (b and c, \(* P < 0.05, \# P > 0.05\)). A significant delay of scab removal was observed in the case of animals bearing tumours expressing VEGF trap compared to wild type and those bearing tumours expressing VEGF sticky-traps (b, d and e, \(** P < 0.01, \# P > 0.05\)). \( n = 9 \) for wild type, \( n = 10 \) for VEGF trap and \( n = 13 \) for VEGF sticky-traps (\( n = 4 \) for *hbdV* trap-68, \( n = 4 \) for *hbdV* trap-78, \( n = 5 \) for *hbdV* trap-678). H&E staining of representative wounds is shown in panel d. Dashed line indicates the wound area and asterisk the scab. Scale bars, 500 \( \mu \text{m} \) (top row) and 50 \( \mu \text{m} \) (bottom row). (f) Tracheal vessels of mice bearing tumours expressing either VEGF trap or *hbdV* trap-678 and control mice fed with *dox*-chow were characterized with confocal microscopy. Co-localization of CD-31 (yellow) and type IV collagen (red) immunoreactivity on vasculature was observed in the vascular bed of tracheas of controls and mice bearing tumours expressing *hbdV* trap-678. In contrast, regressed vessels (vessels of type IV collagen immunoreactivity devoid of CD31 immunoreactivity; arrows) were detected in animals bearing tumours expressing VEGF trap. (g) The number of ghost vessels was significantly higher in mice bearing tumours expressing VEGF trap. (***\( P = 0.0008 \) for w.t. vs VEGF trap and \( P = 0.0009 \) for *hbdV* trap-678 vs VEGF trap, \( \# P > 0.05 \)). \( n = 4 \) for w.t., \( n = 5 \) for VEGF trap and \( n = 5 \) for *hbdV* trap-678.
6. Discussion

Inhibition of angiogenesis is a new area of research that could be applied to a number of diseases including, cancer, blindness, obesity and psoriasis. Currently, a number of approaches have been used in order to inhibit various pathways involved in this process. VEGF and its downstream signaling partners are the main targets. Although many of these approaches show some success and are currently used in the clinic, a number of frequent, unexpected and serious side effects have been reported. These adverse effects indicate that VEGF signaling is essential for proper physiological function of a number of organs during adulthood. In an attempt to ameliorate these setbacks, we further bioengineered an inhibitor of VEGF, i.e. VEGF trap, and derived a soluble short half-life version (VEGF sh-trap) and several variants of VEGF sticky-traps that are retained at the site of expression or introduction.

The intact IgG1 Fc region provides the long half-life of proteins by protecting them from degradation by “recycling”. The removal of the CH2 domain of Fc blocks recycling and consequently shortens the half-life, which has been found to be advantageous in certain application of antibodies. For example, in tumour imaging, rapidly clearing radiolabeled antibodies (known as minibodies) are needed. In VEGF sh-trap, the CH2 domain was removed and consequently decreased the half-life of this protein compared to the original VEGF trap. In effect, VEGF sh-trap was not detectable in the serum or urine and was also less effective in slowing tumour progress in the case of the colorectal HT-29 xenograft model.

VEGF sticky-traps were engineered by fusing the VEGF sh-trap to the VEGF exons coding for HBDs. Similar domains are found in many growth factors and play an important role in their localization and tissue storage as well as the creation of morphogen gradients during development. VEGF contains two regions with HBDs coded by exons 6 and 7. Alternative spicing of these exons results in distinct isoforms. VEGF121 is missing both exons and is therefore completely soluble. VEGF145 and VEGF165 contain exon 6 and 7, respectively and are therefore partially soluble. VEGF189 has both exons and is strictly
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retained at the secretion site\textsuperscript{29, 30}. We took these combinations of VEGF HBDs and inserted them into the VEGF sh-trap which resulted in three new VEGF traps; \textit{hbd}Vtrap-68, \textit{hbd}Vtrap-78 and \textit{hbd}Vtrap-678. Using xenograft assays, we showed that the three new traps (VEGF sticky-traps) localized at the tumour site and were not detectable in the blood circulation. Although VEGF145 and VEGF165 are partially soluble \textsuperscript{29, 30} and therefore detectable in the circulation, we did not identify the corresponding VEGF sticky-traps (\textit{hbd}Vtrap-68 and \textit{hbd}Vtrap-78) in the serum of animals. This may be due to different biochemical properties of the rest of the polypeptide and/or the source of expression.

The ECM of the target tissue may serve as a reservoir for VEGF sticky-traps, similar to growth factors \textsuperscript{30, 496}. In a clinical setting, VEGF sticky-traps could be specifically delivered and released at the target site. Various approaches have been developed for tumour-specific drug delivery, such as liposomes and nanovectors \textsuperscript{498-501}. The ability of VEGF sticky-traps to bind to the ECM could increase their local half-life and might decrease the necessity of frequent administration.

In contrast to targeting the tumour microenvironment, which will require delivery methods via the circulation, the eye is directly accessible. VEGF145 was previously shown to specifically bind to ECM produced by the corneal epithelium \textsuperscript{502}. \textit{hbd}Vtrap78 may also specifically bind and localize at the corneal ECM and may have clinical applicability as a treatment for retinopathies and wet-AMD\textsuperscript{503, 504}. VEGF sticky-traps could be either directly injected into the eye like ranibizumab \textsuperscript{125} or delivered using other approaches such as slow-release implants \textsuperscript{505}, liposomes \textsuperscript{506} or even cells \textsuperscript{507} in future cell-based therapies.

In this study, we showed that VEGF sticky-traps expressed by tumors did not affect wound healing, one of the many but typical side effects of antiangiogenic therapy \textsuperscript{229-231}. In addition, VEGF sticky-traps did not cause vessel regression in the trachea, which has a vascular bed that is sensitive to antiangiogenic agents, implicating its target specificity \textsuperscript{53}. These results strongly indicate that our VEGF sticky-traps hold promise in reducing the side effects observed during the clinical trials of bevacizumab and other antiangiogenic agents \textsuperscript{214, 215}. 

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They may open the possibility of long-term application of antiangiogenic therapy not only for treatment of cancer but other chronic diseases as well.
Chapter 4

Development of VEGF lassos: novel scFv-antibody VEGF-trap fusion molecules
Chapter 4: VEGF lassos

1. Preface

In the previous Chapter, I described the development of VEGF sticky-traps, which allow for specific inhibition of angiogenesis at the target site. These molecules may find clinical applicability in ocular diseases involving neovascularization, such as age-related macular degeneration and diabetic retinopathy, since they can be directly injected into the eye.

However, in the case of solid tumours, such molecules have to be administered systematically in the circulation through the intravenous route. Thus, in this Chapter I aimed to develop bi-functional Ag/V lassos, composed by the variable domain of an antibody, able to recognize cancer specific antigens (Ag), and the VEGF trap-domain (V) responsible for inhibiting angiogenesis. These molecules could potentially be administered in the circulation of cancer patients and target solid tumours.
2. Abstract

Inhibition of angiogenesis is an established (important OR novel) therapeutic approach for cancer treatment. Regimens such as small molecules (sorafenib, sunitinib, pazopanib), antibodies (bevacizumab) and decoy receptors (VEGF trap) have been approved or are currently under phase III clinical trials. Here, we describe the development of second-generation biologics, refer to as lassos, that have the ability to bind to two different targets. Lassos consist of a variable domain of an antibody located at the amino-terminus of the Fc region of an IgG1 immunoglobulin and a VEGF trap-domain located at the carboxy-terminus of the Fc region. In this study we developed two such biologics, HER2/V lasso and EDB/V lasso, using the variable domains of antibodies able to bind to the ectodomain of HER2 receptor, upregulated in breast cancer, and the extra-domain B (EDB) of fibronectin, expressed in the vascular bed of solid tumours. Using genetic approaches we were able to show that these molecules retain the function of both the variable and trap domains, and efficiently inhibit tumour progression in mouse models. In the case of HER2/V lasso a synergistic antitumour activity was achieved through the inhibition of proliferation mediated by blockage of HER2 signaling in addition to the antiangiogenic effect. Furthermore, both lassos could potentially be used for targeted delivery of the VEGF trap-domain to the tumour and minimize current toxicities. The approach used here can be applied in the future for the development of bi-specific biologics for the treatment of complex diseases such as cancer.
3. Introduction

Tumour growth beyond a certain size requires the development of a functional vascular network, angiogenesis, which supplies rapidly proliferating cancer cells with nutrients and oxygen. Thus, inhibition of angiogenesis has proven to be an effective approach to control tumour growth. Various strategies, such as monoclonal antibodies (bevacizumab), decoy-receptors (VEGF trap) and small-molecule tyrosine kinase inhibitors (TKIs, i.e. sunitinib, sorafenib and pazopanib), have been developed and approved by the FDA as monotherapy or in combination with chemotherapy for cancer treatment. Targeting of angiogenesis is now used in the clinic for renal, colorectal, gastrointestinal stromal, non-small-cell lung, breast and glioblastoma cancer. At the same time a large number of clinical trials are examining the therapeutic value of various antiangiogenic agents for a broad range of cancers, such as ovarian, prostate, pancreatic, thyroid, liver, lymphoma, rectal, cervical, sarcomas etc. Thus, in the near future, antiangiogenic therapy may become a standard treatment modality for a number of solid tumours, to be used in conjunction with chemotherapy.

Although inhibition of angiogenesis has shown promise in the clinic, many key questions and challenges remain unanswered. The first has to do with the fact that antiangiogenic strategies result in a broad spectrum of toxicities. While most side effects, such as renal dysfunction and hypertension, are mild and controllable, others, including thromboembolic events, gastrointestinal (GI) perforation, and wound healing problems, can be fatal. These side effects might hinder the potential use of antiangiogenic therapy as a long-term treatment, such as adjuvant therapy. Safer antiangiogenic strategies will at least ensure that the maximal therapeutic value will be applied in the clinic without significant compromise due to toxicities.

The second challenge in the field is associated with the existence of a number of key growth factors that contribute to the development of new vessels. Inhibition of the master regulator, the vascular endothelial growth factor (VEGF) -A, and its signaling pathway has been the focus of current antiangiogenic strategies. However, at the moment only TKIs,
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which inhibit multiple signaling pathways, including the VEGF pathway, have been shown to be effective as monotherapy. In contrast, anti-VEGF specific biological therapies, such as bevacizumab, have to be combined with chemotherapy. Development of resistance to anti-VEGF therapy due to the upregulation of alternative proangiogenic growth factors is another major obstacle. Both the ineffectiveness of anti-VEGF strategies as monotherapy as well as the development of resistance due to alternative growth factors, points into the necessity of developing multitargeted anti-angiogenic strategies that are able to inhibit multiple proangiogenic pathways simultaneously.

Finally, there is evidence that multitargeted therapy can also be applied for the inhibition of pathways that control essential, diverse processes involved in tumour progression such as angiogenesis and proliferation. For example, preclinical studies support the combination of anti-VEGF strategies with either EGFR or HER2 targeting. Such a multitargeted approach can be achieved by TKIs or the combination of biological therapies. An alternative approach would be the development of bifunctional molecules, such as antibodies.

In this study, we developed novel bispecific antibody-based molecules that are composed of an amino-terminal single-chain variable antibody domain (scFv-Fc Ab) and a carboxyl-terminal VEGF-A trap domain. Specific recognition of a cancer-specific antigen by the antibody domain can target anti-VEGF therapy and diminish side effects. Alternatively, binding to a proangiogenic growth factor other than VEGF or inhibition of a pathway involved in cancer progression can increase the therapeutic value. We refer to these molecules as Ag/V lassos, with “x” being the antigen specifically recognized by the antibody domain. We developed and characterized two lassos; EDB/V-lasso and HER2/V lasso. The first one, EDB/V-lasso is composed of an scFv-Fc antibody that specifically recognizes the extra-domain B of fibronectin (EDB). EDB is specifically expressed in the vasculature of solid tumours. The second, HER2/V-lasso is composed of an scFv-Fc antibody that specifically recognizes the ectodomain of the receptor HER2. HER2 is upregulated in patients with breast adenocarcinomas and a specific anti-HER monoclonal antibody,
trastuzumab, is used as a therapeutic in the clinic. Since EDB/V-lasso and HER2/V-lasso both target VEGF and the ECM or HER2, respectively they represent novel multitarget agents to be used for the specific inhibition of angiogenesis in solid tumours.

Using genetic approaches we show that both lassos can be efficiently produced in mammalian cells and most importantly they maintain the function of the individual domains both \textit{in vitro} and \textit{in vivo}. For example, HER2/V-lasso can recognize HER2 and inhibit cancer cell proliferation and also trap VEGF and inhibit angiogenesis. The method developed here can be applied for the design and development of a broad range of bispecific \textit{lassos} able to either target anti-VEGF to the tumour or inhibit alternative pathways involved in cancer progression.
4. Material and methods

Construction of VEGF traps and piggyBac expression system

All transgenes were generated using basic molecular biology techniques. The classic VEGF trap (1479 bp; 492 a.a.; M.W. 54.8 kDa) is composed of: (1) the signal peptide (26 + 5 a.a.; borrowed from human VEGFR-1, NP_002010, a.a. 1-31), (2) domain-2 of human VEGFR-1 (101 a.a.; borrowed from VEGFR-1, NP_002010, a.a. 131-231), (3) domain-3 of human VEGFR-2 (102 a.a.; borrowed from VEGFR-2, NP_002244, a.a. 226-327), and (4) Fc region of human IgG1 (H domain: 15 a.a.; P01857.1, a.a. 99-113 plus CH2 domain: 110 a.a.; P01857.1, a.a. 114-223 plus CH3 domain: 107 a.a.; P01857.1, a.a. 224-330). Two epitope tags (FLAG: DYKDDDDK and His: HHHHHHHH) were added to the carboxyl-terminal end with GS1 linkers (GGGS) in between (Fig 4.1).

For the generation of lassos we first developed VEGF rev-trap (1560bp; 519 a.a.; 57.1kDa) by positioning the trap-domain (domain-2 of human VEGFR-1 and domain-3 of human VEGFR-2) at the carboxyl-terminal end of the Fc region of human IgG1). In order to optimize the length of the GS2 linker we used a series of vectors allowing for cloning of the trap-domain at the carboxyl-terminus of a sequence coding for the signal-peptide followed by the Fc region of human IgG1 a variable length of Gly-Ser linker and the TEV domain (Fig. 4.2a).

HER2/V lasso (2307bp; 768 a.a.; M.W. 83.4kDa) and EDB/V lasso (2268bp; 755 a.a.; 82kDa) were generated by fusion of the VEGF rev-trap with single-chain variable domains (scFv) of the antibodies 4D5 and L19, specifically recognizing HER2 and EDB, respectively. scFv-Fc antibodies, HER2 Ab (1647bp; 548 a.a.; M.W. 59.4kDa) and EDB Ab (1608bp; 535 a.a.; M.W. 58.1kDa), as well as an Fc Ab (900bp; 299 a.a.; M.W. 33.42kDa) were also generated and used as controls (Fig 4.1).

Two epitope tags (FLAG: DYKDDDDK and His: HHHHHHHH) were added to the carboxyl-terminus of all transgenes, with GS1 linkers (GGGS) in between (Fig 4.1).
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The transposons, PB-TAG and PB-rtTA, were generated by modifying PB-TET\textsuperscript{478} and pcDNA-rtTA/neo (IMP, AN unpublished) (Fig 4.3a).

**Cell culture and generation of cancer cell lines**

The human prostate cancer Pc-3, human rhabdomyosarcoma A-673 and human colon adenocarcinoma HT-29 cell lines were purchased from ATCC and maintained in RPMI-1640, DMEM and McCoy’s 5A media, respectively, containing 10% fetal bovine serum (Gibco Invitrogen, Burlington, ON, Canada). For the generation of stable cell lines, 1*10^6 cells were plated in a 6-well plate and transfected 16 hrs after with 0.5 µg of PB-rtTA.neo and 2.5 µg of PB-TAG-GOI (1:5 molar ratio) along with 0.5 µg PBase using ExGen500 (Fermentas) according to the manufacturer's protocol. Stable clones were derived after two weeks of selection using 750 µg/mL G418 (Invitrogen). At the end, all resistant clones were trypsinized and pooled together. For induction of gene expression, we used 2 µg/ml of doxycycline (Sigma). Cell lines were cultured at 37°C in 5% CO\textsubscript{2} containing atmosphere.

**Protein isolation**

Protein from xenografts was isolated from frozen tumour sections. Approximately 0.5g of tissue was quickly homogenized on ice in 3 ml ice-cold RIPA lysis buffer containing protease inhibitors (Roche) using homogenizer. Homogenates were incubated for 1 h at 4°C and centrifuged at 15 000 x g for 30 min at 4°C. The supernatant was collected and frozen at -20°C. Aliquots of supernatant were collected for protein determination by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Richmond, CA).

**Western blot assays**

Cell culture supernatants, tumour protein extracts and plasma were resolved by 4–20% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in TBS-T buffer (10mM Tris pH 7.5, 150mM NaCl and 0.1% Tween 20). A goat anti-human Fc IgG1-HRP conjugated antibody (1 in 5,000; Jackson Immunoresearch, catalog # 109-035-098) was used for detection of VEGF traps. Loading for cell culture and
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tumour extracts was assessed with rabbit antibody against human beta-actin (1 in 10,000; Sigma) followed by anti-rabbit IgG1-HRP conjugated antibody (1 in 10,000; Sigma).

**Flow cytometry analysis**

For flow cytometry analysis, cells were cultured on 6-well plates with or without doxycycline for 48 hrs, trypsinized and suspended into PBS containing 1% v/v of 7-AAD (BD Pharmingen) for detection of apoptotic cells. The FACSARia™ cell sorter (BD Biosciences) was used for single cell analysis.

**ELISA assays**

Human VEGF was measured using a commercially available sandwich ELISA (R&D systems, catalog # DVE00) following the manufacturer’s protocol. One hundred microliters of tissue supernatant was used for each sample.

**Xenografts**

In xenograft assays, we used male nude mice (8-12 weeks old) for the Pc-3 prostate and A-673 rhabdomyosarcoma cancer lines and female nude mice (12-16 weeks old) for the HT-29 colon cancer line. Briefly, cells were trypsinized and suspended into serum-free media containing 33% Matrigel at a final concentration of 3.3*10^7 cells per ml. Mice were anesthetized using isoflurane and injected subcutaneously with 5*10^6 cells per site in both dorsal flanks (150 µl per site). Doxycycline was administered using doxycycline-containing pellets (0.625g/kg, Harlan Laboratories). Tumour size was monitored using calipers and the volume was calculated using the formula V= (L*W*H)π/6.

**Plasma collection**

Blood samples were collected in Microtainer plasma separating tubes (Becton Dickinson, Franklin) from retro-orbital sinus during the study and by cardiac puncture of mice under anesthesia with isoflurane at the end of the study. Samples were centrifuged,
aliquoted, and stored at -70°C until assayed. All capillary tubing, syringes, and needles used for bleeding were heparin-coated to avoid clotting.

**Immunostaining**

Blood vessels were detected by staining for endothelial cells using hamster anti-CD31 (PECAM) monoclonal antibody (1:200; Chemicon, catalog # MAB1398Z). Vascular basements were examined with rabbit polyclonal antibody against type-IV collagen (1:5000; Cosmo Bio CO., Tokyo, Japan). Pimonidazole adducts were detected using hypoxyprobe rabbit antisera (1:50, Natural Pharmacia International Inc.). Proliferation was assessed by phospho-Histone H3 (PH3) antibody (1:200; Upstate, catalog # 06-570). Secondary antibodies were anti-hamster Cy-3 conjugated for CD31 (1:500), anti-rabbit Cy-5 conjugated for collagen, PH3 and pimonidazole (1:500).

Tumour samples were frozen using O.C.T. compound (Sakura Tissue-Tek) immediately after dissection and stored at -70°C. Cryostat sections were cut 18 µm in thickness and stored at -70°C until assayed. For immunostaining, sections were fixed with cold methanol for 10 min at room temperature (RT) and left to dry. Tracheas were fixed with 1% paraformaldehyde in PBS for 1 hr at RT. Specimens were incubated in 5% goat serum in PBS containing 0.3% Triton X-100 (PBS-T) for 1 hr at room temperature, in order to permeabilize them and block any nonspecific antibody binding. Primary antibodies were incubated at 4°C overnight, and secondary antibodies for 2 hrs at RT.

**Image acquisition and quantification**

Sections were visualized using Zeiss LSM510 Meta two-photon microscope. Images were captured with a Zeiss Axiocam camera connected to the microscope using AxionVision software. Images of six fields were taken for each tumour section (from at least three different tumours), while images of 8 to 12 fields were taken for each trachea specimen. Images were analyzed and quantified using ImageJ (http://rsbweb.nih.gov/ij/).
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**Histopathology**

Tumour samples were fixed in 10% neutral buffered formalin prior to paraffin embedding followed by H&E staining. Digital slides were created from microscope slides using ScanScope CS and images were acquired using ImageScope viewing software (Aperio Technologies, Inc.).

**Statistical analysis**

Results are reported as mean ± SD. Statistical significance of differences were assessed by one-way ANOVA followed by the Newman-Keul test, using PRISM, Ver. 4.0 (Graph-Pad, San Diego, CA)
5. Results

**Design of VEGF lassos**

For the generation of VEGF lassos we first generated a novel reverse form of the original VEGF trap, *i.e.* VEGF rev-trap (Fig 4.1 and 4.2). We derived VEGF rev-trap by fusing the VEGF trap domain, composed of IgG-like domains 2 and 3 of VEGF-R1 and VEGF-R2, to the carboxy-terminus of the constant region (Fc) of immunoglobulin IgG1. For the derivation a functional VEGF rev-trap we had to optimize the length of the linker between the Fc domain and the trap domain so that: (a) both domains retain their functions, (b) do not compromise the levels of expression due to incorrect folding and precipitation, and (c) the protein will be stable in order to prevent degradation by proteases.

For optimization of the linker’s length between the trap and Fc (GS2), we used a series of vectors that contain an Fc followed by variable length of Gly-Ser (5, 10, 20, 30, 40 and 50 a.a.) and the TEV domain. The VEGF trap region was cloned between the restriction sites *Not*I and *Fse*I (Fig. 4.2a). Transient expression of VEGF rev-traps with GS2 length between 12 and 37 amino acids in 293 cells revealed that all molecules had similar expression levels and were stable (Fig. 4.2b and c). However, VEGF rev-traps containing a GS2 linker with length > 37 amino acids, *i.e.* 47 and 57, were subjected to degradation (Fig. 4.2b and c). Subsequently, we determined whether or not VEGF rev-traps were functional. After adding exogenous recombinant hVEGF protein into conditioned media of 293 cells expressing the VEGF rev-trap with GS2 between 12 and 37 amino acids, we determined the levels of free hVEGF with a competitive ELISA. We found that VEGF rev-traps with linker size between 12 and 37 amino acids were able to trap hVEGF and thus were functional (Fig. 4.2 d).

In order to derive VEGF lassos, we fused the single-chain variable domains (scV\textsubscript{L} along with scV\textsubscript{H}) of scFv-Fc antibodies to the amino-terminus of VEGF rev-trap. We used two different antibodies: the first one (4D5) specific for HER2 and the second one (F19) recognizing the extra domain B (EBD) of fibronectin. Both antibodies were previously
**Figure 4.1: Schematic representation of VEGF lassos, traps and antibodies design.**

VEGF trap is composed by the VEGF trap region (domain 2 and 3 of VEGFR-1 and -2, respectively) at the carboxyl-terminal of the Fc region of IgG1 (hinge, CH2 and CH3 domains). A poly-glycine-serine linker (GS1) is included between CH3 and FLAG tag and between FLAG and His tag. VEGF rev-trap is composed by the VEGF trap region at the amino-terminal of the Fc region of IgG1. The tags, FLAG and His, are located at the carboxyl-terminal of the trap region, separated by GS1 linkers. HER2 and EDB antibody variable domains (scV_L and scV_H) located at the amino-terminus of VEGF rev-trap composed HER2/V lasso and EDB/V lasso, respectively. The original order of the two variable domains, V_L and V_H, was kept for each of the antibodies. scFv-Fc HER2 and EDB antibodies as well as Fc alone were also made.
Figure 4.2: Generation of VEGF rev-trap.

(a) In order to optimize the length of the linker between the Fc and trap domains we used a series of vectors composed by an Fc domain followed by variable length of Gly-Ser linker along with the TEV domain. At the carboxyl-terminal, two unique restriction enzymes, NotI and FseI, allowed for the cloning of the VEGF trap domain and the generation of a series of VEGF rev-traps with GS2 length varying from 12 to 57 amino acids. Transient expression of the aforementioned VEGF rev-traps in 293 cells allowed us to monitor the expression levels in conditioned media by western blotting using an anti-human FcIgG1-HRP antibody. Both non-reducing (b) and reducing (c) conditions revealed that VEGF rev-traps with GS2 up to 37 amino acids were secreted in comparable levels and were stable. (d) Using an ELISA assay as a competitive method we examine if the VEGF rev-traps were functional, i.e. were able to trap VEGF. Indeed, exogenous hVEGF added to conditioned media with VEGF rev-traps was trapped and were undetectable by ELISA indicating the VEGF rev-traps with GS2 between 12 and 37 amino acids are functional.
rearranged as single-chain variable antibodies, thus in this study we used the same order of domains for their structure. In the case of 4D5 the scV\textsubscript{L} is located at the amino-terminus and the scV\textsubscript{H} at the carboxyl-terminus, while in the case of L19, the opposite configuration was used. Thus, we generated two VEGF lassos, HER2/V lasso and EDB/V-lasso (Fig. 4.1). In addition, we generated constructs coding for the scFv-Fc HER2 Ab and scFv-Fc EDB Ab, as well as for the Fc (Fc Ab), used as a negative control (Fig. 4.1).

\textit{Utilization of the transposon-mediated transgene delivery system in cancer cell lines for the characterization of novel VEGF lassos}

In order to characterize the function of the unique VEGF lasso molecules we took advantage of the \textit{piggyBac} transposon described in Chapter 3 (Fig. 3.2 and 4.3). We derived stable cancer lines (SKOV-3, HT-29 and A-673) expressing the various molecules in a doxycycline dependent manner. In the absence of doxycycline, none of the transgenes were detected in conditioned media of the cancer cell lines, showing that this system has zero background expression. On the other hand, in the presence of doxycycline, all transgenes were detected at high levels (Fig. 4.3b-d). Flow cytometry for EGFP in the induced (+ dox) state showed that 85% of cells expressed the transgene, indicating that a very high proportion of the resistant cells became double transgenic and responded to doxycycline (Fig. 4.3f and Table 4.1). Taken together, these results indicate that the molecules and especially VEGF lassos are correctly folded and secreted into the conditioned media of the cancer cell lines.

\textit{VEGF lassos are bi-functional in vitro: sequestering VEGF and inhibiting cell proliferation}

All cancer lines used in this study express and secrete VEGF into the conditioned media. Therefore, we used a competitive ELISA that only detects free VEGF in order to test the functionality of VEGF traps\textsuperscript{487, 488}. Media was collected after 48 hrs of culture with or without the addition of doxycycline. After addition of doxycycline, the VEGF rev-trap as well as the VEGF lassos (HER2/V lasso and EDB/V lasso), similar to the original VEGF trap, blocked the appearance of free VEGF in the supernatant, indicating that they retain their ability to bind VEGF (Fig. 4.4a and b).
Figure 4.3: Schematic representation of piggyBac transposon system and characterization of piggyBac transposon-based expression from stable lines.
Figure 4.3: Schematic representation of piggyBac transposon system and characterization of piggyBac transposon-based expression from stable lines.

(a) PB-TAG is composed of a tetracycline-regulated promoter (TRE) that controls the expression of the bicistronic transgene of the gene of interest (GOI) with EGFP. The GOI is inserted into the Gateway cassette with reading frame A (GatRFA) using standard recombineering techniques. PB-rtTA includes the reverse tetracycline transactivator (rtTA) and the neomycin-resistance gene (neo), driven by CMV and PGK promoter, respectively. Stable lines were generated for three human lines, ovarian cancer SKOV-3, rhabdomyosarcoma A-673 and colon adenocarcinoma HT-29 (b, c and d). The expression of the transgene was monitored in conditioned media by Western blot analysis using an anti-human Fc IgG1-HRP antibody. In the absence of doxycycline, none of the transgenes were expressed, while strong expression was observed after 48 hrs doxycycline treatment. (f) Flow cytometry analysis of the expression of EGFP from cancer stable cell lines (SKOV-3, A-673 and HT-29) after induction with doxycycline. EGFP expression was analyzed by flow cytometry 48 hrs after doxycycline addition to the media. A high percentage of the cells expressed EGFP in an inducible manner (Table 4.1). No expression was detected in the absence of doxycycline (data not shown).

Table 4.1

Percentage of cells expressing GFP after 48hrs doxycycline determined by single-cell flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>SKOV-3</th>
<th>A-673</th>
<th>HT-29</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc</td>
<td>92</td>
<td>95</td>
<td>86</td>
<td>91</td>
</tr>
<tr>
<td>VEGF rev-trap</td>
<td>88</td>
<td>89</td>
<td>83</td>
<td>86.67</td>
</tr>
<tr>
<td>HER2 Ab</td>
<td>94</td>
<td></td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>HER2/V-lasso</td>
<td>83</td>
<td></td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>EDB Ab</td>
<td>90</td>
<td>93</td>
<td>77</td>
<td>86.67</td>
</tr>
<tr>
<td>EBD/V-lasso</td>
<td>87</td>
<td>80</td>
<td>63</td>
<td>76.67</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>87.25</td>
<td>91.2</td>
<td>76.9</td>
<td></td>
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</tbody>
</table>
In order to show that VEGF lassos also retained the function of the Ab domain, we examined the ability of the HER2 Ab and HER2/V lasso to inhibit the proliferation of the SKOV-3 cells, known to express HER2 in high amounts (ref). After addition of doxycycline into the media, expression of either HER2 Ab or HER2/V lasso resulted in slower proliferation compared to expression of Fc Ab, indicating that the function of the Ab is retained in the lasso molecules (Fig. 4.5a). Furthermore, using an anti-human HER2-Cy5 conjugated Ab, we were able to show that expression of either HER2 or HER2/V lasso blocks its ability to recognize HER2 expressed on the cell surface of SKOV-3 cells (Fig. 4.5a). Collectively, these data imply that VEGF lassos are able to both trap VEGF and retain their Ab function.

Figure 4.4: Functional characterization of VEGF lassos: VEGF inhibition.

The ability of VEGF rev-trap and VEGF lassos to bind VEGF was assessed using a binding assay (ELISA) that measures unbound-free VEGF in the conditioned media of cancer transgenic cell lines (SKOV-3 (a) and A-673 (b)) before and after doxycycline. White bars show the levels of VEGF secreted by various cell lines in the absence of doxycycline. Addition of doxycycline induces the secretion of the corresponding transgene. Secretion of VEGF traps sequestered VEGF within the supernatant and therefore VEGF was undetectable by ELISA (grey bars).
Figure 4.5: Functional characterization of HER2/V lasso: inhibition of proliferation.

(a) The function of the scFv HER2 domain of HER2/V lasso was tested by SKOV-3 proliferation assay. SKOV-3 lines expressing Fc, HER2 Ab and HER2/V lasso were plated at a density of $1 \times 10^5$ cells per well of a 24-well plate. Doxycycline was added into the media and cells were counted every two days. Expression of either HER2 Ab or HER2/V lasso resulted in slower proliferation rate compared to the Fc control. (b) Confocal immunostaining for HER-2 receptor expression on the cell surface of SKOV-3 cell lines expressing Fc, HER2 Ab and HER2/V lasso. All lines were expressing HER-2 receptor before doxycycline, while addition of doxycycline into the media had as a result the cells expressing either HER2 Ab or HER2/V lasso to abolish the signal for HER2 receptor. An anti-human HER-Cy3 conjugated antibody was used for immunostaining.
**VEGF lassos block tumour growth and inhibit angiogenesis in vivo**

To explore the in vivo characteristics of our novel biologics, we established xenograft models for all transgenic cancer cell lines by injecting them subcutaneously into nude mice. Once the xenografts reached a volume of 0.5 to 0.75 cm$^3$, we induced the expression of the transgene by feeding the mice with doxycycline containing food pellets (dox-chow). We were able to monitor the expression of the transgene by visualizing the expression of EGFP within the xenograft using a 388 nm wavelength (blue) LED light source, the appropriate cut-off filters and a conventional camera. Before per os administration of doxycycline, none of the SKOV-3 or A-673 xenografts expressed EGFP (Fig 4.6a and b). In the A-673 xenografts, strong EGFP expression was detected as early as 2 days post doxycycline administration while the onset of strong EGFP expression occurred around day 4 in tumors formed by SKOV-3 cells. A-673 and SKOV-3 lines expressed intense EGFP at day 4 and 8, respectively, post doxycycline (Fig. 4.6a and b). Using the SKOV-3 lines we established an intraperitoneal model of ovarian cancer dissemination. We were able to detect the organs in which cancer cells established foci by EGFP (Fig. 4.6c). The expression of all VEGF traps was also monitored by Western blot analysis of tumour extracts and serum collected pre and post administration of dox-chow (see below).

The doxycycline induced expression of VEGF lassos had considerable effects on tumour growth. In the case of the HER2/V lasso, growth inhibition of SKOV-3 xenografts was more significant compared to VEGF rev-trap or HER2 Ab alone or in combination (Fig. 4.7a). In the case of EDB/V lasso growth inhibition was comparable to that of VEGF rev-trap for both SKOV-3 and A-673 xenografts (Fig. 4.7b and c).

We analyzed the effect of VEGF lassos and the other molecules on tumour angiogenesis (Fig. 4.8). SKOV-3 and A-673 xenografts were sectioned and immunostained with antibodies recognizing the endothelial marker CD31 (PECAM). Expression of VEGF rev-trap as well as HER/V lasso and EDB/V lasso resulted in a dramatic decrease in microvascular density in the SKOV-3 and A-673 tumours compared to control tumours expressing Fc (Fig. 4.8). In agreement with previous studies, expression of HER Ab also
resulted in decreased microvascular density. Histological analysis revealed extensive areas of necrosis in both SKOV-3 and A-673 tumour types (Fig. 4.9).

**Figure 4.6: Xenograft models.**

Bright field and EGFP images of subcutaneous xenografts of SKOV-3 (a) and A-673 (b) transgenic lines before and after addition of doxycycline. EGFP expression was monitored using an appropriate filter set and a conventional camera after excitation with 388nm wavelength light source. No EGFP was detected before feeding the tumour-bearing mice with dox-chow (Day 0). Exposure to doxycycline resulted in high EGFP expression levels as shown at “day 8” and “day 4” for SKOV-3 and A-673, respectively, which was maintained until the animals were euthanized at “day of dissection”. SKOV-3-Fc tumour bearing mice were euthanized at day 12 and the rest at day 18 or 24 (see Fig. 4.7). A-673-Fc tumour bearing mice were euthanized at day 4 and the rest of the groups at day 20. Dissected tumours are also shown. Note the absence of red colour due to blood vessels of the dissected A-673 tumours in the case of the VEGF rev-trap and EDB/V lasso groups in contrast to the Fc and EDB Ab groups (red arrowheads); indicating the antiangiogenic activity of the latter. (c) SKOV-3 intraperitoneal model of ovarian cancer dissemination. SKOV-3 lines expressing VEGF rev-trap, HER2 Ab, HER2/V lasso and Fc were intraperitoneally injected and dox-chow was added after one week. Mice bearing SKOV-3/Fc cells develop ascites (white arrowhead) while the rest of the groups did not. Mice bearing SKOV-3/HER Ab cells showed signs of persistent anorexia, weakness and fatigue (black arrowhead). Wild type, non-tumour bearing, animals are marked with a white star. Bright field and EGFP images of dissected mice are shown. Tumour foci are indicated with white arrows.
Figure 4.7: Tumour growth kinetics after induction of transgene expression.

SKOV-3 (a, b) and A-673 (c) cells expressing Fc, HER2 Ab, EDB Ab, VEGF rev-trap, EDB/V lasso and HER2/V lasso were implanted subcutaneously. Expression of VEGF rev-trap, HER2 Ab, EDB/V lasso and HER2/V lasso resulted in slower tumour growth compared to Fc and EDB Ab. Error bars represent standard error of the mean. \( n = 10-12 \) tumours for SKOV-3, and \( n = 8 \) for A-673 (***\( p < 0.001 \), *\( p < 0.05 \), #\( p > 0.05 \)).
Figure 4.8: In vivo characterization of VEGF lassos anti-angiogenic activity.

Confocal images of SKOV-3 (a) and A-673 (b) xenograft sections were immunostained for vessels using an anti-CD31 antibody. Expression of VEGF lassos and rev-trap resulted in vessel regression. Green represents the EGFP expression from the xenografts. Blue represents DAPI staining. Quantification of microvascular density (MVD) is shown in panels b and d. Area density positive for CD31 immunoreactivity was quantified using ImageJ. (***p<0.001 and * p<0.05).
Figure 4.9: H&E staining of SKOV-3 (a) and A-673 (b) xenograft sections.

Viable tumour tissue with nuclei stained purple is clearly distinguishable from diffusely pink stained necrotic regions (a and b, asterisks). Arrows indicate blood vessels supporting tumour survival.
Figure 4.10: Western blot analysis of transgene expression at the tumour site (a, b, and c), and the circulation of tumour bearing mice (d-h).

All transgenes were expressed and detected at the tumour site of SKOV-3 (a, b) and A-673 (c) xenografts (arrowheads show dimers). All molecules were also detectable in serum circulation of SKOV-3 (d, e and f) and A-673 (g, and h) tumour bearing mice. For Western blot analysis two µl of serum were loaded for the HER2Ab trap group and ten µl for the rest. Serum from non-tumour bearing animals, fed with dox-chow, was used as “w.t.” control. The tumour extracts and serum samples are from the matching samples. Recombinant hIgG was used as Western blot control. Mouse IgG was also detectable by the anti-human IgG1-HRP antibody (arrow).
In order to characterize the expression of the various molecules we isolated protein extract from xenografts as well as serum from tumour bearing animals before and after administration of doxycycline. All of the molecules were highly expressed in high levels at the tumour site (Fig. 4.10a, b and c). VEGF rev-trap, HER2/V lasso, EDB/V lasso, HER2 Ab and EDB Ab were expected to circulate and have long serum half-life owing to their intact Fc. Indeed, we were able to detect these molecules in the serum after addition of dox-chow, further confirming their tightly regulated expression in vivo (Fig. 4.10d-h).
6. Discussion

VEGF trap was one of the first antiangiogenic biologics to be developed by fusing the combined extracellular domains of the VEGF-R1 and -R2 (VEGF trap-domain) to the amino-terminus of the Fc region of an IgG1 immunoglobulin. Here, we report the generation of the second-generation of VEGF traps. We first generated VEGF rev-trap by positioning the VEGF trap-domain at the carboxy-terminus of the Fc region. By doing this, the amino-terminal end is free and able to be fused to any existing antibodies or traps, leading to the generation of molecules with dual-specificity. Subsequently, we generated fusion proteins comprised of VEGF rev-trap with single-chain variable domain antibodies (scFv-Fc Abs) and named these novel, dual-specificity molecules “Ag/V lassos” (Ag being the antigen recognized by the Ab). We developed and characterized two such lassos, the EDB/V lasso and the HER2/V lasso.

EDB is an isoform of fibronectin that is essentially undetectable in healthy adult tissues but is found abundantly in the vascular bed of many aggressive solid tumours. Using phage display technology, a high affinity anti-EDB human antibody L19 was developed and shown to efficiently localize to tumour vasculature in mouse models as well as in patients with different cancer types. L19 was used for the development of a large number of therapeutic derivatives, such as conjugates with fluorophores and photosensitizers, liposomes, procoagulant agents (i.e. tTF), cytokines (i.e. L2 and L12), enzymes (i.e. human propyl endopeptidase) and other proteins (i.e. TNFα). Among the latter, three, i.e. L19-L2, L19-TNFα and L19, are currently under clinical development.

The aforementioned L19 derivatives were able to successfully target the active conjugate to the tumour site and thus increase the therapeutic index by increasing the local concentration and decreasing systemic side effects. Accordingly, in a similar manner, we expect that the EDB/V lasso will also show higher therapeutic value compared to VEGF trap. Moreover, in contrast to VEGF trap, which can freely diffuse inside the tumour, EDB/V lasso should target the VEGF trap domain to the vascular bed of tumours. This feature should also
contribute toward a higher therapeutic index. Finally, EDB/V lasso might also be more efficient than VEGF trap in the setting of an adjuvant therapy since: 1) a lower dose will be required thereby reducing side effects and allowing for long-term administration; and 2) due to the ability of EDB/V lasso to localize to areas of neovascularization, this biologic molecule should be more efficient in the prevention of relapse of the primary tumour as well as the growth of metastatic foci.

HER2 is amplified and overexpressed in 20-30% of breast cancers, as well as in subsets of patients with ovarian, gastric, lung and prostate cancers. Trastuzumab (Herceptin), the humanized form of the 4D5 murine anti-HER2 monoclonal antibody, blocks HER2 signaling and has been approved for the treatment of breast cancer. It improves survival in the first-line setting when combined with chemotherapy in patients with advanced disease. A number of antibody derivatives targeting HER2 have been described. These include conjugates with cytokines (e.g. IL12 and G-SCF) as well as bispecific antibodies targeting HER2 and either a NK cell antigen (e.g. CD16) or a mature T cells antigen (e.g. CD3) aiming to bring immune cells into close proximity with tumour cells. The latter trispecific antibody, ertumaxomab, showed promising preliminary results during Phase I clinical trials. HER2 antibodies have also been conjugated with drugs, such as geldanamycin and DM1, a maytansine derivative. The latter, T-DM1, has shown encouraging preclinical and early clinical anti-tumour activity with limited toxicity. Likewise, we expect that HER2/V-lasso will exhibit a lower toxicity profile compared to VEGF trap.

Recent studies have shed light into another important role of HER2 signaling as a modulator of the equilibrium between pro- and antiangiogenic factors. The proangiogenic factors VEGF, IL-8, TGF-α, Ang-1, Ang-2 and PAI-1 (plasminogen-activator inhibitor-1) were all reduced, whereas expression of the anti-angiogenic factor TSP-1 (thrombospondin-1) was increased in trastuzumab-treated tumours relative to control-treated tumours in vivo. Clinical studies showed a positive association between HER2 and VEGF expression, implicating VEGF in the aggressive phenotype exhibited by HER2.
overexpression. These data support the use of combination therapies directed against both HER2 and VEGF for treatment of breast cancers that overexpress HER2 \(^{397-399}\). This combination can have a number of synergistic effects: 1) anti-VEGF will increase the already antiangiogenic activity of anti-HER2, and 2) augmentation of the antiangiogenic effect will further sensitize the cancer cells to the anti-proliferative effect of anti-HER2 and increase its antitumour efficacy. Finally, it is worth mentioning that a recent study using a radiolabeled anti-HER2 antibody described a predominantly perivascular accumulation of the injected antibody in tumours, indicating a vascular pattern of HER2 \(^{327}\). Thus, HER2 could be used as a vascular “zip-code” for specific targeting of tumour vasculature. A number of on-going clinical trials are currently evaluating the combination therapy of trastuzumab and bevacizumab for HER2-positive breast cancer.

Various bi-targeting antibody formats have been previously described that typically assemble the variable domains of two different antibodies into one protein. Recent studies allowed the development of (a) a dual-variable-domain immunoglobulin (DVD-Ig), able to simultaneously bind to IL-12 and IL-18 \(^{335}\), (b) a derivative of trastuzumab that can bind and neutralize VEGF as well as HER2 (two-in-one Ab) \(^{337}\), and (c) a derivative of VEGF-trap that can also bind to angiopoietins (double-antiangiogenic protein; DAAP) \(^{512}\). All of the above molecules have both binding domains located at the amino-terminus in tandem. “Two-in-one” Abs \(^{337}\) can only bind to one antigen at a time since binding of one of the antigens masks the binding site for the other. The currently developed DVD-Ig and DAAP can bind both of their targets simultaneously, however it is likely that this feature will not be applicable for every combination of two different targets \(^{335, 512}\). Binding of one antigen might cause conformational changes in the binding site of the second antigen or prohibit its binding due to steric hindrance. In contrast, the Ag/V lasso molecules developed in this study probably allow for the simultaneous binding of both antigens for the current combination. Since the binding sites are located at the opposite ends of the Fc region, substitution of any one can be done without the risk to abolish the ability to bind both antigens simultaneously.
Chapter 4: VEGF lassos

Similar to VEGF trap, the Ag/V lassos developed in this study have the ability to trap VEGF-B and PlGF in addition to VEGF-A. Recent studies suggest that PlGF might also be an important factor for tumour angiogenesis. Upregulation of alternative proangiogenic growth factors, such as PlGF, was shown to be a prevalent mechanism of resistance to anti-VEGF therapy. Thus, the ability of Ag/V lasso to inhibit more than one proangiogenic growth factor may delay or abolish the development of resistance. Furthermore, substitution of the current scFv domains with ones that are specific for other proangiogenic factors can increase even more the therapeutic value.

In summary, we have created a new class of bi-targeting molecules, lassos, consisting an antibody variable region recognizing either HER2 or EDB and a trap region recognizing VEGF-A, VEGF-B and PlGF. These second-generation VEGF trap molecules retain intact Fc domains and thus are expected to retain the antibody effector functions as well as the pharmacokinetic profile of antibodies. The development of such multi-targeting, multi-functional biologics can be envisioned as a successful strategy for targeting of multiple mediators of complex diseases such as cancer.
Chapter 5

Summary and future directions
Chapter 5: Summary

Cancer is responsible for 30% of all deaths in Canada and the United States\textsuperscript{516, 517}. Chemotherapy and radiation are two traditional modalities used for cancer treatment\textsuperscript{518}. During the past three decades, accumulating evidence support the notion that antiangiogenic therapy may also be used in cancer management\textsuperscript{107, 108, 178, 185}. This first became evident with the approval of bevacizumab by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic colon cancer in 2004\textsuperscript{118, 474, 519}. This was followed by the approval of bevacizumab for additional cancer types as well as other antiangiogenic regimens in the clinic\textsuperscript{114, 115}.

The decade during which antiangiogenic therapy was proposed also witnessed the concurrent emergence of the field of monoclonal antibodies and biological therapies. This field introduced a novel class of drugs into the clinic that revolutionized the treatment of many conditions, such as autoimmune and infectious diseases as well as cancer\textsuperscript{321, 520}. The FDA approved the first antibody with antitumour properties, trastuzumab, in 1998\textsuperscript{521, 522}.

In this thesis, we modified the basic protein structure of VEGF trap in order to derive novel antiangiogenic biologics. The first class, VEGF sticky-traps, was derived by fusion of a short isoform of VEGF trap with sequences borrowed by VEGF-A, encoding for heparin-binding domains. The second class, VEGF lassos, was derived by fusion of single chain variable regions of monoclonal antibodies with a reverse form of VEGF trap. Development of fusion proteins can be very difficult and of high risk due to possible misfolding, which can lead to protein aggregation or abolishment of functionality. Traditional methods include the production and purification of the candidate proteins. Instead, we opted to develop a genetic system that would allow us to evaluate the functionality of the new biologics in a high-throughput manner.

Our major findings are summarized and future directions are proposed in the following paragraphs.
1. Generation of a genetic system for high-throughput screening of novel biologics

**Summary**

The aim of this part of the thesis was to develop a novel genetic system that would allow for rapid evaluation of the biochemical characteristics, biological activity and pharmacokinetics of novel biologics, both *in vitro* and *in vivo*. It was necessary that this system has the following two properties:

(a) High-throughput generation of cancer lines stably transfected with the desired transgene, and

(b) Inducible expression of the transgene.

Two different systems were explored (Table 5.1). The first one, ΦC31-IMSI, utilizes the ΦC31 recombinase for the specific integration of the gene of interest (GOI) into a predetermined genomic locus. Our experiments in Chapter 1 demonstrate that this system can be successfully used in cancer cells. Establishment of cancer lines with a single-copy of the docking site allowed for high-throughput ΦC31-mediated integration of the incoming vector. Stable transfection of rtTA in the same line as the docking site allows for inducible expression of the GOI.

Even though the ΦC31-IMSI harbored both of the desired characteristics; it has a number of limitations. First, the establishment of cancer lines with a single copy of the docking site demands the screening of a high number of colonies. Second, an additional round of transfection and selection of resistant colonies is required in order to introduce the rtTA. Thus, complete establishment and characterization of a cancer line containing both a single copy of the docking site as well as the rtTA can take up to six months.
Table 5.1

Comparison of plasmid, ΦC31-IMSI and piggyBac transposon methods for generation of transgenic cancer cell lines

<table>
<thead>
<tr>
<th></th>
<th>Linear plasmid</th>
<th>ΦC31-IMSI</th>
<th>piggyBac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copy number</td>
<td>- Multiple</td>
<td>- Single</td>
<td>- Multiple</td>
</tr>
<tr>
<td>Integration fidelity</td>
<td>- Not always</td>
<td>- Intact, “clean”</td>
<td>- Intact, “clean”</td>
</tr>
<tr>
<td>Integration sites</td>
<td>- Multiple</td>
<td>- One</td>
<td>- Multiple</td>
</tr>
<tr>
<td>Multiple copy at the same site (concatameres)</td>
<td>- Yes</td>
<td>- No</td>
<td>- No</td>
</tr>
<tr>
<td>Expression levels</td>
<td>- Variable</td>
<td>- Comparable</td>
<td>- Variable</td>
</tr>
<tr>
<td>Characterization of gene variants (e.g. mutations) &amp; transcriptional elements (e.g. promoters)</td>
<td>- No</td>
<td>- Yes</td>
<td>- No</td>
</tr>
<tr>
<td>Number of plasmids per transfection</td>
<td>Single</td>
<td>- Single</td>
<td>- Multiple</td>
</tr>
<tr>
<td>Time</td>
<td>- Four weeks</td>
<td>- Six months (initial)</td>
<td>- Two weeks</td>
</tr>
<tr>
<td>Ability for subsequent transfections in an already transfected cell line</td>
<td>- Yes</td>
<td>- No</td>
<td>- No</td>
</tr>
</tbody>
</table>
Chapter 2 describes the generation of a system based on the piggyBac transposon. piggyBac transposition allows for the stable integration of multiple copies of desired transposons in a single step. This feature is unique and allows for the development of an exceptionally efficient and rapid system. High-throughput generation of stable lines expressing the GOI in an inducible manner can be established within a two-week period. As shown in both Chapters 2 and 3, the expression was very tight; GOI was only expressed in the presence of doxycycline.

The piggyBac transposon system is ideal, especially for screening secreted protein-based biologics, since variation between clones will not affect their further characterization. Moreover, pooling of cancer cell colonies, instead of selecting single colonies, may also be biologically relevant since the heterogeneity of the cancer line is preserved. This system is ideal especially for screening of protein-based biologics that are secreted from the cells, since the variation between clones will not affect their further characterization. Moreover, pooling of cancer cell colonies, instead of picking single colonies, might be better since the heterogeneity of the cancer line is preserved.

**Future directions**

The piggyBac transposon system has been widely used in various applications such as establishment of inducible pluripotent stem cells. Another future area of interest could be the establishment of mammalian lines for protein production. Our preliminary data indicate that this system can be successfully used for generation of stable 293 cell lines growing in suspension, expressing the GOI in an inducible manner. In order to achieve the latter we used the combination of transposons illustrated in Figure 5.1a.

The inducible expression of the GOI is an important aspect of this system. Since the selection for stable lines occurs in suspension, the final culture contains a mixture of colonies with a broad range of transgene expression levels. Constitutive expression of the transgene can select for those colonies that express low levels since they will grow faster. On the other hand, high expressers tend to grow slower and may disappear during subsequent passaging.

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steps. In contrast, inducible expression allows for maintenance of the initial population by keeping part of the culture in the non-induced stage. Inducibility with doxycycline is applied once, for a small period of time, for each culture and then the cells are discarded.

A combination of the two systems, ΦC31-IMSI and piggyBac, can also be established. This will allow us to select for clones with single integration of the docking site. This can be achieved using the vectors shown in Figure 5.1b. During the first step, after transfection using a molar ratio for PB/rtTA/dock:PB/toxin of 1:10, stable lines resistant for

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**Figure 5.1: Diagram showing potential development of the genetic systems used during the course of the PhD thesis.**

(a) The piggyBac transposons PB/rtTA.neo and PB/GOI/puro can be used in a similar way as those described in Chapters 3 and 4 for the establishment of mammalian lines for expression of recombinant protein. (b) Combination of the docking site, described in Chapter 2, and the piggyBac system, described in Chapters 3 and 4, can facilitate the easier isolation of clones with single copy of the docking site.
puromycin are selected. Due to this biased molar ratio, clones that contain multiple transposon integrations are likely to contain a copy of the PB/toxin transposon. So addition of doxycycline will induce the expression of those clones and mainly the clones that have a single copy of the PB/rtTA/dock will survive. The addition of doxycycline can take place during the second week of the two-week period of puromycin selection and thus shorten the period required for establishment of the desired lines.

This system will allow for tremendous enrichment for colonies with single copy integration and thus decrease the number that have to be screened in order to identify those that allow for high ΦC31-mediated integration.
2. Generation of novel antiangiogenic molecules

Summary

As shown in Chapters 2 and 3, two novel classes of antiangiogenic molecules were derived and characterized during the course of this thesis.

(1). **VEGF sticky-traps**: This class of molecules has two functions: (a) binds to VEGF and inhibits angiogenesis, and (b) binds to the ECM of the target tissue and thus act only in a local manner (Figure 5.2 and 5.3).

VEGF sticky-traps are composed of the trap-domain at the amino-terminus of a modified Fc region of an IgG1 immunoglobulin and a heparin-binding domain at the carboxy-terminus. Using the *piggyBac* transposon transgene delivery system described in Chapter 2 we showed that VEGF sticky-traps are at least as efficient as soluble VEGF trap in their ability to inhibit tumour growth. Furthermore, we demonstrated that in contrast to the soluble VEGF trap, VEGF sticky-traps remain at the tumour site and are not detectable in the circulation, verifying our initial hypothesis that addition of HBD will allow for local inhibition of angiogenesis (Table 5.2).

(2). **VEGF lassos**: This class of molecules is also bi-functional: (a) they are able to bind to VEGF and thus inhibit angiogenesis, and (b) they are able to bind to a cell membrane or stroma antigen and thus target their antiangiogenic effect within the tissue/tumour of interest (Figure 5.2 and 5.3).

Two major advances are described in Chapter 3. First, the development of **VEGF rev-trap**. For the first time we demonstrated that the trap domain can be successfully positioned at the carboxy-terminal end of the Fc region of an IgG1 immunoglobulin. Using the *piggyBac* transposon transgene delivery system we show that VEGF rev-trap has the same characteristics as VEGF trap. Both traps have similar expression levels, protein stability and antiangiogenic effect (Table 5.2).
Table 5.2
Comparison of biologics generated during the PhD thesis

<table>
<thead>
<tr>
<th></th>
<th>VEGF trap</th>
<th>VEGF sh-trap</th>
<th>VEGF rev-trap</th>
<th>VEGF sticky-traps</th>
<th>Ag/V lassos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum half-life</td>
<td>- 2-3 wks</td>
<td>- Hours</td>
<td>- 2-3 wks</td>
<td>- N/A (zero)</td>
<td>- 2-3 wks</td>
</tr>
<tr>
<td>Route of administration</td>
<td>- Direct injection / Intravenous</td>
<td>- Direct injection / Intravenous</td>
<td>- Direct injection / Intravenous</td>
<td>- Direct injection only</td>
<td>- Direct injection / Intravenous</td>
</tr>
<tr>
<td>Binding domains</td>
<td>- One</td>
<td>- One</td>
<td>- One</td>
<td>- Two</td>
<td>- Two</td>
</tr>
<tr>
<td>Target specificity</td>
<td>- No</td>
<td>- No</td>
<td>- No</td>
<td>- Yes (+++ )</td>
<td>- Yes (+)</td>
</tr>
<tr>
<td>Ability to inhibit other pathways</td>
<td>- No</td>
<td>- No</td>
<td>- No</td>
<td>- No</td>
<td>- Yes</td>
</tr>
<tr>
<td>Effector functions</td>
<td>- Yes</td>
<td>- No</td>
<td>- Yes</td>
<td>- No</td>
<td>- Yes</td>
</tr>
<tr>
<td>Flexible exchange of domains / specificity</td>
<td>- No</td>
<td>- No</td>
<td>- Yes</td>
<td>- Yes</td>
<td>- Yes</td>
</tr>
</tbody>
</table>

The second major advance was the fusion of VEGF rev-trap with single chain variable regions of monoclonal antibodies and derivation of VEGF lassos. The data described in Chapter 4 demonstrate that VEGF lassos are characterized by appropriate biochemical properties, *i.e.* they are stable and can be expressed in good levels from mammalian cells. Furthermore, according to our initial hypothesis, the data from Chapter 4 provide convincing evidence that VEGF lassos retain dual-functionality, since both the antibody variable and trap regions retain their ability to recognize their primary antigens (Table 5.2).
Figure 5.2: Novel antiangiogenic biologics generated during the course of this PhD thesis.

**Potential limitations**

1. VEGF-sticky traps

The novel VEGF sticky-traps contain high affinity heparin binding domains (HBDs) which might compromise their pharmacokinetics and decrease their efficacy in a number of ways. For instance, direct delivery into the eye via injection might result in localization of VEGF sticky-traps only at the point of the injection leading to limited distribution via perfusion to the rest of the eye. Ultimately, this may limit their antiangiogenic activity. This drawback might be important especially in the case of AMD since the injection site, vitreous, is anatomically different from the site of abnormal angiogenesis, i.e. choroid. However, in the case of diabetic retinopathy, since the abnormal angiogenesis occurs in the vitreous compartment, this might not affect their efficacy.
A similar problem might occur in the case of delivery of VEGF sticky-traps at the tumour site using nanoparticles, such as liposomes. These molecules tend to extravasate and stay close to the vessels due to their limited ability to penetrate because of their large size. Release of VEGF sticky-traps and strong binding at the ECM at the site of release will limit their perfusion and antiangiogenic efficacy. However, in the case of homogeneous distribution of the nanoparticles in all vessels of the tumour, the strong binding might be beneficial since it will lead to strong anti-VEGF activity at the site of new sprouting vessels.

In order to circumvent these problems, the existing HBDs could be replaced with alternative ones with weaker affinity to heparan sulphate proteoglycans. Since retention at the ECM will also depend on the characteristics of the rest of the molecule, i.e. VEGF sh-trap, it is difficult to predict which HBD will allow for the best distribution. In the case of eye diseases, an alternative solution will be to deliver the VEGF sticky-traps using vehicles such as cells or nanoparticles that can diffuse and reach the site of neovascularization.

2. Ag/VEGF lassos

Lassos will potentially allow for targeting of VEGF-trap at the tumour site and thus increase anti-VEGF activity, in combination with anti-proliferative activity in the case of HER2/V lasso. However, this approach might have some limitations compared to the administration of soluble VEGF trap alone or in combination with soluble anti-HER2 Ab.

The first limitation might be due to the increased toxicity of lassos. Expression of EDB in sites of physiological angiogenesis, i.e. uterine lining and wound healing, might lead to complications in the case of EDB/V lasso. In a similar way, it is known that both anti-HER2 Ab and anti-VEGF Ab have cardiotoxicity, i.e. impairment of left ventricular ejection fraction, thus combination and simultaneous administration of the two agents through HER2/V lasso might lead to increased cardiac toxicity.

One of the prevailing concepts in oncology is the sequential administration of various anti-cancer therapies. For example, chemotherapy and anti-VEGF therapy might be more effective when given in alternate cycles rather than simultaneously. In a similar way, anti-
HER2 and anti-VEGF therapy might less effective if given simultaneously during the same cycle.

The use of alternative antigens recognized by the antibody domain of lassos may eliminate the limitations described above. It is possible that some antigens are overexpressed only during tumour angiogenesis and thus using them will not lead to increased toxicity due to inhibition of angiogenesis during physiological processes. In a similar way, different

**Figure 5.3: Diagram showing the specificity of the various antiangiogenic biologics.**

VEGF trap is completely soluble and can have side-effects. VEGF sticky-traps remain at the site that are delivered at, e.g. tumour. *Lassos* can be specific for either tumour vascular (vAg/V lasso) or tumour cancer cell (tAg/V lasso) antigens, and thus target the antiangiogenic activity at the tumour site. However, in case that the vascular antigen or the tumour antigen is also expressed in other sites (e.g. wound site or heart), distribution at these sites can lead to side-effects.
cancer cell antigens can be utilized allowing for specific delivery of VEGF trap at the tumour site.

One of the major anti-cancer mechanisms of antibodies is their ability to activate host immune defense mechanisms such as CDC and ADCC. Both of these processes are mediated through the binding of the Fc domain with receptors located on immune cells or proteins of the complement system. The position of VEGF trap at the carboxy-terminus of the Fc region of lassos might create steric hindrance and compromise their ability to activate ADCC and CDC. Increasing the distance between the Fc CH3 and trap VEGFR1/D2 domains, i.e by using a longer linker of 30 a.a., might solve this problem.

Finally, the expectation is that both VEGF sticky-traps and Ag/V lassos will have increased efficacy and antiangiogenic activity compared to the existing soluble VEGF trap. This might result in two problems. The first is related to increase hypoxia at the tumour site and upregulation of the HIF1 pathway. In this case, a number of tumour responses such as upregulation of alternative angiogenic factors, increased invasion and metastasis might lead to a worst outcome. The second problem is associated with the fact that inhibition of two important pathways, i.e. HER2 and VEGF, might lead to faster development of tumour resistance and/or “survival of the fittest”, in this case of the most aggressive cancer cells. In both cases lassos will have to be combined with other anticancer drugs such as inhibitors of the HIF1 pathway or inhibitors of invasion and metastasis, like anti-c-met Abs, in order to avoid the development of more aggressive tumours.

**Future directions**


The genetic model used in this thesis allowed for completion of proof of principle studies. However, in order to further characterize the biochemical and pharmacological characteristics of the novel antiangiogenic biologics, recombinant protein production and purification must first ensue. Purified proteins have to be further characterized using BIACORE assays and functional assays such as *in-vitro* tube formation. BIACORE assays
will be very important in the case of the Ag/V lassos in order to show that they can bind both antigens simultaneously.

The pharmacokinetic and biodistribution profile are very important aspects for biologics. VEGF-sticky traps are expected to have negligible serum half-life, while Ag/V lassos and VEGF rev-trap are predicted to have a similar profile as VEGF trap, i.e. 15-20 days. With regards to biodistribution, VEGF sticky-traps are expected to remain at the site of injection, e.g. tumour or eye, while Ag/V lassos should show higher accumulation at the tumour site compared to VEGF trap.

Finally the therapeutic value and toxicity remains to be further characterized. In the case of VEGF-sticky traps, models of AMD and DR will be employed. Their activity will be compared to VEGF trap. It is expected that VEGF-sticky traps will preferentially remain in the eye and thus their activity will be prolonged. Thus, the interval between injections will be longer. In addition, VEGF-sticky traps are not expected to go into the circulation and thus will have negligible, if any, side effects.

In the case of Ag/V lassos, the xenograft and wound healing assays described in Chapter 3 can be used. A number of important questions regarding the antitumour and potential side effects have to be explored at this point.

The first question is related to the efficacy of the antiangiogenic biologics. The dose required in order to achieve the same therapeutic benefit as VEGF trap, i.e. inhibition of tumour progression, has to be determined. Of course a direct comparison between Ag/V lasso and VEGF trap will be unreasonable due to the possible antitumour activity of the antibody component, especially in the case of HER2/V lasso. Thus Ag/V lasso should be compared to Ag antibody with VEGF trap. It is expected that a lower dose will be required for Ag/V lassos since they are targeted therapies. If this is the case, then this will lead to two major benefits. First, a decreased dose will automatically imply fewer side effects, which has to be further evaluated (see below). The second benefit is directly associated with lower cost of treatment which has been the barrier to approval of bevacizumab in the clinic in some cases.
For example, even though the U.S. FDA approved bevacizumab for treatment of metastatic colorectal cancer in 2006, the National Institute for Clinical Excellence (NICE, UK) has not approved it as it is not cost-effective (high cost relative to the benefits).

The second question is related to the possible synergistic effect of HER2/V lasso and how this compares to co-administration of HER2 Ab along with VEGF rev-trap. The preliminary results presented in Chapter 4 (Fig. 4.7a) imply that there is a greater synergistic effect of HER2/V lasso compared to co-administration of HER2 Ab with VEGF trap. This might be due to the fact that HER2/V lasso allows to targeting of the VEGF trap domain and thus a higher therapeutic index.

The third intriguing question is related to the anatomical location and mechanism of action of HER2/V lasso compared to EDB/V lasso at the tumour site. HER2/V lasso will target the VEGF trap domain mainly in the intratumour cancer cell area, the source of VEGF, while EDB/V lasso will target the VEGF trap domain mainly in the vascular bed, where the VEGF receptors are located. Thus, it will be very important to show that inhibition of VEGF is more effective in one or the other anatomical location. For this experiment, a tumour model that overexpresses an “inactive” HER2, without proliferative activity will be needed, in order to avoid the antiproliferative effect due to inhibition of HER2 signaling.

Another question, related to the latter, is whether the combination of HER2/V lasso with EDB/V lasso is more effective compared to either agent alone. This might be the case since they could inhibit VEGF both at the source as well as the point of biological action.

Evaluation of the toxicity and side effect profiles of these novel antiangiogenic biologics is another important aspect. This can be evaluated with the wound-healing assay established and described in Chapter 3. It is expected that the degree of side effects caused by Ag/V lasso will be less compared to VEGF rev-trap. However, from the available literature it is not clear if EDB is also expressed in areas of physiological neovascularization, such as during wound healing. If this is the case, then EDB/V lasso might actually increase the wound healing complications compared to VEGF rev-trap; unless there is an optimal dose
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that will allow for antitumour effect to be achieved without compromising the wound healing process. Furthermore, it is known that both trastuzumab (anti-HER2 Ab) and bevacizumab (anti-VEGF Ab) have cardiotoxic side effects. The cardiac toxicity of the combined treatment is currently evaluated in clinical trials (BETH study). The possible effect of HER2/V lasso to the cardiovascular system has to be examined in detailed.

2. Effector functions

One of most important features of antibodies is their ability to direct the immune system against tumour cells by activating ADCC and CDC (Fig 1.8). Activation of the immune system is responsible for the clinical therapeutic effect of a number of antibodies, including trastuzumab. Both ADCC and CDC are initiated through interactions of the Fc region of the antibody with either cell surface receptors on immune cells or proteins such as the C1q complement factor. It will be important to determine if the Ag/V lassos are still able to interact with these components and initiate immune responses. The fact that the carboxy-terminus of the Fc region is occupied by the VEGF trap-domain might create steric hindrance and prevent this ability. In that case it will be important to determine if this affects their therapeutic value.

3. Adjuvant therapy

The therapeutic value of antiangiogenic biologics as an adjuvant therapy is one of the emerging questions in the field. This will imply that administration of antiangiogenic agents after surgical removal of the primary tumour will delay the recurrence of cancer. A number of clinical trials are currently evaluating the possible therapeutic benefit of adjuvant bevacizumab (BETH and BEATRICE trials).

A few questions are of interest regarding the possible advantages of Ag/V lassos versus VEGF trap in the setting of adjuvant therapy. The first is related to the time point of initiation of antiangiogenic therapy. Since the removal of the primary tumour is a surgical procedure, a 30 day period after the surgery must past in order to be able to start any of the currently approved antiangiogenic agents. This period is crucial since it may allow for any
remaining cancer cells, either at the primary or metastatic sites, to establish new vessels and undergo regrowth. It is possible that treatment with Ag/V lassos can be initiated immediately after surgery due to the targeted nature of these agents. This may lead to beneficial patient outcomes since it will prevent tumour regrowth. Low dose EDB/V lasso is expected to specifically target sites of angiogenesis, such as metastatic sites or cancer cells at the primary site undergoing proliferation. Moreover, low dose of HER2/V lasso can target any circulating HER2 positive cells and prevent them from establishing metastatic foci.

The second question is related to the duration of adjuvant therapy and the side effects. In the case of breast cancer, trastuzumab is given as adjuvant therapy for a period of 5 years post-surgery. Prolonged treatment with either of the current antiangiogenic agents may lead to increased toxicity, which in turn, may result in discontinuation of therapy. However, in the case of Ag/V lasso this can possibly be avoided since a lower dose may likely be required in order to achieve the same therapeutic index as the current biologics.

4. Development of double-traps

Resistance to antiangiogenic therapy and tumour relapse is another important aspect of current agents. One of the main reasons is the upregulation of alternative angiogenic factors. The existence of many angiogenic pathways might be also the reason for

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**Figure 5.4: Double antiangiogenic biologics.**
which TKIs are successful as monotherapy in contrast to biologics that target only one pathway.\textsuperscript{113}

Using the VEGF rev-trap, it is possible to develop double-traps, which are able to inhibit other factors such as FGF and PDGF-C (Figure 5.4). It is plausible that inhibition of multiple angiogenic factors by double-traps will allow for their use in the clinic as monotherapy as well as delay any relapse secondary to antiangiogenic treatment.
3. Concluding remarks

In summary, throughout the course of my thesis I was able to advance two major fields of research related to molecular biology and the development of biologics.

I thoroughly examined and characterized two genetic systems, ΦC31-IMSI (Chapter 2) and piggyBac transposons (Chapters 3 and 4), for high-throughput generation of human (cancer) cell lines stably transfected with the transgene of interest. The combination of these systems with the second-generation doxycycline-inducible system allows for robust control of transgene expression. Using the ΦC31-IMSI system, it is possible to generate a library of co-isogenic human cell lines containing a single copy of full-length cDNA library, for example. The utility of the piggyBac transposon system for screening biologics in drug discovery has been extensively used. This system allowed for the *in vitro* and *in vivo* characterization of novel potential protein-based drugs, including stability, activity and pharmacokinetics. In conclusion, from the body of work generated in this thesis, strong evidence is provided that both systems can be utilized for both basic as well as translational research.

The main body of my thesis describes the successful development of two novel classes of biologics, “sticky-traps” and “lassos” (Chapters 3 and 4). I was able to design, generate and characterize a series of novel molecules and provide convincing evidence supporting their biological and pharmacological properties. Both of these new entities, “sticky-traps” and “lassos”, can trap soluble growth factors at the site of interest. During my thesis, VEGF was targeted and demonstrated the bifunctionality of VEGF sticky-traps and VEGF-lassos. These novel molecules can be useful both for basic and translational research.

At present, VEGF sticky-traps represent the only molecules available capable of inhibiting VEGF specifically at the site of interest. VEGF-lassos comprise a new and promising entity of bifunctional proteins with a unique structure and can easily be modified for the development of new “lasso” molecules inhibiting other signaling pathways. In addition to their potential therapeutic value for treatment of diseases with pathological
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angiogenesis, both VEGF sticky-traps and lassos can also be used in basic research in order to elucidate the role of VEGF in different organs and physiological conditions. We envision that the same approaches could be used for the generation of similar biologics that will aid in the understanding and treatment of various diseases.
Chapter 6

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